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Interaction of embryonic chicken lung cell with different strains of infectious laryngotracheitis virus infections

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ABSTRACT

The economic losses of infectious laryngotracheitis virus (ILTV) are prevented using attenuated live vaccines. Differential diagnosis of ILTV strains is still a critical problem in controlling programs. In this study, the embryonated chicken liver cell (ECL) serves as a host model to study virulence characteristics of ILTV strains. The permissivity of ECL cells to ILTV infection was investigated by assessing susceptibility of the cells to vaccine strain and virulent strain infections, analyzing the impact of viral infection on cell viability, and determining the host cellular factor X (FX) and cyclophilin A (CypA) at three passages. To evaluate the lytic replication dynamics of ILTV in infected cells the collected suspension of last passage of each strain was inoculated onto the dropped chorio-allantoic membrane of specific pathogen free eggs then checked for observing characteristic lesions. The results indicated that ECL cells are highly susceptible to attenuated vaccine strain ILTV infection. Upon infection, the strain showed faster replication kinetics in cell culture and marked cytopathic effects. Virulent strain was able to enter ECL cells but no infectious virus was produced at 3rd passage. The establishment of latency state was not confirmed by reactivation assay. In contrast to vaccine strain, cellular FX was also traced following virulent strain infection. The difference expression pattern of FX in ILTV strains-infected cells is most closely with the presence of cytopathic effects in culture. The embryonated chicken lung cell system may potentiate the relevant tool for differential diagnosis of ILTV strains.

Key words: Infectious laryngotracheitis virus, embryonic chicken lung cell, cyclophilin A, factor X

1. INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is an economically significant avian pathogen prevented by attenuated live vaccines. ILTV belongs to the genus Iltovirus of Herpesviridae has a linear double stranded DNA genome consist of a unique long region and a unique short region which flanked by two inverted repeats (1). Entry of the virus into respiratory cells and mucosa depends upon host cell surface receptors and viral surface glycoproteins. The viral nucleocapsid is released into the cytoplasm and migrated into the nucleus following fusion of the viral envelope to the cell membrane (2, 3). ILTV genome replication and transcription seems to be similar to other alpha-herpesvirus. Three classes of genes include immediate early, early, and late genes are expressed during transcription of ILTV DNA. The immediate-early gene, infected cell polypeptide 4 (ICP4), has regulatory functions and involve in the cascade transactivation of other genes (4, 5). After assembly of viral particles, the enveloped virions are released by cell lysis. ILTV strains are propagated efficiently in embryonated chicken egg and primary cell culture derived from the embryo tissues (1, 6). The viruses can further diagnose using serological test including fluorescent antibody technique, indirect immunofluorescence, serum neutralization, and agar gel immunodiffusion which are less sensitive and laborious. Molecular techniques are preferred for rapid for detection and quantitation of ILTV DNA in clinical samples especially when the virus established lifelong latency state in trigeminal ganglia (7-10). Reversion of virulence after reactivation of the latent virus and evident of possible

outbreak are the major problems following vaccination with the live vaccines (11, 12). Due to the high antigenic and genetic similarity among ILTVs, characterization of the circulated virus from vaccine strain is complicated (13). Previously PCR/RFLP analysis based on a small region of the viral genome and/or a combination of genes has been used to genetically characterize ILTV isolates, but in many cases the field isolates' restriction profiles were not distinguish from those of vaccine strains (7-9, 14-16). To date, a variety of avian and mammalian cells have been investigated for their potential application in differential diagnosis of ILTV strains. None of the cell line can serve as a suitable substrate for this purpose. Zhao et al (17) have been shown that the levels of ILTV DNA in larynx and lung were relatively higher than those in other tissues in both natural and experimental infections. Moreover, microarray analysis of lung cell responses against virulent and vaccine ILTV infections revealed that bone morphogenetic protein 2 (BMP2), chromosome 8 open reading frame 79 (C8orf79), coagulation factor X (FX), and neuropeptide Y (NPY) were expressed in the distinct direction (18). Considering this study, FX and cyclophylin A (CypA), as a response protein to virus stimulation might be useful elements for differential diagnosis of ILTV strains. Here, we designed a sensitive assay for targeting the pathogenicity of ILTVs. In this regard we candidate embryonic chicken lung (ECL) as natural host in a cell culture system. By using the technique, levels of the cellular factors were quantified in the ILTV strains-infected cells at various passages. The potential of the ECL cell line for the propagation and diagnosis the virus strains was also evaluated.

