Diagnosis of Infectious Laryngotracheitis Virus Using Routine and Advanced Techniques under Local Egyptian Field Conditions

Kawther S. Zaher and Sohier M. Syame

Department of Microbiology and Immunology, Veterinary Research Division, National Research Centre, Giza, Egypt

Abstract: Infectious laryngotracheitis (ILT) is one of the most important diseases affecting poultry industry worldwide. This virus belongs to the family alphaherpesviridae. In this study 100 blood and tracheal samples were taken from affected birds showing signs of the disease manifested as a moderate to severe upper respiratory disease. The samples were subjected for routine examination such as isolation on Chicken Embryo Rough cells (CER) cells and identification by cultural behavior and Electron Microscopy (EM) as well as advance techniques such as rough protein analysis using poly acrylamide gel, Fingerprinting using restriction enzymes (RE), HindIII and BamHI and PCR using primers encoding the 1.1 kb BamHI fragment of ILTV as a trial for the purpose of finding some methods for analysis which would be used successfully in a diagnostic setting for all ILTV local strains. This study demonstrated a promising results for the utility and potential of fingerprinting using digestion by RE and PCR, based on primers encoding the 1.1 kb BamHI fragment of ILTV, to detect different isolates from field outbreaks. This may enable earlier detection in the event of an outbreak if employed directly on material obtained from the field.

Key words: ILTV • Fingerprinting • RE • Protein analysis • EM • PCR

INTRODUCTION

Infectious laryngotracheitis (ILT) in poultry has been diagnosed on an ongoing basis in Egypt [1], as well as in other parts of the world [2-5]. The causative agent is infectious Laryngotracheitis virus (ILTV) which is an alphaherpesvirus. The infection is manifested as a moderate to severe upper respiratory disease which may result in significant losses to the producer, both from mortality [6-8] and production losses [3, 4, 6, 7, 9]. Currently, diagnosis of ILTV requires histological examination of post mortem specimens for the demonstration of nuclear inclusion bodies post mortem specimens for the demonstration of nuclear inclusion bodies and virus isolation in embryonated chicken eggs [10]. Antigen detection assays have been developed but appear to lack sensitivity [11-13]. The recent development of molecular based diagnostic methodologies, such as DNA probes [14-16] and the polymerase chain reaction (PCR) have been applied to ILTV [17-19]. PCR development has been described to detect ILTV by amplifying DNA from a 1.1 kb BamHI fragment of the genome [20]. It was found to be homologous to the immediate–early protein (ICP-4) gene of ILTV [21]. The specificity of the reaction was determined by successful amplification of the DNA from a number of different ILTV strains with different virulence levels and the inability to amplify nucleic acid from fowl pox virus, avian adenovirus, herpes virus of turkeys, Newcastle disease virus, infectious bronchitis virus and Marek's disease virus [20, 22]. It was not known how well these primers would perform on field cases of ILTV. Although strains of ILTV are indistinguishable serologically [5], it has been shown that certain isolates from various geographical areas may differ slightly in their restriction endonuclease (RE) patterns [22, 23]. Additionally, vaccine strains have different RE patterns from established reference strains [24]. Although the 1.1 kb BamHI fragment was successfully used as a probe to detect different ILTV strains and a number of ILTV strains maintained in the laboratory were amplified by PCR [25], it was necessary to determine whether the primers selected from this area of the genome would amplify all isolates encountered. In the current study isolates
from outbreaks of ILT which occurred in Egypt between 2006 and 2007 were analyzed by cultural behavior, electron microscopy, fingerprinting, protein analysis and PCR. This study was designed to imitate the methods of analysis which would presumably be employed in a diagnostic setting for all ILTV local strains.

**MATERIALS AND METHODS**

The current work was carried out during a period of May 2006 – December, 2007.

**Samples**: Blood samples on EDTA and tracheal samples were taken from live Broilers and layers birds suffered from respiratory symptoms and bloody coughing at all ages. The blood was centrifuged at X 1500g for 10 min using cooling centrifuge and the buffy coat was separated for virus isolation.

**Reference Virus**: Egg adapted live attenuated virus vaccine (TAD ILT vaccine 10000ds, Lohman animal GMH & Co/kg, Heinz-Lohman str. 4-2-7471 Cuxhaven Germany, Batch no 1262251) was used. Also a reference virus strain is available at our lab which was previously identified [12]

**Virus Isolation and Identification**: The virus was isolated and propagated on Chicken Embryo Rough cells (CER) which were purchased from VACSERA institute, Agosa. 10% of the tracheal tissue was performed and concentrated using poly ethylene glycol (PEG). The suspected virus was isolated from both buffy coats of the blood samples and tracheal tissue and titrated according to Montanha et al. [26] and Madbouly et al. [12].

