# POTENTIAL OF AN INFECTIOUS LARYNGOTRACHEITIS (ILT) RECOMBINANT VIRUS DEPLETED OF OPEN READING FRAME (ORF) C GENE AS A LIVE ATTENUATED VACCINE FOR *IN OVO* USAGE

By

GUSTAVO HENRIQUE SCHNEIDERS

(Under the Direction of Maricarmen García)

## ABSTRACT

Infectious laryngotracheitis (ILT) is an upper-respiratory disease of poultry of worldwide distribution. The disease is caused by infectious laryngotracheitis virus (ILTV), a member of the family *Herspesviridae*, is characterized by acute respiratory signs, and is common in areas of intense poultry production. Currently, the main method of control of the disease is by vaccination with live attenuated vaccines. *In ovo* vaccination is a highly effective mass of vaccination commonly utilized in the United States, and is characterized by reduced costs in labor associated with high protective index of the chickens. In an effort to enable *in ovo* vaccination with ILTV, attenuation of the virus by deletion of genes associated with virulence has been performed. Recently, a recombinant ILTV depleted of open reading frame C (ORF C) gene induced protection similar to that of the TCO vaccine when delivered via eye drop in three week old SPF chickens. The objectives of this study are to evaluate the attenuation and protection efficacy of a

recombinant ΔORF C ILT virus when delivered *in ovo* in the absence and presence of maternally derived antibodies; and to evaluate the protection efficacy of the recombinant ΔORF C virus when administered singly via *in ovo*, spray, or nasal-oral, and when administered *in ovo* followed by either spray or nasal-oral routes at eight-days of age in commercial layers. The results of this work indicate that the ΔORF C recombinant virus is capable of eliciting protection against ILT in chickens, however is still not sufficiently attenuated for *in ovo* vaccination; the protection efficacy of ΔORF C recombinant virus was affected by maternally derived antibodies; and priming by *in ovo* immunization with ΔORFC was essential to elicit a strong protective response to ILTV challenge.

INDEX WORDS: Infectious laryngotracheitis virus; ILTV; Open reading frame C; *In ovo* vaccination, Attenuation, Protection efficacy.

# POTENTIAL OF AN INFECTIOUS LARYNGOTRACHEITIS (ILT) RECOMBINANT VIRUS DEPLETED OF OPEN READING FRAME (ORF) C GENE AS A LIVE ATTENUATED VACCINE FOR IN OVO USAGE

By

GUSTAVO HENRIQUE SCHNEIDERS

DVM, Universidade Federal de Santa Maria, Brazil, 2013

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2016

© 2016

Gustavo Henrique Schneiders

All Rights Reserved

# POTENTIAL OF AN INFECTIOUS LARYNGOTRACHEITIS (ILT) RECOMBINANT VIRUS DEPLETED OF OPEN READING FRAME (ORF) C GENE AS A LIVE ATTENUATED VACCINE FOR IN OVO USAGE

By

GUSTAVO HENRIQUE SCHNEIDERS

Major Professor: Maricarmen García

Committee: Guillermo Zavala

Holly Sellers

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2016

# ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor and major professor, Dr. Maricarmen García not only for the research opportunity, continuous support and encouragement, but also for her contributions and guidance throughout this entire research project.

I also would like to express my sincere appreciation to each professor that served on my advisory committee, Dr. Holly Sellers and Dr. Guillermo Zavala for their expert input and all the time they have spent reviewing this work.

To my PDRC and UGA colleagues and co-workers; I would like to thank everyone who helped with the development and progression of this research. Their assistance, suggestions and comments have been invaluable.

Finally, many thanks to my family and friends near and far for their confidence in my ambitions and for their continuous support throughout this journey.

This experience has been both challenging and immensely rewarding both personally and professionally and to all who have contributed to its completion in so many ways, I am and will always be especially grateful.

