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ORIGINAL ARTICLE

Molecular detection and characterization of infectious laryngotracheitis virus (*Gallid herpesvirus-1*) from clinical samples of commercial poultry flocks in India

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Abstract Although the existence of infectious laryngotracheitis virus (ILTV) in India was first reported in 1964, no reports are available regarding its molecular detection and characterization. The present study was aimed to detect and characterize ILTV from recent respiratory disease complex (RDC) outbreaks of commercial poultry flocks in different parts of the country by using envelope glycoprotein G gene (US4 gene) based PCR and sequencing. A total of thirty two flocks with a history of RDC were investigated. Overall, all the strains/breeds of birds and all ages of birds are equally susceptible and depending on the severity, the clinical signs and gross lesions were varied. Out of 32 flocks investigated 10 were found positive for ILTV infection by PCR. The phylogenetic analyses of eight representative sequences in the present study deciphered that Indian ILT viruses are closely related to chicken embryo origin vaccine strains of Italy, USA, China and Brazil.

Keywords Infectious laryngotracheitis · PCR detection · Phylogenetic analyses · Molecular epidemiology · India

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Introduction

The etiology of respiratory diseases is complex in poultry, often involving more than one pathogen at the same time [20]. A wide variety of pathogens have been associated with respiratory infections in poultry including Avian pneumovirus (APV), Avian influenza virus (AIV), Infectious bronchitis virus (IBV), Newcastle disease virus (NDV), *Avibacterium paragallinarum, Ornithobacterium rhinotracheale* (ORT), *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Avian pathogenic Escherichia coli* (APEC) [4, 28]. These pathogens are of major importance because they can cause disease independently, or in association with each other [26]. Mixed infections of different respiratory agents may occur because of extensive use of multiple live vaccines, high geographic population, and housing densities [10]. Among the respiratory diseases of poultry Infectious laryngotracheitis (ILT) is a highly contagious, acute respiratory disease of chickens. This disease causes economic loss in poultry industries worldwide and is a significant concern for poultry health and welfare [2]. The causative agent is infectious laryngotracheitis virus (ILTV), also designated *Gallid herpesvirus 1* (GaHV-1), which belongs to the genus *Iltovirus*, subfamily *Alphaherpesvirinae* of the family *Herpesviridae* [6]. ILTV possesses a linear 155-kb linear double-stranded DNA consists of a unique long (UL), region of 113 kb and a unique short region (US) region of 13 kb, which is flanked by inverted repeat sequences (IR, TR) permitting formation of two isomers [19]. The unique short genome region contains a cluster of six conserved alphaherpesvirus genes encoding membrane proteins, including gB, gC, gG, gJ, gM and gN [32]. The gG gene US4 has been predicted to form a co-terminal transcription unit with the translocated UL47 gene of ILTV [32], and were only found in infected cells [8, 27]. The differentiation of vaccine and ‘‘wild-type’’ ILT viruses based

on sequencing of UL47 and glycoprotein G genes could serve as a useful tool for future investigations [22].ILTV infection causes respiratory disease symptoms in chickens, pheasants, partridges, and peafowl [5, 13]. Clinical signs include extension of the neck, gasping, gurgling, rattling, and coughing of clotted blood [11, 17]. ILTV usually causes a reduction in egg production and variable mortality ranging from 0 to 70 % [9]. The existence of ILT was first reported in India in 1964 [29], and recently, based on clinical and pathological observations presence of ILT virus infection in commercial layer flocks has been reported [30]. However, the existence of ILTV in Indian poultry flocks was not confirmed by molecular methods such as PCR and sequencing, which are highly essential to find out the molecular epidemiology of the disease and to trace the origin of virus. Therefore, the present work was aimed to investigate the involvement of ILTV in respiratory disease complex (RDC) in Indian poultry flocks using PCR-based detection and nucleotide sequencing methods. Poultry farms situated in different parts of the country, covering seven states, with the history of respiratory disease complex (RDC) were investigated.

Materials and methods

Case history and sample collection

Thirty two commercial poultry flocks with a history of respiratory disease complex (RDC) were investigated during the period between May 2010 and September 2011. The study covered seven Indian states viz. Uttar Pradesh, Uttarakhand, Haryana, Rajasthan, Andhra Pradesh, Karnataka and TamilNadu. The flocks consisted of multi-aged layers, breeders and broilers reared under cage and deep litter system, respectively. The flock size of the poultry farms ranged between 1,000 and 3.5 lakh (0.35 million) birds. The age of the flocks under investigation ranged between 4 and 72 weeks. The strains included Babcock, Lohmann, Bovans, Hy-Line, Cobb, Ross and Synthetic color broiler. All the birds were reared under standard managemental conditions recommended by the breeding companies. Necropsy examination was carried out on dead and ailing birds. Tissue samples such as trachea, lungs and conjunctiva were collected for direct tissue PCR. Pieces of trachea were preserved in 10 % buffered formal saline for histopathology. The formalin fixed tissue samples were processed and paraffin sections were stained with Haematoxylin and Eosin (H&E) staining for microscopic examination.