**Electron Microscopy**: The isolated virus was examined at Electron Microscope Unite, National Research Centre using Electron microscope Model EM 10 Zesiss, West Germany at 60 kv and resolution of 10Å according to Wu et al [27] and Madbouly et al. [12].

**Protein Analysis**: The test was performed on both buffy coats and infected cells. The isolated virus was purified according to Madbouly et al. [12]. Electrophoresis was performed using poly acrylamide gel. The gel was then stained by silver nitrate [28].

**Finger Printing Using Restriction Enzymes (RE)**: CEF cells were infected at a multiplicity of infection (MOI) of 1 and the infected cell monolayer was monitored for cytopathic effect (CPE) until it had completely developed. The cells and medium were then freeze-thawed through three cycles and sonicated on ice, with two bursts of 30 s each. After low speed clarification the virus in the supernatant fraction was concentrated by pelleting and ultra filtrated according to Madbouly et al. [12]. Samples from the resultant bands were diluted in HBS and concentrated by pelleting and resuspended in HBS. For DNA extraction, samples were treated with protease K (500 µg/mL) and 0.5% sodium dodecyl sulphate (SDS) at 37ºC for 2 hr and extracted with phenol/chloroform, precipitated with 70% ethanol and the DNA pellet resuspended in TE buffer (10 mM Tris, 1 mM EDTA; pH = 7.2). DNA extracted from ILTV as described above was digested with the restriction enzymes HindIII and BamHI according to the manufacturer’s (BRL: Bethesda Research Laboratories) instructions. Agarose gel electrophoresis was at 5 volts/cm in 0.75% agarose gels made up in TEA buffer (40 mM tris-acetate, 2 mM EDTA and pH 8.00). A 1 kb marker DNA preparation from BRL was used as a size reference in agarose gels [29].

**Polymerase Chain Reaction (PCR)**: PCR primer design and reaction parameters are as described by Alexander [30] and Alexander and Nagy [20]. The primers, from the sequence information of the 1.1 kb BamHI fragment, were synthesized by GenoSys Biotechnologies (The Woodlands, Texas, USA) with the sequences: "ILTV1": 5'-AGAACGAGACAATTCCCTCC-3'; "ILTV2": 5'-GTTCTGTTGTCATGATGTA- 3'. All reactions were in 50, uL. The following parameters were used for the generation of the 443 bp product: an initial denaturation at 94ºC for 1 min, followed by 35 cycles of denaturation at 95ºC for 20 s, annealing at 60ºC for 90 s and extension at 72ºC for 90 s. At the completion of all cycles, a final extension at 72ºC for 5 min was used. Controls used included a PCR positive control (ILTV-infected cell culture), a negative control (uninfected cell culture) and reagent controls (PCR master mix with water in place of the DNA). Products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

**RESULTS**

**Samples**: Infected birds showed hemorrhagic tracheas as shown in Fig. 1.

**Virus Isolation**: Typical picture of ILTV CPE was demonstrated such as cytoplasmic fusion which results information of multinucleated cells and dispatched areas on later stage of infection (Fig. 2).
Fig. 1: Hemorrhagic tracheas of infected birds

Fig. 2: Vero cells infected with the isolated virus after 27 hours. Very clear syncytial and protoplasmic threads (A). Vero cells infected with ILT virus after 48 hours. Cell detachment and destruction of cells and the cells appears in island of small number. (Crystal violet stain, X 200)

Fig. 3: Electron micrograph of ILT virus showing the size approximately 200 nm and the fine projection on the surface. (Sodium phosphotungstate stain, X 75000)

Fig. 4: Electrophoresis using poly acrylamide gel. Protein marker on the right followed by isolated virus from infected cells then isolated virus from buffy coats, then reference virus at the left

Fig. 5: Agarose gel electrophoresis of ILTV DNA extracted from sucrose density gradient of ILTV. DNA was extracted from buffy coat (lanes 2), infected CER cells (lanes 3) and from reference vaccine ILTV (lanes 4) each containing ILTV. The extracted DNA was analyzed by agarose gel electrophoresis after digestion with BamHI.
Electron Microscopy (EM): The observed virus particles by EM examination reveal that the virus has an irregular envelope of a diameter of 195-250 nm. The surface of the envelope contains projections (Fig. 3).