TABLE OF CONTENTS

Page

# LIST OF TABLES

Page

# LIST OF FIGURES

Page

# CHAPTER 1

# INTRODUCTION

Infectious Laryngotracheitis virus (ILTV) is a highly contagious and economically significant avian alphaherpesvirus, genus *Iltovirus*, species *Gallid Herpesvirus 1* (GaHv-1) and causative agent of infectious laryngotracheitis (ILT), an upper respiratory tract infection that may result in severe production losses due to mortality, decreased egg production and weight-gain. The virus has a limited host range. Natural infections are restricted to galliform birds. It is most commonly described as an infection of chickens, although there are reports of natural infections in pheasants, peafowl and partridges (5, 10). Experimental (61) and natural (44) infections were reported in turkeys. Quail and guinea fowl, as well as non-galliform birds and mammals are refractory to infection, (6, 54).

In addition to biosecurity measures, the main method to control the disease is through mass vaccination with live-attenuated vaccines or recombinant viral vector vaccines. Live-attenuated vaccines originated from virulent field strains that circulated during the late 50's to early 60's in the United States and have been attenuated by sequential passage in embryonated eggs (chicken embryo origin [CEO]) (50) and cell culture (tissue culture origin [TCO]) (22). Live attenuated vaccines are preferred in many vaccination programs against ILT due to the rapid onset of immunity, conferring partial protection against challenge by three to four days post exposure, and complete protection

after one week (27, 30). High levels of protection occur between 15 and 20 weeks post-vaccination, with degree of protection varying over the year. Early studies have demonstrated that revaccination may improve the protection levels against ILTV (28), as the infectivity of the vaccine virus might be neutralized, preventing viral replication in the host (4, 14, 65). These two live-attenuated vaccines can stimulate a strong and lasting immune response, however, side effects such as the ability to transmit and infect non-vaccinated birds (49) and establishment of latency in the trigeminal ganglia (60) and trachea (3) of carrier birds have been attributed to these vaccines. It is speculated that the selections of vaccine subpopulations aided by poor mass vaccination, particularly with CEO vaccines, have contributed to the persistence of vaccine-derived strains in the field (2, 20, 21, 41). Subsequently, these vaccine strains have the potential to revert to higher levels of virulence through recombination events with other strains of ILTV (31), or as a result of consecutive passages in chickens, vaccine strains (23, 40) become source of ILT outbreaks (34). In the case of the CEO vaccine strain, 10 consecutive passages in SPF chickens caused severe respiratory disease and mortality, whereas 20 consecutive passages of TCO vaccine strain chickens caused mild respiratory reaction in SPF chickens (23). Worldwide, CEO vaccine-related strains have been frequently isolated from severe ILT outbreaks (9, 36, 38-40, 64), while outbreaks related to TCO immunizations are fairly rare (40, 55).

To avoid introducing live attenuated virus in the field, especially in areas where ILT challenge is not considerable, recombinant viral vector vaccines have been developed using the fowl poxvirus (FPV) expressing the ILTV glycoprotein B (gB) and a membrane associated protein ($U_L32$) (11); and the turkey Herpesvirus (HVT) vector

expressing the ILTV glycoproteins I (gI) and D (gD) (25). Both vaccines are commercially available in the United States. The hallmark of these recombinant vaccines is their safety, as they do not transmit from vaccinated to non-vaccinated chickens (1) and can be administered *in ovo* (29). It has been experimentally demonstrated that recombinant viral vector vaccines reduce clinical signs of ILTV but fail to prevent challenge virus shedding, whereas live attenuated vaccines are quite effective in reducing clinical signs of the disease and viral shedding (13, 29, 56).