Polymerase chain reaction and sequencing

Tissue samples were cut into small pieces and 5 % homogenates were prepared in phosphate buffered saline (pH 7.2). DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA) as per manufacturer's instructions. The primer pair targeting the envelope glycoprotein-G gene (US4 gene) of GaHV-1 as described by [25] viz. ILTp32 U2-CTA CGT GCT GGG CTC TAAT CC and ILTp32 L2-AAA CTC TCG GGT GGC TAC TGC were used in the PCR. The reaction was carried out using HotStar Taq® Master Mix (Qiagen, Germany), with an initial denaturation at 95 ° C for 15 min, followed by 35 cycles at 95 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. Two micro litres of the PCR product were analyzed by electrophoresis in 1.5 % agarose gel. PCR products were purified employing ExoSAP-IT® (Affymetrix, USA) and sequenced through commercial source (Scigenom, Cochin).

Phylogenetic analysis

The nucleotide sequences were trimmed using Sequencing Analyses Software v5.3 (Applied Biosystems, USA) and aligned in MEGA 5 [31] with reference sequences which were downloaded from GenBank. Phylogenetic analyses and evolutionary associations were inferred in MEGA 5.0 [31] using the Maximum Likelihood algorithm with Kimura-2P correction and 1,000 bootstrap replications.

Results

Epidemiological and clinical findings

All the flocks had frequent movement of vehicles and service personal. The mortality rate ranged between 1.0 and 6.0 %. The disease outbreaks were reported around the year and course of disease extended up to 45 days. The egg production dropped up to 10 %, birds recovered to their production after 2 weeks, but the peak production was not attained. The epidemiological details of the ILT positive flocks are presented in Table 1. The affected birds showed varied clinical signs including dullness, reduced feed and water intake, purulent conjunctivitis watery to tenacious oculo-nasal discharge and decrease in egg production. Depending on the severity of the disease, the degree of respiratory signs ranged from mild respiratory distress to difficulty in breathing, gasping, coughing up blood or bloody mucus and pump handle-type of respiration.

The lesions were confined only to the respiratory system. Grossly caseous plugs in the larynx, catarrhal or haemorrhagic or fibrinous exudates or combinations of these were noticed in the tracheal lumen. Catarrhal tracheitis, crumbly casts in trachea, pulmonary congestion and oedema, and highly congested thecal blood vessels in the ovaries were observed in majority of the affected birds.

Table 1 Epidemiological findings of flocks positive for ILTV

S. no	State	Farm size	Bird type	Age (weeks)	Season	ILT vaccination history	Mortality (%)	No. of samples collected	No. of samples positive by PCR
1.	Tamil Nadu	1,50,000	Layer	16	Winter	Not vaccinated	2.4	22	19
2.	Tamil Nadu	70,000	Layer	35	Winter	Not vaccinated	4.0	14	14
3.	Tamil Nadu	25,000	Layer	70	Summer	Not vaccinated	0.7	08	07
4.	Tamil Nadu	1,00,000	Layer	12	Rainey	Not vaccinated	5.2	28	23
5.	Tamil Nadu	1,20,000	Layer	6	Summer	Not vaccinated	3.0	16	15
6.	Haryana	2,500	Broiler	3	Winter	Not vaccinated	6.2	06	06
7.	Haryana	4,000	Broiler	2	Spring	Not vaccinated	6.0	11	08
8.	Haryana	700	Broiler	4	Spring	Not vaccinated	5.4	03	03
9.	Rajasthan	1,200	Broiler	4	Summer	Not vaccinated	4.8	04	04
10.	Utter Pradesh	9,000	Breeder	70	Rainey	Not vaccinated	3.4	12	11

Tissue collected: Trachea, conjunctiva and lungs

Histopathologically, trachea revealed diffuse haemorrhagic inflammation leading to accumulation of fibrino-haemorrhagic and necrotic debris which resulting in obliteration of tracheal lumen. Bronchial lumen was filled with casts of sloughed off epithelial cells. Deciliation of epithelium covering the bronchi associated lymphoid tissue and severe vascular engorgements were also observed.