2. MATERIALS AND METHODS

2.1. Virus and cell line

The virulence and modified vaccine strains of ILTV at a multiplicity of infection (MOI) of 0.01 were used. The ECL cells were maintained in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and antibiotics (Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2. Monolayers of the cells at a concentration of 1×10^6 cells/ml were infected with each of the ILTV strain. For each strain three different sets of culture flask were infected. Up to 96 hours post-infection (hpi) and at 24-h intervals, cultures observed daily by inverted light microscope (Nikon Eclips TS100) for cytopathic effect (CPE). In each experiment, cells for the purpose of mock infection were considered. Cell viability following viral infection was determined by Trypan blue 0.4% (Sigma-Aldrich). Cell survival was expressed as the ratio of virus infected to uninfected control. The supernatants were collected and stored at −70°C and then thawed for three subsequent passages.

2.2. DNA fragmentation assay

Induction of apoptosis in ECL cells following infection with ILTV was applied if the cell viability decreased. DNA fragmentation was monitored by extraction of DNA (GeneAll, Korea) from virus-infected cells and visualization through a 2% agarose gel using Bio Dac-IT™ Imaging System.

2.3. Molecular analysis virus-host interaction

The replication of virus was screened by PCR assays using specific primer pairs (ICP4F: 5′-TTTGAGGGAGTGGGTCGAAA-3′ and ICP4R: 5′-CCCGTACGGTGACACAGATA-3′). The primers were designed based on a highly conserved region of the ICP4 gene to amplify a 1500 bp fragment. The PCR reaction mixture contained 2 µl of Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark), 1 µl of each primer, and 1 µl of DNA template was incubated in thermocycler (Mastercycler epgradient eppendorf) at 94°C for 5 min, then subjected to 30 cycles of 94°C for 30 s, 57°C for 45 s and 72°C for 60 s, and finally incubated at 72°C for 10 min. Amplification of cellular FX and CypA from mock and infected cells were analyzed by PrimeScript™ One Step Ver.2 RT-PCR (Takara, Japan) at various times after infection. The assay was carried out using FXF: 5′-GATGAGTGTCGTCCTGGTGA-3′, FXR: 5′-AGCCACGCCACTACTACTTT-3′ and CypAF: 5'-GAGCTCTTCGCTGACAAGGT-3' and CypAR:5'-GGATGAAGTTCTCGTCGGCA-3' specific primers for 30 cycles with the thermal profile stage 1: 50°C for 30 min; stage 2: 95°C for 15 min; stage 3: 94°C for 15 sec, 55°C for 30 sec; and 72°C for 30 sec.

2.4. Lytic replication determination

To evaluate the dynamics replication of ILTV in infected lung cells the collected suspension of each passage was inoculated onto the dropped chorio-allantoic membrane (CAM) of 10-day-old specific pathogen free eggs (Venkey, India). Eggs were incubated at 37°C for five days then checked for observing characteristic lesions on the CAM.

2.5. Statistical analysis

Statistical correlation of data expressed as mean ± SD was checked for significance (P<0.05) by one-way ANOVA and Student's t test.

3. RESULTS AND DISCUSSION

The ILTV vaccine strain was efficiently grown in ECL cell and produced multi nucleated cells or *syncytia* as a marked CPE of ILTV compared to uninfected mock (Figure 1). The infected cells were gradually rounded at 24 hpi, fused with adjacent cells to form syncytia, and detached at 72 hpi.

