

# Molecular Classification of Infectious Laryngotracheitis Virus from Chick Embryo Origin Vaccine, Tissue Culture Origin Vaccine and Field Isolates

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## *Abstract*

The molecular classification of the infectious laryngotracheitis virus from chicken embryo origin (CEO) vaccine, tissue culture origin (TCO) vaccine and field isolates by the sequence of ICP4 genes from CEO and TCO vaccines in the GenBank was performed for the different sequence analysis. The sequences of TCO vaccine virus (accession no. EU104908) were used as a prototype for a primer design to cover the region of 450 basepairs (bp) by Primer3 software. The simulation sequences of CEO vaccine virus (accession no. EU104900) and TCO vaccine virus (accession no. EU104908) from PCR reaction were selected for restriction enzyme. *Hga* I could separate 3 isolates of the viruses from simulation model and in vitro model. The PCR products from CEO, TCO and field isolates were 428, 440 and 450 bp, respectively. The PCR products were cut by restriction enzyme, *Hga* I, and the results revealed the DNA of CEO vaccine virus equal to 414 and 14 bp and TCO vaccine virus equal to 250 and 176 bp. The field virus has no restriction sequences according to *Hga* I, so no RFLP production occurred and the PCR product remained at 450 bp.

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**Keywords:** CEO vaccine, *Hga* I, ILTV, RFLP, TCO vaccine

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## บทคัดย่อ

### การจำแนกทางโมเลกุลของไวรัสกล่องเสียงอักเสบติดต่อจากวัคซีนที่เตรียมจากคัพพะโก่ วัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง และไวรัสในพื้นที่

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การจำแนกไวรัสกล่องเสียงอักเสบติดต่อจากวัคซีนที่เตรียมจากคัพพะโก่ วัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง และไวรัสในพื้นที่ จากลำดับเบสของยีน ICP4 ของวัคซีนที่เตรียมจากคัพพะโก่ และวัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง จาก GenBank แล้วนำมาวิเคราะห์ลำดับเบสที่แตกต่างกันของวัคซีนทั้งสอง จากนั้นใช้ลำดับเบสของไวรัสจากวัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง (Accession no. EU104908) เป็นต้นแบบในการออกแบบไพรเมอร์ที่ครอบคลุมลำดับเบสขนาด 450 bp โดยใช้ Primer3 software แล้วจำลองลำดับเบสของวัคซีนที่เตรียมจากคัพพะโก่ (Accession no. EU104900) และวัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง (Accession no. EU104908) ที่ได้จากปฏิกิริยาลูกโซ่โพลีเมอร์เรส เพื่อใช้ในการคัดเลือกเอนไซม์ตัดจำเพาะ พบว่าเอนไซม์ตัดจำเพาะ *Hga* I สามารถแยกความแตกต่างของไวรัสทั้ง 3 ชนิดได้ ทั้งจากแบบจำลองและการตัดจริง โดยได้ผลิตภัณฑ์พีซีอาร์ของไวรัสจากวัคซีนที่เตรียมจากคัพพะโก่ ไวรัสจากวัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง และไวรัสจากพื้นที่ คือ 428, 440 และ 450 คู่เบส ตามลำดับ จากนั้นนำผลิตภัณฑ์พีซีอาร์ที่ได้มาตัดด้วยเอนไซม์ตัดจำเพาะ *Hga* I พบว่าไวรัสจากวัคซีนที่เตรียมจากคัพพะโก่ได้ DNA ขนาด 414 และ 14 คู่เบส ไวรัสจากวัคซีนที่เตรียมจากเซลล์เพาะเลี้ยงได้ DNA ขนาด 250 และ 176 คู่เบส แต่ไวรัสจากฟาร์มไม่มีลำดับเบสจดจำของเอนไซม์ *Hga* I ดังนั้นจึงไม่เกิดปฏิกิริยา RFLP และให้ผลผลิตขนาด 450 คู่เบส เท่าเดิม