PCR detection of ILTV and phylogenetic analyses

ILTV was detected in 10 out of 32 flocks by PCR assay. The positive flocks were belonging to Tamil Nadu, Haryana, Rajasthan and Utter Pradesh states. Amplification of partial glycoprotein-G gene (US4 gene) from the tissue samples yielded expected product size of 589 bp (Fig. 1). On sequencing eight samples yielded clear reads and two samples yielded poor reads. Therefore, only eight sequences were used for further phylogenetic analysis. All the eight Indian ILTV sequences showed 100 % nucleotide identity. The percent identity matrix calculated for Indian ILT viruses shared 99.8 % homology with chicken embryo origin (CEO) vaccine strains of Italy, USA and China, and 98.4 % with Brazilian strain. The phylogenetic tree was constructed using 8 sequences of partial glycoprotein-G gene with 26 reference sequences from various countries. Indian ILTV sequences were clustered with CEO vaccine strains of Italy, USA, China and Brazil (Fig. 2).

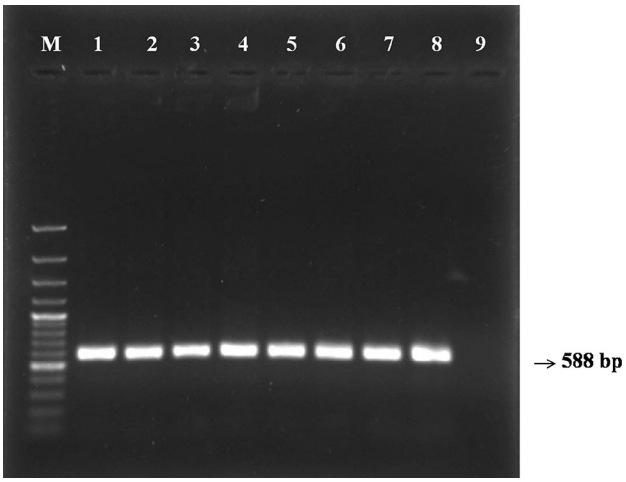

Fig. 1 ILT virus-specific PCR amplicons visualized by agarose gel electrophoresis. *Lane M* molecular weight marker, *1–8* field samples showing the ILT virus-specific 589 base pairs product, *Lane-9* negative control

Discussion

At this point, very few reports are available on the existence of the ILTV in Indian poultry population, and this is the first description on the molecular detection and characterization of Indian ILT virus (GaHV-1). It was reported that males are more susceptible than females and the heavier breeds more susceptible than light ones [16]. In our study, the virus was detected in all stains/breeds suggesting that all breeds are equally susceptible to ILTV infection. Further, both young and adult birds exhibited severe respiratory disease. This controverts the general assumption that the most characteristic signs are observed in adult birds [12]. The strain circulating in Indian poultry might have virulence for all ages. Recent work has confirmed considerable variation among ILTV strains in their tropism, capacity to induce mortality, clinical signs and lesions in different tissues [18]. In the current investigation, all the ILT affected farms had the past history of similar clinical disease in the same or neighboring farms indicating that virus persistence in farm environments leading to spread of disease. ILTV can survive away from the host for several weeks under farm conditions, especially in the presence of organic material such as faces [16].

PCR detection of ILTV is correlates with the characteristic clinical signs and pathology of ILT observed in birds. Thus, the PCR assay would be a useful tool to confirm the ILTV. PCR

