

Rapid detection of infectious laryngotracheitis virus by standardization of polymerase chain reaction targeting a relatively conserved region of the thymidine kinase gene

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Abstract: Infectious laryngotracheitis is an acute upper respiratory tract infection of chickens caused by infectious laryngotracheitis virus. The study was conducted to standardize the polymerase chain reaction targeting a relatively conserved region of the thymidine kinase gene for the rapid detection of infectious laryngotracheitis virus. The vaccine samples were collected from two renowned company of Bangladesh. DNA was extracted from diluted vaccine samples by using Wizard[®] Genomic DNA purification kit and thymidine kinase gene was amplified by using PCR system 9600 Thermocycler. Two vaccine samples were positively amplified by polymerase chain reaction. A procedure was developed for rapid detection of infectious laryngotracheitis virus by polymerase chain reaction of the conserved region of viral thymidine kinase gene containing DNA fragments. The results obtained in this study suggested that the polymerase chain reaction procedure could serve as a fast and sensitive method for the detection of vaccine strains of infectious laryngotracheitis viruses.

Key words: Infectious laryngotracheitis virus, viral thymidine kinase (TK) gene, polymerase chain reaction

Introduction

Infectious laryngotracheitis (ILT) is a worldwide distributed upper respiratory disease of chickens (Guy & Bagust, 2003) and included within the list E of the Office International Des Epizooties (OIE) (Hidalgo, 2003). Chicken is the only significant primary host species for infectious laryngotracheitis virus (ILTV) and no other reservoir species have been recognized, even though pheasant and peafowl can sometimes be naturally infected by contact with chickens actively shedding ILTV (Guy & Bagust, 2003). Infection is acquired via the upper respiratory tract and transmission occurs most readily from acutely infected birds but clinically inapparent infection can persist for long periods with intermittent re-excretion of the virus, and these recovered carrier birds are also a potential means of transmission of the disease (OIE, 2008). The disease is common in areas of intense poultry production and causes great economic losses due to moderate to severe mortality, and drop in egg production (Callison *et al.*, 2007).

Strains of infectious laryngotracheitis virus may vary considerably in their virulence and there was

evidence that vaccine derived strains have become established in the field (Graham *et al.*, 2000). This ability of ILTV vaccine strains to re-circulate may also be responsible for some outbreaks in susceptible birds, as passage in birds has been reported to result in increasing virulence (Guy *et al.*, 1991), while stress in latently infected birds has also been demonstrated to be responsible for the re-excretion of ILTV (Bagust *et al.*, 2000).

Conventional laboratory diagnosis of ILT is based on virus isolation in specific pathogen free chicken embryos inoculated via the chorioallantoic route or in primary chicken embryo kidney cells, chicken embryo liver cell (Tripathy, 1998). However, it is time consuming and labor intensive in spite of its high sensitivity and specificity. The use of direct fluorescent-labeled polyclonal antibodies (FA) to detect viral antigens in tracheal and conjunctival smears (Goodwin *et al.*, 1991), or monoclonal antibodies to detect viral antigens in frozen tracheal sections have been successfully utilized in the rapid diagnosis of the disease, although those had comparatively poor sensitivity.

Therefore, a requirement for the development of rapid and sensitive diagnostic techniques for the verification of clinical diagnosis of ILTV for the improvement of the quality of surveillance systems. As such, the present study was carried out to standardize the polymerase chain reaction (PCR) targeting a relatively conserved region of the thymidine kinase (TK) gene for the rapid detection of ILT virus.

Materials and Methods

Collection of samples for DNA extraction

The Nobilis[®] ILT and Gallivac LT freeze dried vaccine samples were collected respectively from the Intervet, Bengal Overseas Limited and Advance Animal Science Company Limited, Dhaka, Bangladesh, and carried in an ice box to the Department of Pathology, Faculty of Veterinary Science, Bangladesh Agricultural

University, Mymensingh. The vaccine was diluted with the given sterile buffered and colored solvent and stored at 2-8^oC.

DNA extraction

DNA was extracted from diluted vaccine samples by using Wizard[®] Genomic DNA purification kit (Promega Corporation, 2800 Woods Hollow Road, Madison, USA). The extracted DNA was quantified using a spectrophotometer's (Spectronic[®] Genetics[™] New York, USA) and expressed in ng/ μ l.

Selection and synthesis of primers

An appropriate primer sequence for PCR was selected to amplify thymidine kinase (TK) gene (Griffin & Bournsnel, 1990) of ILT virus. The primer sequences (Table 1) were synthesized commercially by Sangamo Biosciences, Inc., Singapore.