**คำสำคัญ:** วัคซีนที่เตรียมจากคัพพะโก่ *Hga* I ไวรัสกล่องเสียงอักเสบติดต่อ RFLP วัคซีนที่เตรียมจากเซลล์เพาะเลี้ยง

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## Introduction

Infectious laryngotracheitis (ILT) is an important infectious disease in chickens. Infected chickens show severe clinical signs including respiratory distress, dyspnea, gasping, coughing, excretion of bloody mucus, high mortality and production loss. Chickens that are infected with ILT virus (ILTV) show clinical signs similar to other respiratory disease infection (Albicker-Rippinger and Hoop, 1998). ILTV is classified as a member of Gallid herpesvirus 1 (GaHV-1), subfamily Alpha herpesvirinae, family Herpesviridae (Roizman, 1982). Normally, vaccines originating from chicken embryo origin (CEO) or tissue culture origin (TCO) have been used for disease prevention. Many techniques have been used for ILTV identification such as polymerase chain reaction (PCR) (Abbas and Andreasen, 1996; Abbas et al., 1996), virus isolation and identification (Alexander and Nagy, 1997; Bagust and Guy, 1997) and pathology (Bagust and Guy, 1997). A molecular identification of ILTV that is more sensitive than cellular identification has been developed (Keam et al., 1991; Key et al., 1994; Williams et al., 1994; Alexander and Nagy, 1997). Alexander and Nagy (1997) revealed that PCR could detect ILTV infection in the middle and late stage of infection similar to virus isolation. Moreover, PCR was sensitive to ILTV

detection also in the first stage of infection. However, the main problem of PCR is that this technique cannot reveal the differences of vaccine and field strains of virus (OIE Terrestrial Manual, 2008). Restriction fragment length polymorphism (RFLP) has been used to separate the vaccine and field strains. Genes that are frequently used include ICP4, TK, glycoprotein G (gG), glycoprotein E (gE) and UL47 (Chang et al., 1997; Graham et al., 2000; Han and Kim, 2001; Sellers et al., 2004; Creelan et al., 2006; Kirkpatrick et al., 2006). The DNA genome of ILTV consists of a linear 155-kb, double-stranded molecule consisting of unique long and short segments flanked by inverted repeats (Lieb et al., 1987; Johnson et al., 1991). Five genes of ILTV have been studied using PCR and RFLP revealing that ICP4 was the best to separate the molecular pattern between vaccine and field strains. It may cause a wide variation of ICP4 gene sequence (Chang et al., 1997). ICP4 gene consists of 4,386 nucleotides (Michael et al., 1995). The aim of this study is to separate the ILTV between the CEO, TCO and field isolates by PCR-RFLP technique.

## Materials and Methods

**Primer designs:** The sequences of the ICP4 gene of ILTV from CEO and TCO vaccine strains were analyzed. Primers were designed using Primer3

software (Rozen and Skaletsky, 2000). The forward primer was ILTF 5' CCT CGA CGC CGA GTA ATT T 3', 19 bp, T<sub>m</sub> = 58.0, %GC = 52.63 and the reverse primer was ILTR 5' GAG CGA GTC GAT GAC CGT AT 3', 20 bp, T<sub>m</sub> = 62.0, %GC = 55.00 (Sigma-Proligo, Proligo Singapore Pty Ltd, Singapore). They were checked for specificity and accuracy using the BLASTN 2.2.19 program in National Center for Biotechnology Information (NCBI).

**Restriction enzyme selection:** The sequences of the CEO strain (accession no. EU104900) and the TCO strain (accession no. EU104908) were analyzed. The restriction enzymes (*Hga* I) (<http://www.thelabrat.com/restriction/HgaI.shtml>) were selected to separate the difference between CEO and TCO vaccine strain through <http://tools.neb.com/REBsites/index.php>.

**DNA extraction and PCR products:** DNA was extracted from the CEO vaccine (Fowl laryngotracheitis vaccine, Broilertrake® M, Schering-Plough Animal Health, USA) and TCO vaccine (Fowl laryngotracheitis vaccine, LT-IVAX®, Schering-Plough Animal Health, USA) and field isolates using DNA Trap (Biotech, Thailand). Amplification reactions were carried out. The reaction mixture composed of 1xPCR buffer, 2.0 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1.0 μM Primer F, 1.0 μM Primer R and 1.25U recombinant Taq polymerase (Fermentas life science, USA) and 3 μl of DNA templates of CEO, TCO and field isolates were added to each vial. The PCR reactions were performed in 3 replications. Firstly, the PCR was conducted at 95°C for 3 min 1 round. Secondly it went through 30 cycles of denaturation at 94°C for 30 sec. Thirdly, it was annealed at 56°C for 45 sec. Forthly, it was polymerized at 72°C, 60 sec. Finally, it went through an elongation step of 7 min at 72°C. The amplification products were analyzed in 1.0% agarose gel in 0.5xTBE buffer and ethidium bromide and visualized under Gel Documents (UVI Tec, Cambridge, UK). The amplified products were measured using UVI gelstart MW (UVI Tec, Cambridge, UK). The PCR products were stored at -20°C until further analysis.