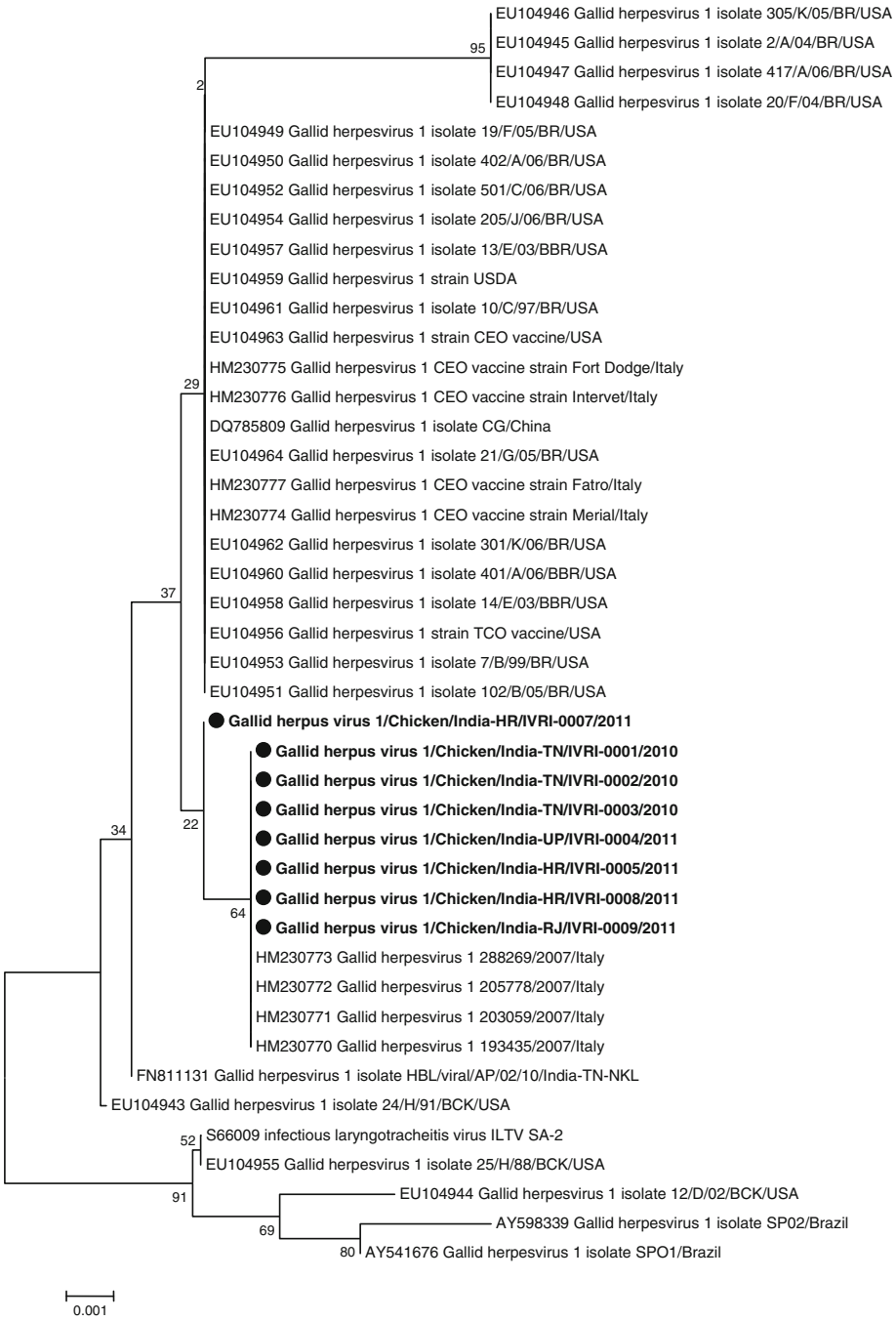

Fig. 2 Phylogenetic tree constructed based on the partial sequences of envelope glycoprotein-G gene (US4 gene) of ILT virus

has been found to be more sensitive than virus isolation from clinical samples [33], especially when other contaminant viruses such as adenoviruses are present [1]. All the eight Indian ILTV sequences were closely related with CEO vaccine strains of Italy, USA, Brazil and China. Evidence is mounting that most field outbreaks are caused by viruses indistinguishable from chicken-embryo-origin vaccine strains and, for that reason, broiler outbreaks are often referred to in the field as "vaccinal laryngotracheitis" (VLT) [7]. A number of previous studies demonstrated that many ILTV strains circulating in the fields were genetically related to the vaccine strains [3, 21]. In USA, [23, 24] investigated ILTV isolates from commercial poultry that were collected between 1988 and 2007 and observed that most of the ILT outbreaks have caused milder clinical signs and have been associated with the vaccine-like strain virus. In Europe, 104 field isolates were collected during 35 years from eight different countries and it was shown that 98 of these field isolates had the same RFLP patterns as vaccine strains [21]. In Australia, some field isolates were closely related to vaccine strains [3].

After its first episode during 1970s the incidence of ILTV was not reported in India. The probable origin of virus could be

from legal or illegal trade of CEO vaccinated birds, fighting cocks from neighbor countries and/or lack of surveillance of ILTV in India. In 2004, ILT live vaccines are imported in Bangladesh for GP farms and the birds of these farms are applied with the vaccine following the vaccination regimen of the manufacturer [15] and ILTV cases were confirmed in 2010 [14]. Porous Indo-Bangladesh border stretches across 4095 kms, still remains critical factor in introduction of diseases like HPAI between India and Bangladesh through Legal and illegal trade of live birds/migration. Our findings suggest that ILTV is one of the re- emerging diseases in poultry flocks of the country and nation-wide epidemiological studies are needed to know the magnitude of this virus along with isolation and characterization of field viruses. These studies will clearly help defining the need, if any, for the inclusion of ILT vaccination programmes in India.

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