In an effort to improve the control of ILT, deletion of non-essential genes related to virus virulence has been pursued in order to develop more stable, Live attenuated strains. Twenty individual genes have been successfully removed from ILTV genome resulting in recombinant viruses with a wide range of growth defects *in vitro* (15, 35, 42, 43). Among the twenty gene-deleted ILTV recombinants generated, only eight have been evaluated *in vivo* for their level of attenuation and their protection efficacy. The mutants contain individual gene deletions such as the $U_L0$ gene, encoding for a nuclear protein of unknown function (57); $U_L$ 23 gene, encoding for thymidine kinase (17, 24, 53); $U_L50$, encoding for dUTPase (17, 24, 53); $U_S4$ gene, encoding for glycoprotein G (12); $U_S5$ gene, encoding for glycoprotein J (16, 35); $U_L44$ gene, encoding for glycoprotein C (42); $U_L47$ gene, encoding for a tegument protein (26) and open reading frame C (ORF C), encoding for a protein of unknown function (19). Growth kinetic analysis has indicated that the deletion of genes encoding for UL0, thymidine kinase, dUTPase, glycoprotein G, glycoprotein J, glycoprotein C, tegument protein and open reading frame C were non-essential for virus replication in cell culture (15, 19, 35, 42, 43). Attenuation of the gene deleted

recombinants in chickens showed that the $U_L50$ (17) and $U_L44$ (42) gene-deleted recombinants preserved some degree of virulence, while ORF C (19), Us4 (gG) (12), $U_L47$ (tegument protein) (26), $U_L23$ (thymidine kinase) (24), $U_L0$ (nuclear protein) (57), and $U_S5$ (gJ) (16) gene deleted recombinants presented moderate to marked degrees of attenuation.

The ILTV genome contains a cluster of five ORFs (A-E) located at the 5'end of its genome, ranging from 334 to 411 residues, and unique to the genus *Iltovirus*. These ORFs have been successfully deleted from the ILTV genome and shown to be individually dispensable for viral replication in cell culture (58). However, recombinant viruses lacking three ORFs (A-C or ORFs C-E) exhibited significant growth defects *in vitro,* and attempts to generate an ILTV lacking all five unique ORFs was not successful. Although the precise functions of these ORFs remain unknown, it was concluded that ORFs A through E possess significant but redundant functions critical for virus replication (58). Among the ORFs that are not essential for propagation of the virus *in vitro* is the ORF C (58). The ORF C gene encodes a 37.4kDa protein mainly localized in the cytoplasm with a small fraction localized to the nucleus of infected cells (58). Full genome analysis of the TCO vaccine strain revealed that the TCO ORF C gene contains a point mutation that introduces a premature stop codon, predicting that TCO expresses a truncated ORF C polypeptide (21). The ORF C sequence was deleted from the United States Department of Agriculture ILTV challenge strain (USDAch) through homologous recombination. The deletion of the ORF C gene from USDAch strain did not affected viral replication *in vitro*, although it has contributed to virus attenuation *in vivo* as indicated by lack of

tracheal pathology after intratracheal inoculation of SPF chickens. Protection efficacy after eye-drop vaccination with ΔORF C virus was similar to that elicited by TCO vaccination (19).

As mentioned above, some of the limitations in vaccination against ILTV are the poor coverage of mass vaccination methods, labor intense vaccination methods (individual application) and late onset of immunity elicited by recombinant viral vector vaccines. To minimize these problems and to optimize early onset of immunity in the chickens, *in ovo* vaccination has been considered. *In ovo* vaccination is a highly effective mass vaccination method that results in uniform coverage, fast delivery, reduction in bird handling, and lower costs when compared to other field vaccination methods (46). In addition, *in ovo* vaccination is widely used method for immunization of broilers and layers in the United States against Marek's disease (MD) (18, 37, 59). Embryo vaccination against MD with the three serotypes (MDV1, MDV2 or HVT) (63), a combination of serotypes 2 and 3 (SB1 + HVT) (7, 52, 62), or attenuated serotype 1 (CVI-988) (47, 48) are considered safe and are commercially licensed MD vaccines for use *in ovo* have been commercially available in the US since 1992 (51). The advances in genetic engineering allowed for the construction of recombinant vaccines utilizing the HVT and fowl poxvirus (FPV) as vectors to express proteins of ILTV, Newcastle disease virus (NDV), infectious bursal disease virus (IBDV) and avian influenza virus (AIV) (1).