**RFLP analysis of vaccine and field strain of ILTV:** The PCR products of the CEO, TCO and field isolate were analyzed by RFLP reaction with selected restriction enzymes. The reactions were performed with 1xNE buffer and 2.0 mM restriction enzyme and 3 μl of the PCR products of CEO, TCO and field isolates. The mixtures were incubated at 37°C for 120 min and the reaction was stopped at 65°C for 20 min. The RFLP products were analyzed in electrophoresis with 1.0% agarose gel in 0.5xTBE buffer, 100 volts of electricity for 40 min and visualized with ethidium bromide in Gel documentation (UVI Tec, Cambridge, UK).

## Results

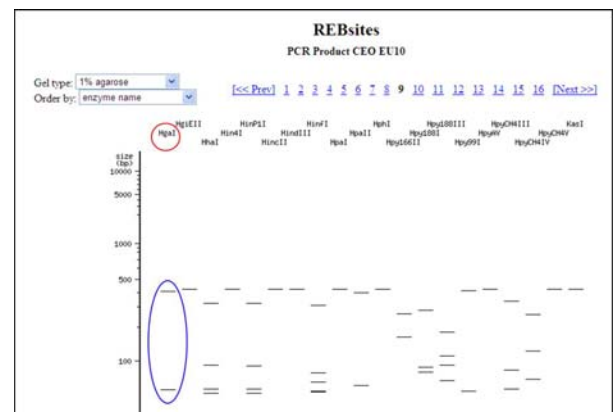
**PCR amplification:** According to the sequences of CEO and TCO vaccine strains from the GenBank, ICP4 genes of both vaccine strains had differences at

the position of 272-283. The nucleotides of the TCO vaccine strain at the position of 272-283 are CGG CCC AAG ACG but those nucleotides were missing in the CEO vaccine strain (Figure 1). This nucleotide position was used to differentiate between the 2 types of vaccine strains. Primers were designed to cover these different nucleotides. TCO vaccine nucleotides (Accession no. EU104908) were used to design the primers using Primer3 software. The primers were amplified the nucleotides of 440 bp at the position of 14-453 (Figure 1). BLASTN 2.2.19 in NCBI confirmed that the primers have the specificity and accuracy to ILTV.

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TCO EU104908: 1 ATGTCCTCTG ATTCTCTGAC GCCGAGTAAT TTGGCCGCTC CAATTGTTGA 50
CEO EU104900: 1 ATGTCCTCTG ATTCTCTGAC GCCGAGTAAT TTGGCCGCTC CAATTGTTGA 50

TCO: 51 TGAGTCGGGA TTCTCAACTG GCCCGCCCTC AGATCTATTT ACCTTTTTCG 100
CEO: 51 TGAGTCGGGA TTCTCAACTG GCCCGCCCTC AGATCTATTT ACCTTTTTCG 100
TCO: 101 GCGCCGATAA GAGTGGGCGC TACGATTCTT TGGGTTCTCC CATCCCGAAC 150
CEO: 101 GCGCCGATAA GAGTGGGCGC TACGATTCTT TGGGTTCTCC CATCCCGAAC 150
TCO: 151 CCTCCAAACG CACCTACCTG TGAGACCACT ACTGATAGCT TTTCGTACAG 200
CEO: 151 CCTCCAAACG CACCTACCTG TGAGACCACT ACTGATAGCT TTTCGTACAG 200
TCO: 201 CACGCCACCT CTTTITTTCA GCGAGGACGA GCTCTTTTCA TCTGTACATG 250
CEO: 201 CACGCCACCT CTTTITTTCA GCGAGGACGA GCTCTTTTCA TCTGTACATG 250
TCO: 251 CTGAGAATGC GGCCCAAGAC GCGGCCCAAG ACGTGGACGG GCGAGAAATG 300
CEO: 251 CTGAGAATGC GGCCCAAGAC G----- TGGACGG GCGAGAAATG 300
TCO: 301 TCTCTCCGCG ACCTTATAGC GATGTTAAAC GACATGGATC CCGAAAAACAT 350
CEO: 301 TCTCTCCGCG ACCTTATAGC GATGTTAAAC GACATGGATC CCGAAAAACAT 350
TCO: 351 AAACCTCTATA GAACCGTGTC CGAACGCTAC CAGTGATGTC GTGGCACTAG 400
CEO: 351 AAACCTCTATA GAACCGTGTC CGAACGCTAC CAGTGATGTC GTGGCACTAG 400
TCO: 401 ATATTAACGT GGATACCGAT CAGCCCGGAG TTTATACGGT CATCGACTCG 450
CEO: 401 AT ATTAACGT GG ATACCGAT CAGCCCGGAG TTTATACAGTCA TCGACTCG 450
TCO: 451 CTCGCACATC 460
CEO: 451 CTCGCACATC 460
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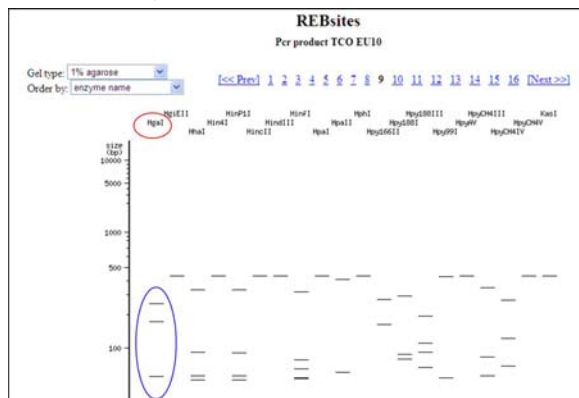
**Figure 1.** Comparison of TCO and CEO vaccine strains of ICP4 gene. The position of 272-283 is the different region of the ICP4 gene. Primers are the underlined nucleotides.