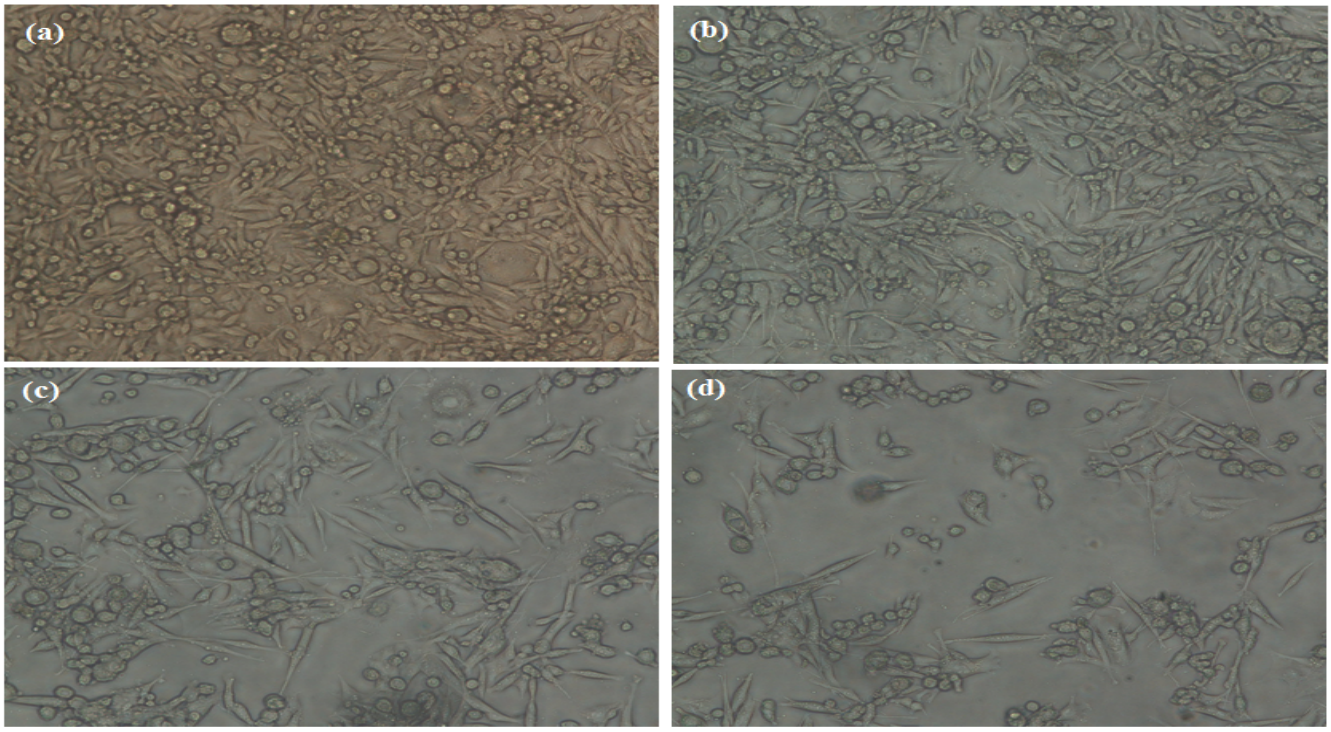

Figure 1. Cytopathogenecity of embryonated chicken lung cells to attenuated vaccine infectious laryngotracheitis virus infection at 24, 48 and 72 hours post infection; a) Mock, b) 24 hpi, c) 48 hpi, d) 72 hpi; (100x magnification)

A significant (P<0.05) decrease in cell viability percentage from 78.2 ±2.10 to 54.3 ±1.62 was detected at the end of the period trial. The impact of programmed cell death on cell viability was examined by cellular DNA laddering assay. Neither smeared nor fragmented DNA was detected in infected ECL cells indicated that the strain did not trigger apoptosis early in infection. Unlike the vaccine ILTV strain, virulent strain is not capable of producing CPE until the end of incubation period (Figure 2), while entry of the virus and initiation infection procedure were confirmed by detection of ICP4 DNA in PCR assay. Thus, viral replication has taken place in all collected samples at three passages.