The use of live attenuated virus for vaccination against respiratory disease is still limited for commercial *in ovo* vaccination, for instance, still restrained to experimental studies with a recombinant infectious bronchitis virus (IBV) and a recombinant NDV. The recombinant IBV was prepared through reverse genetics system, where the insertion

of IBV Beaudette strain`s spike protein in the IBV M41-CK strain (8) was considered safe and induced high protection (90%) post *in ovo* vaccination of SPF chickens. Furthermore, two recombinant NDV viruses have been engineered utilizing the parental NDV Clone 30 vaccine strain possessing alterations in the fusion (F) and hemagglutinin-neuraminidase (HN), phosphoprotein (P), matrix (M), and large-RNA-dependent-RNA-polymerase (L) genes. Although both recombinant NDV were not considered safe for *in ovo* vaccination, they induced 100% protective efficiency post challenge with a virulent NDV strain (45). Therefore, it is believed that the *in ovo* vaccination of chickens with recombinant live attenuated ILTV will elicit early immunity to ILT. *In ovo* vaccination with an ILTV lacking glycoprotein G (gG) has been reported as safe and efficacious for SPF chickens (32). Another ILTV recombinant lacking the glycoprotein J (gJ) was developed and applied *in ovo* resulting in high mortality in commercial broilers during the first week of age (33).

Our hypothesis is that infectious laryngotracheitis virus attenuated by deletion of the ORF C gene (ΔORF C) is a safe and efficacious alternative for *in ovo* and/or early vaccination against ILTV in SPF and maternal antibody positive chickens. The first objective of this study was to evaluate attenuation and protection efficacy of the virus when administered *in ovo* in specific pathogen free (SPF) and commercial leghorn chicken embryos. The second objective was to evaluate the level of attenuation of the ΔORF C recombinant virus and its protection efficacy in commercial leghorn chickens after a prime immunization *in ovo*, followed by revaccination at eight days-of-age via spray or nasal-oral routes.

## REFERENCES


1. Armour NK, García M. 2014. Current and Future Applications of Viral-Vectored Recombinant Vaccines in Poultry. The Poultry Informed Professional:1-9.
2. Avila AR. 2007. Replication, transmission, and protection of live-attenuated infectious laryngotracheitis virus (ILTV) vaccines. [electronic resource]. 2007.
3. Bagust TJ, Calnek BW, Fahey KJ. 1986. Gallid-1 herpesvirus infection in the chicken. 3. Reinvestigation of the pathogenesis of infectious laryngotracheitis in acute and early post-acute respiratory disease. Avian Dis 30:179-190.
4. Bermudez AJ. 2013. Principles of Disease Prevention: Diagnosis and Control, p 3-16. *In* Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair V (ed), Diseases of Poultry, 13th ed. Blackwell Publishing, Ames, Iowa.
5. Brandly CA. 1936. Studies on the Egg-Propagated Viruses of Infectious Laryngotracheitis and Fowl-Pox. Journal of the American Veterinary Medical Association 88:587-599.
6. Brandly CA, Bushnell LD. 1934. A Report of Some Investigations of Infectious Laryngotracheitis. Poultry Science 13:212-217.
7. Calnek BW, Schat KA, Peckham MC, Fabricant J. 1983. Field trials with a bivalent vaccine (HVT and SB-1) against Marek's disease. Avian Dis 27:844-849.
8. Casais R, Dove B, Cavanagh D, Britton P. 2003. Recombinant Avian Infectious Bronchitis Virus Expressing a Heterologous Spike Gene Demonstrates that the Spike Protein Is a Determinant of Cell Tropism. Journal of Virology 77:9084-9089.
9. Chacon JL, Nunez LF, Vejarano MP, Parra SH, Astolfi-Ferreira CS, Ferreira AJ. 2015. Persistence and spreading of field and vaccine strains of infectious laryngotracheitis

virus (ILTV) in vaccinated and unvaccinated geographic regions, in Brazil. Trop Anim Health Prod 47:1101-1108.