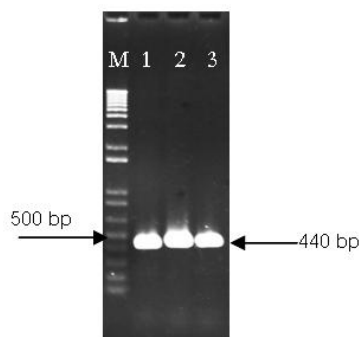
**Figure 2.** The restriction model of gel electrophoresis of the CEO vaccine strain analyzed in <http://tools.neb.com/REBsites/index.php>. The small circle indicates the *Hga* I restriction enzyme. The big circle indicates the expected RFLP fragments.

**Restriction enzyme selection:** The restriction enzyme selection to separate the CEO and TCO vaccine strains was simulated from the nucleotide sequences of CEO (Accession no. EU104900) and TCO (Accession no. EU104908) after the designed primers were used to PCR amplification. The primers provided the amplicons of 428 and 440 of CEO and TCO vaccine

strains, respectively. The amplicons were analyzed by <http://tools.neb.com/REBsites/index.php>. The results revealed that *Hga* I separated the CEO vaccine strain into 2 fragments of 414 (position 15-428) and 14 (position 1-14) bp (Figure 2) and separated the TCO vaccine strain into 3 fragments of 250 (position 15-264), 176 (position 265-440) and 14 (position 1-14) bp (Figure 3). The *Hga* I restriction enzyme was separated from *Avibacterium paragallinarum* (Takanami, 1974) and had the restriction position GACGCNNNNN/NNNNNN and CTGCGNNNN NNNNNN/N. The enzyme reaction performed at 37°C and stopped at 65°C for 20 min (<http://www.thelabrat.com>).



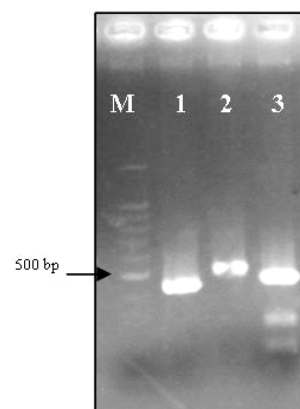
**Figure 3.** The restriction model of gel electrophoresis of the TCO vaccine strain analyzed in <http://tools.neb.com/REBsites/index.php>. The small circle indicates *Hga* I restriction enzyme. The big circle indicates the expected RFLP fragments.



**Figure 4.** PCR products of 428, 450, and 440 bp from CEO, field isolate, and TCO ILTV, respectively. Lane M: 1 kb plus DNA Ladder, Lane 1: CEO vaccine; Lane 2: field isolate; Lane 3: TCO vaccine.

**In Vitro testing of primer and restriction enzyme selection:** The designed primers amplified the DNA templates of ILTV from CEO, TCO and field isolates. The results revealed that the primers could amplify all ILTV isolates. The CEO, TCO and field isolates provided the amplicon of 428, 440 and 450 bp, respectively. The CEO vaccine strain provided amplicons which had 12 bp less than the TCO vaccine strain because of the missing nucleotides at the position of 272-283. The field virus showed the longest amplicon of 450 bp. When the PCR products of the CEO, TCO and field isolates were run and

visualized in electrophoresis, there was no clear difference between the amplicons of the 3 templates due to the minor difference in the molecular weight (Figure 4). The PCR products were further analyzed by RFLP using *Hga* I, restriction enzyme. The results revealed clearly different fragments among the 3 isolates. The CEO vaccine strain showed one DNA fragment of 414 bp. The TCO vaccine strain showed 3 DNA fragments of 440, 250 and 176 bp.