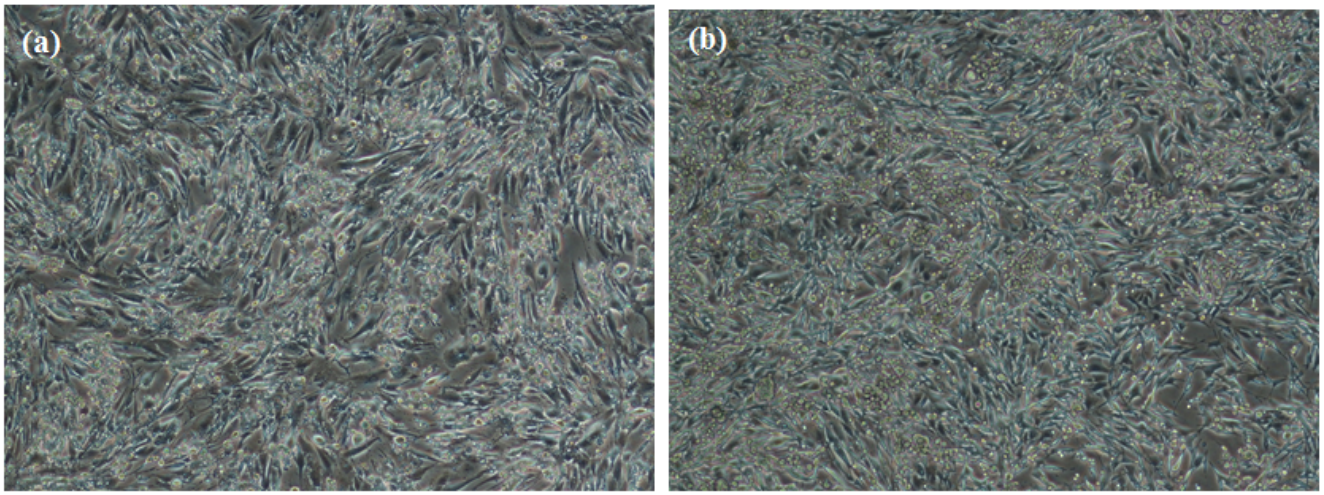

Figure 2. Cytopathogenecity of embryonated chicken lung cells to virulent infectious laryngotracheitis virus infection at 24, and 72 hours post infection; a) Mock, b) 72 hpi (40x magnification)

To ensure replication of virulent strain in ECL cells, the culture suspensions of each passage was injected onto the CAM of embryonated eggs. Various white pocks were observed five days post inoculation indicating the virulent strain produced progeny while CPE is not appeared in infected cells. To understand the impact of viral pathogenicity on the integrity of the lung cell during ILTV infection, the virus-host interaction was evaluated by targeting the cellular CypA and FX. CypA was detected in all ILTV strains-infected cell samples even when the ICP4 was amplified. In contrast different pattern of FX was detected in response to ILTV vaccine and virulent strains infections. FX DNA was amplified from ECL infected-vaccine strain, whereas no products were found in the cell infected-virulent strain at 2nd and 3rd passages (Figure 3).

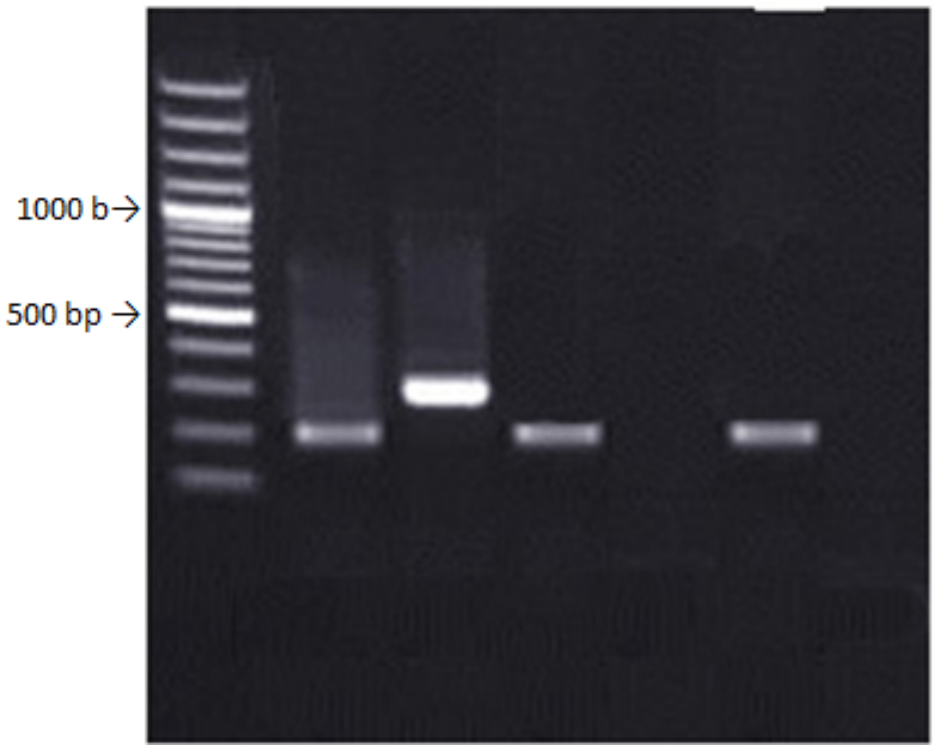

Figure 3. Detection of embryonated chicken lung cells CpyA and FX in response to infectious laryngotracheitis virus vaccine and virulent strains

