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ASSERTION AND DEVELOPMENT OF A POLYMERASE CHAIN REACTION TO DETECT CONSERVED THYMIDINE KINASE GENE OF INFECTIOUS LARYNGOTRACHEITIS VIRUS FROM CLINICAL OUTBREAKS^

Puvarajan B[1], K. Sukumar[2], J. Johnson Rajeswar[3], T.J. Harikrishnan[4] and G.A.B. Balasubramaniam[5]

[1]Assistant Professor, Department of Veterinary Microbiology, VCRI, Namakkal
[2]Professor and Head, Department of Veterinary Microbiology, VCRI, Namakkal
[3]Professor and Head, Department of Veterinary Microbiology, VCRI, Tirunelveli
[4]Director of Research, TANUVAS, Chennai-51
[5]Dean (i/c), VCRI, Namakkal
Department of Veterinary Microbiology, Veterinary College and Research Institute, Orathanadu

^ Part of Ph.D thesis submitted to Tamilnadu Veterinary and Animal Sciences University (TANUVAS), Chennai

E-mail: vetpuva@yahoo.co.in (**Corresponding Author*)

Abstract: Infectious laryngotracheitis (ILT) is an important respiratory disease of chickens and annually causes significant economic losses in the poultry industry world-wide. ILT virus (ILTV) belongs to alphaherpesvirinae and the *Gallid herpesvirus 1* species. The transmission of ILTV is *via* respiratory and ocular routes. The study was conducted to identify directly infectious laryngotracheitis virus from field outbreaks by polymerase chain reaction. DNA was extracted from field isolates and vaccine sample by using One tube DNA Extraction kit® Genomic DNA purification kit and thymidine kinase gene was amplified by using PCR system 9600 Thermocycler. Both the samples were positively amplified by polymerase chain reaction. A procedure was developed and standardized 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 field and vaccine strains of infectious laryngotracheitis virus. In this study, a rapid and sensitive PCR assay for detection of ILTV was developed and evaluated by using ILTV DNA in the tissues of the chickens infected with ILTV from clinical cases.

Keywords: Infectious laryngotracheitis virus, thymidine kinase (TK) gene, polymerase chain reaction

INTRODUCTION

Infectious laryngotracheitis (ILT) is a worldwide distributed upper respiratory disease of chickens (Guy & Bagust, 2003) (Ou and Giambrone, 2012) and included within the list E of the Office International Des Epizooties (OIE) (Hidalgo, 2003). Chicken is the only significant

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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. 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 Merile® ILT vaccine sample were collected respectively from the Merile, USA Company Limited. The vaccine was diluted with the given sterile buffered and colored solvent and stored at -80^{0}C.

DNA extraction

DNA was extracted from diluted vaccine samples by using Bio-basic® Genomic DNA purification kit (Bio-basic, Canada, USA). The extracted DNA was quantified using Nanaodrop (Thermo Fisher, USA) and expressed in 722.50 ng/µl.

Selection and synthesis of primers

An appropriate primer sequence for PCR was selected to amplify thymidine kinase (TK) gene (Chacon and Ferriera, 2009) of ILT virus. The primer sequences were synthesized commercially by Bio serve Inc., Hyderabad, India.

Forward Primer

5'-ACG ATG ACT CCG ACT TTC-3'

Reverse Primer

5'-CGT TGG AGG TAG GTG GTA-3'

Amplification of DNA by polymerase chain reaction (PCR)

PCR amplification was performed in a final volume of 50µl containing 2 µl l of extracted DNA template, Taq DNA polymerase Red Dye Mastermix 30µl (Bio-basic, Canada), 0.5 µl of Forward and Reverse primers each and17 µl nuclease free water (Hi-Media, Mumbai, India). Nuclease free water (2 µl) was added instead of DNA to the water control tube. Amplification was carried out in Gene amplification PCR system 9600 Thermocycler (Eppendorf, Germany), using condition modified from Pang et al., (2002). The pre-mix was then mixed well through spinning. The thermal conditions are initial denaturation was at 94°C for 1 min, again denaturation was at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes, with a final extension at 72°C for 5 minutes for total 35 cycles and held for 40C in refrigerator until electrophoresis.

Agarose gel electrophoresis amplified PCR products were separated by electrophoresis on 1.5% agarose gel containing0.5 µl ethidium bromide with a 100 bp ladder (Bio-basic, Canada, USA) as molecular weight marker (Chacon and Ferraira 2009).

After electrophoresis, the gel was taken out carefully from the gel chamber and placed on the Gel Doc Image Documentation System (Bio-Rad Inc., USA).

III. RESULTS AND DISCUSSION

The affected birds clinically showed matted eyelid and difficult to breathe with typical pump and handle respiration in the layer farms (Fig1).

Fig 1 ILT affected layers with matted eyelid

Polymerase chain reaction was optimized and evaluated by the amplification with primers made from targeted portion of thymidine kinase (TK) gene. The expected fragment of 647-bp of the thymidine kinase gene of ILTV was obtained from two samples (Fig 2). The positive result in the PCR directed to the thymidine kinase gene of infectious laryngotracheitis virus (ILTV) is in accordance with the findings of Chakma et al. (2010).

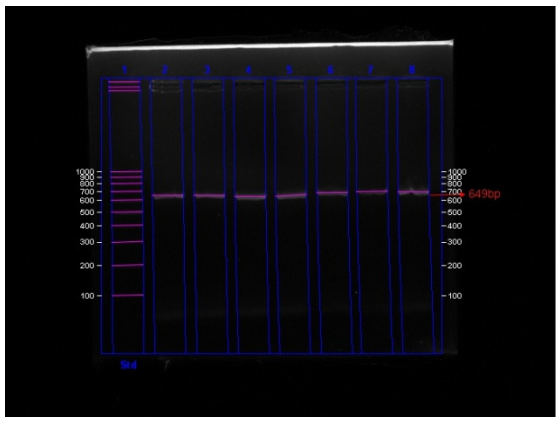

FIGURE 1 VISUALIZATION OF THE 647-BP PCR PRODUCT FROM THE THYMIDINE KINASE GENE OF ILTV BY AGAROSE GEL ELECTROPHORESIS (1.5%) AFTER STAINING WITH ETHIDIUM BROMIDE

Lane-1: molecular marker; Lane 2-6: ILTV Field Isolates

Lane 7: ILT Vaccine (Merile)

Molecular based diagnostic methodologies, such as DNA probes (Key et al., 1994) and the polymerase chain reaction (Abbas *et al*., 1996; Creelan *et al.,* 2006) 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. The 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 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 spite of the use of vaccines for the control of ILTV, the disease continues in commercial poultry (Clavijo & Nagy, 1997). 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 and Jones, 1988).

To conclude, these findings provide support for the requirement of continuous monitoring of the vaccine strains of ILT, an economically important disease by development of molecular techniques that may allow simple and reliable identification of different vaccine strains for prevention of vaccine reversion and latent infection.

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