Table 1. Primers used in PCR for infectious laryngotracheitis detection

Primer	Target Gene	Sequence	Product Size	Location
ILTV PCR5'	TK gene	5'-ACGATGACTCCGACTTTC-3'	647bp	The ILTVPCR5' primer starts at base 222 in the keeler sequence and base 4501 in the Griffin sequence. The ILTPCR3' primer starts at base 847 in the keeler sequence and base 5130 in the Griffin sequence.
ILTV PCR3'		5'-CGTTGGAGGTAGGTGGTA-3'		

Amplification of DNA by polymerase chain reaction (PCR)

PCR amplification was performed in a final volume of 10 μ l containing 2 μ l of extracted DNA template, Taq DNA polymerase buffer (10x) 1 μ l, dNTPs 1 μ l, primer-F (50n mol) 0.5 μ l, primer-R (50n mol) 0.5 μ l, Taq DNA polymerase 0.2 μ l and 4.8 μ l nuclease free water (Science Park Rd.#01-23, The Geni S'pore). 2 μ l water was added instead of DNA to the water control tube. Amplification was carried out in Gene amplification PCR system 9600 Thermocycler (ependorf, Germany), using condition modified from Abbas *et al.*, (1996). The pre-mix was then mixed well through spinning. Initial denaturation was at 94^oC for 1 min, again denaturation was at 95^oC for 1 minute, annealing at 50^oC for 1 minute and extension at 72^oC for 1.5 minutes, with a final extension at 72^oC for 5 minutes for total 35 cycles and held for 4^oC in refrigerator until electrophoresis.

Agarose gel electrophoresis

The amplified PCR products were separated by electrophoresis on 1.5% agarose gel containing 5 μ l ml⁻¹ ethidium bromide with a 100 bp ladder (Promega, Madison, WI, USA) as molecular weight marker (Oliveira *et al.*, 2003).

Documentation of the PCR products

After electrophoresis, the gel was taken out carefully from the gel chamber and placed on the UV Transilluminator in the dark chamber of the Image Documentation System (Labortechnik, Germany).

Results and Discussion

Polymerase chain reaction was optimized and evaluated by the amplification with primers made from targeted portion of thymidine kinase (TK) gene of the ILTV. The PCR was performed at different annealing temperatures and products of 647-bp were successfully amplified at 50^oC

annealing temperature. The expected fragment of 647-bp of the thymidine kinase gene of ILTV was obtained from two samples, as well as no band was detected in the negative control (Fig 1). The positive result in the PCR directed to the thymidine kinase gene of infectious laryngotracheitis virus (ILTV) agrees with the findings of Abbas *et al.* (1996).

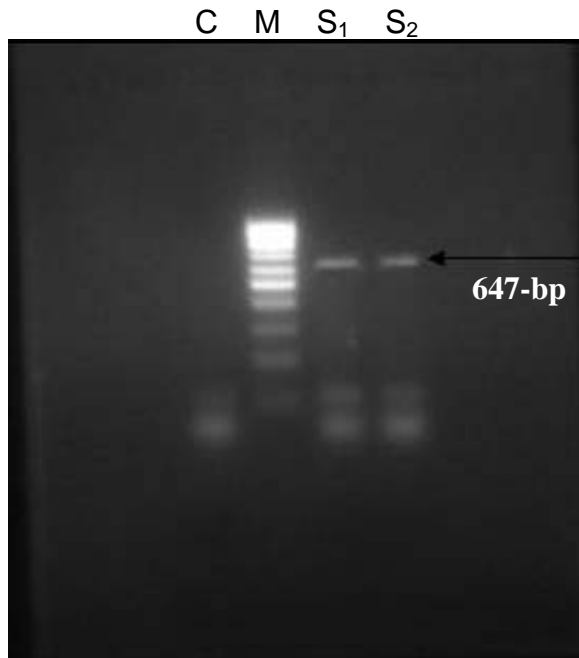


Fig 1. Visualization of the 647-bp PCR product from the thymidine kinase gene of ILTV by agarose gel electrophoresis (1.5%) after staining with ethidium bromide. Lane-S₁: viral vaccine (Nobilis® ILT); Lane-S₂: viral vaccine (Gallivac LT); Lane-M: molecular marker; Lane -C: negative control

Molecular based diagnostic methodologies, such as DNA probes (Key *et al.*, 1994) and the polymerase chain reaction (Williams *et al.*, 1994; Abbas *et al.*, 1996) have been developed to diagnose the infectious laryngotracheitis virus. The positive results in the PCR directed to the thymidine kinase gene of ILTV is a useful tool to confirm the diagnosis of birds suspected to infectious laryngotracheitis. Results can be obtained in less than 24 hours which is an essential point in outbreaks when fast decisions are required. The application of the procedures described herein must be evaluated for field sample collected from suspected birds considering that PCR applied to viral diagnosis is a highly sensitive technique (Forghani & Erdman, 1994) that allows the detection of infection in a very early phase when compared to serological reactions (Pang *et al.*, 2002).

In spite of the use of vaccines for the control of ILTV, the disease continues to be a problem in commercial poultry (Clavijo & Nagy, 1997). The use of live and modified live vaccines has been demonstrated to lead to spread of the virus, particularly within 7-10 days of vaccination. Carriers may result, and it is possible that the infection become indigenous and thus the vaccine strains of the virus may be involved in ILTV outbreaks (Hughes *et al.*, 1987). These findings provide support for the requirement of continuous monitoring of the vaccine strains and the development of molecular techniques that may allow simple and reliable identification of different vaccine strains.

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