ILTV is primarily an upper respiratory and ocular pathogen. Tracheal, oropharyngeal or conjunctival swabs, exudate and epithelial cells scraped from the trachea are used for virus isolation. Viral suspension is inoculated on to the dropped CAM of embryonated chicken eggs or cultivated on chick embryo liver or kidney cell monolayers (19). The growth of ILTV on some available cell lines originated from different animal tissues has been evaluated due to the easier maintenance condition in diagnostic laboratory (6) but the impact of cell type on virus infectivity was not consider. ILTV may induce apoptosis and latency in a variety of primary cultured cells or cell lines as futures of herpesviruses infections. Herpesviruses encode US5, ICP27, and LAT anti-apoptotic genes to prevent apoptosis by evolving various strategies (20-22). Depend on cells for their replication, the viruses can affect various signaling pathways to block the caspase-8-dependent apoptosis pathway (23), however, the exact mechanism of action is still poorly understood. Our data revealed that the sensitivity of ECL cells to ILTV strains infection did not induced cell death signaling. It seems that the ILTV strains block apoptosis in infected ECL cells. ICP4 represents the major regulatory protein and synthesis of the immediate early gene is obviously plays a key role in efficient transcription of early and late viral genes during productive infection (4). Here, we have detected the ICP4 DNA of the virulent ILTV strain-infected cells *without* showing obvious cytopathology. The frequencies of detection in the attenuated and virulent strains-infected cell specimens were not different. Clearly, establishment the lytic infection as the cause of virus replication interacts with cell cycle regulation pathways. Despite small DNA tumor viruses, herpes viruses encode a viral DNA polymerase and accessory factors which are not promote entry into S phase of the cell cycle (24). They induce cell cycle arrest at the G1/S transition in dividing cell cultures by involving multiple viral factors. Such alter cellular environment is related to the expression of ICP0 which elicit cell cycle arrest signaling in more than one checkpoint pathway (25, 26). The immediate early gene expression is responsive to G0/G1 signaling events and expressed prior to the G0 or G1 checkpoint. It seems that the virulent ILTV strain induces cell cycle arrest in chicken lung cells to support own replication. The interaction with the cell cycle cyclin-cdk complexes is needed to be studied in future. The inhibitory effect of CypA on replication of avian influenza virus has been demonstrated. The cellular protein is interfered by translocation of newly synthesized viral M1 protein into nucleus at the early stage of infection (27). The anti-ILTV infection potential of CypA was assayed for three passages. These data suggested that CypA is not an interacting protein of ICP4 to promote and/or inhibit the replication of ILTV. Previously the host responses to infection of virulent and vaccine strains of ILTV were evaluated by chicken oligo microarrays (18). Of the 273 genes; BMP2, C8orf79, FX, and NPY showed a different regulation pattern at 1 to 3 days post infection with the ILTV strains. Over expression of the coagulation factor has been detected during the early phase of severe acute respiratory syndrome infection and during influenza virus infection lately. However, the functional role of FX in ILTV infection is unknown, the distinct detection profiles during ILTV infections may related to hemostasis and coagulation roles in maintaining cellular morphology.

4. CONCLUSION

We described a more tractable biologic system based on

cultivation of attenuated and virulent ILTV strains in embryonated chicken lung cell cultures that can be easily applied for viral pathogenicity screening. The cell is susceptible to lytic infection of both virus strains; however, in contrast to virulent strain the vaccine virus showed mark CPEs which extended to 96 hpi. We also showed that different viral-cellular FX interactions related to the ILTV strains infections. More examinations of various ILTV strains isolated from different geographic regions will help us to validate this simple and practical model.

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AUTHORS CONTRIBUTION

This work was carried out in collaboration among all authors.

CONFLICT OF INTEREST

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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