Protein Analysis: Protein analysis performed to compare the content of the reference vaccine virus (figure 3, lane 2) and the isolated virus from the buffy coats (figure 3, lane 3) and from infected cells (figure 3, lane 3) showed very clear similarity between the virus isolated from infected cells and that of vaccine (Fig. 4).

Finger Printing Using RE: Of the several restriction enzymes which were used to analyze the ILTV DNA, BamH I gave a reasonable number of well resolved fragments and in a suitable size range (0.5 kb to 15 kb, Fig. 5).

Polymerase Chain Reaction (PCR): Amplification products from the sequence information of the 1.1 kb BamHI fragment were predicted for ILTV at 443 bp. By using DNA extracted from blood samples and infected CER cells with the isolated virus, products after extraction of DNAs, consistent with those predicted were obtained (Fig. 6). A total of 100 ILTV isolates were typed by PCR.

DISCUSSION

ILTV is belonging to Herpes virus family. This is considered as one of the most important diseases affect poultry industry worldwide causing great economic losses, mortality may reach 70% and recovered birds fail to gain desired weights [1]. Tracheal samples showed hemorrhagic lesions which comes in agreement with [7]. In the current study 100 blood and tracheal samples were taken from clinically affected birds and the samples were subjected for routine and advanced techniques for ILTV diagnosis, for the purpose of finding the most ultimate methods of analysis which would presumably be employed in a diagnostic setting for all ILTV local strains. Data of cultural behavior such as cytoplastic fusion which results information of multinucleated cells and dispatched areas on later stage of infection, also that of EM examination comes in agreement with Madbouly et al. [13].

Rough Protein analysis by poly acrylamide gel electrophoresis revealed very close similarities between the reference vaccine strain and the field strain of ILTV virus. The process of purifying the virus from infected CER and the process of culturing the virus for many passages in CER cells may affect the infectivity and protein content of the virus and this may be the reason for unclear protein picture in Figure 4 lane 3 and this comes in agreement with Nagy [15].

There are some problems with any serological approach for the detection of ILTV, such as cross-reactivity with normal serum and lack of antibodies at certain times during the infection or in the latent stage of the virus [11]. In particular, sample preparation could be simplified and the overall time required to complete the assay could be reduced dramatically. However, for the effective ILTV diagnosis, it is necessary to develop a readily available and reliable sequence that will identify a variety of ILTV isolates. This problem may be circumvented with advanced techniques such as fingerprinting with digestion by RE, PCR and cloned ILTV DNA based probe [22]. Therefore, it was necessary to demonstrate specific area of ILTV genome which would not react with other common avian viral pathogens. The 1.1 kb BamH I fragment of ILTV DNA failed to react with nucleic acid from cells infected with FPV, HVT, MDV or NDV [22]. The current study tested the efficacy of digestion by RE BamH I and also by PCR using primers specific for 1.1 kb BamHI fragment which are predicted for ILTV at 443 bp. Both methods have shown promising results for detection of local strains of ILTV. The results come in agreement with Nagy [15]. With additional sequence information for ILTV genes [31] it may be possible to select more conserved regions of the ILTV genome for use as a broad range for different ILTV isolates diagnosis. Alternatively the use of a larger DNA fragment may increase the sensitivity of the detection of ILTV, as shown in this study and might also be more likely to react with DNA from several isolates. The latter may not be possible using a serological approach, especially if the degree of virulence cannot be distinguished serologically. However, when the ILTV infected samples were used for PCR, all of the isolates were amplified. This indicates that the primers from the 1.1 kb BamHI fragment are appropriate for use in detection of isolates obtained from outbreaks. The ability of these primers to also amplify all of the isolates may indicate that they have widespread applicability to other geographical areas.

This study demonstrates the utility and potential of fingerprinting using digestion by RE and PCR, based on primers encoding the 1.1 kb BamHI fragment of ILTV, to detect different isolates from field outbreaks. This may enable earlier detection in the event of an outbreak if employed directly on material obtained from the field.
Studies by Alexander and Nagy [20] and Neff et al. [22] reported the ability of this PCR to amplify ILTV DNA from conjunctival swabs or blood samples.

It was concluded that there are promising results for the utility and potential of fingerprinting using digestion by RE and PCR, based on primers encoding the 1.1 kb BamHI fragment of ILTV, to detect different isolates from field outbreaks. This may enable earlier detection in the event of an outbreak if employed directly on material obtained from the field.

REFERENCES