**Figure 5.** The RFLP pattern of CEO, field isolate, and TCO ILTV, respectively cut by *Hga* I endonuclease. Lane M: 100 bp DNA Ladder (Promega, USA), Lane 1: CEO 414 bp, Lane 2: field isolate 450 bp, Lane 3: TCO 440, 250, 176 bp.

## Discussion

Molecular techniques have been useful tools for diagnostic detection and differentiation of many pathogens. These techniques promote sensitivity and specificity compared to cellular techniques (Chansiripornchai et al., 2001). DNA sequencing is the gold standard of genetic identification. However, this technique is labouring and needs much equipment that is not generally available in general laboratories. RFLP is a molecular technique beneficial for rapid differentiation of specific DNA sequences. This method digests the target DNA which is separated by gel electrophoresis and visualized by staining. The separation and identification of poultry pathogens between vaccines and field isolates promote the epidemiological studies and the disease prevention. In this study, the ICP4 gene was utilized to differentiate between the CEO, TCO and field strains of ILTV. Comparing the RFLP reaction model (<http://tools.neb.com/REBsites/index.php>) and in vitro testing, one fragment of 14 bp was missing because a small fragment of 14 bp could not be seen in 1% agarose gel. Also, the fragment of 440 bp could be seen on the gel meaning an incomplete restriction of *Hga* I on TCO vaccine strain. The RFLP fragments of 250 and 176 from TCO and 414 bp from CEO vaccine strains were accorded to the RFLP model of the DNA template and *Hga* I restriction enzyme. Anyhow, the *Hga* I could not cut the PCR product of the field isolate where one fragment of 450 bp was seen in the gel electrophoresis because there is no restriction site

of *Hga* I in the PCR product of the field isolate (Figure 5). In conclusion, the designed primers and *Hga* I could be used to identify ILTV and differentiate among CEO, TCO and field isolate strains. Vaccination with live attenuated vaccines has been the principal tool used to control the spread of the disease (Guy and Bagust, 2003). Traditionally, 2 types of live attenuated vaccines have been widely used; the vaccines generated by multiple passages of embryonated eggs-CEO (Samberg et al., 1971) and the vaccine generated by multiple passages in tissue culture-TCO (Gelenczei and Marty, 1965). Generally, the CEO vaccine is applied by mass vaccination via drinking water or by spraying whereas the TCO vaccine is generally individually vaccination by eye-drop for breeders and layers (Hilbink et al., 1987; Fulton et al., 2000). In contrast to mass vaccination techniques, the individual vaccination provides the flock immunity necessary to decrease the number of susceptible birds within a vaccinated flock and consequently minimizes the potential for reversion to virulence to live attenuated vaccines (Guy et al., 1991). Vaccination programs vary widely; commercial layers, layer breeders and broiler breeders are usually vaccinated twice with CEO and/or TCO vaccines. Broilers are vaccinated only in cases of outbreaks with the CEO vaccines. The increased frequency of outbreaks in broilers has been associated with dense poultry populations, mixing of different types of poultry in the same geographical area, shorter down times and lax biosecurity. Previous studies confirmed that most ILTV outbreaks were shown to be closely related to CEO vaccines and only a few outbreaks were related to the TCO vaccine (Oldoni and Gracia, 2007; Oldoni et al., 2008). CEO vaccine isolates persist in long lived bird operations and spill-over broiler populations (Davison et al., 2005). Moreover, particularly the CEO vaccines can easily revert to virulence after bird-to-birds passage (Guy et al., 1991) or after reactivation from latency (Hughes et al., 1991). Once vaccine strains have been introduced in the field, the identification of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses (Guy and Bagust, 2003). RFLP analysis provides evidence that ILTV field isolates are closely related to the CEO vaccine strains. However, routine use of RFLP analysis of the viral genome is limited due to the difficulties in obtaining high yields of pure viral DNA. RFLP of PCR products greatly facilitate the differentiation of ILTV. In this experiment, PCR-RFLP could differentiate between the CEO, TCO and field virus isolates. A simulation model on computer bases can guide the practical work in vitro. Identification of vaccines and field strains helps select the suitable vaccine application in farms and can also be a useful tool for epidemiological linkage between the outbreak of strains of ILTV.

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