10. Crawshaw GJ, Boycott BR. 1982. Infectious laryngotracheitis in peafowl and pheasants. Avian Dis 26:397-401.

11. Davison S, Gingerich EN, Casavant S, Eckroade RJ. 2006. Evaluation of the efficacy of a live fowlpox-vectored infectious laryngotracheitis/avian encephalomyelitis vaccine against ILT viral challenge. Avian Diseases 50:50-54.

12. Devlin JM, Mahmoudian A, Noormohammadi AH, Gilkerson JR, Browning GF, Hartley CA, Kirkpatrick NC. 2006. Glycoprotein G is a virulence factor in infectious laryngotracheitis virus [electronic resource]. Journal of general virology 87:2839-2847.

13. Esaki M, Noland L, Eddins T, Godoy A, Saeki S, Saitoh S, Yasuda A, Dorsey KM. 2013. Safety and Efficacy of a Turkey Herpesvirus Vector Laryngotracheitis Vaccine for Chickens. Avian Diseases 57:192-198.

14. Fahey KJ, York JJ. 1990. The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chickens. J Gen Virol 71 ( Pt 10):2401-2405.

15. Fuchs W, Veits J, Helferich D, Granzow H, Teifke JP, Mettenleiter TC. 2007. Molecular biology of avian infectious laryngotracheitis virus. Vet Res 38:261-279.

16. Fuchs W, Wiesner D, Veits J, Teifke JP, Mettenleiter TC. 2005. In vitro and in vivo relevance of infectious laryngotracheitis virus gJ proteins that are expressed from spliced and nonspliced mRNAs. J Virol 79:705-716.

17. Fuchs W, Ziemann K, Teifke JP, Werner O, Mettenleiter TC. 2000. The non-essential UL50 gene of avian infectious laryngotracheitis virus encodes a functional dUTPase which is not a virulence factor. J Gen Virol 81:627-638.

18. Gagic M, St Hill CA, Sharma JM. 1999. In ovo vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis 43:293-301.

19. García M, Cheng Y, Spatz SJ, Riblet SM, Schneiders GH, Volkening J. 2016 Submitted. Attenuation and Protection Efficacy of Open Reading Frame C (ORF C) Gene Deleted Strain of The Alphaherpesvirus Infectious Laryngotracheitis Virus (ILTV)

20. García M, Riblet SM. 2001. Characterization of Infectious Laryngotracheitis Virus Isolates: Demonstration of Viral Subpopulations within Vaccine Preparations, p 558. American Association of Avian Pathologists, Inc.

21. García M, Volkening J, Riblet SM, Spatz S. 2013. Genomic sequence analysis of the United States infectious laryngotracheitis vaccine strains chicken embryo origin (CEO) and tissue culture origin (TCO). Virology 440:64-74.

22. Gelenczei EF, Marty EW. 1964. Studies on a Tissue-Culture-Modified Infectious Laryngotracheitis Virus, p 105. American Association of Avian Pathologists, Inc.

23. Guy JS, Barnes HJ, Smith L. 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis 35:348-355.

24. Han MG, Kweon CH, Mo IP, Kim SJ. 2002. Pathogenicity and vaccine efficacy of a thymidine kinase gene deleted infectious laryngotracheitis virus expressing the green fluorescent protein gene. Arch Virol 147:1017-1031.

25. Hein R. 2008. Recombinant HVT/ILTV Vaccine (Innovax-ILT): Field Application Issues. NATIONAL MEETING ON POULTRY HEALTH AND PROCESSING EDIT 43:73.

26. Helferich D, Veits J, Teifke JP, Mettenleiter TC, Fuchs W. 2007. The UL47 gene of avian infectious laryngotracheitis virus is not essential for in vitro replication but is relevant for virulence in chickens. J Gen Virol 88:732-742.

27. Hitchner SB. 1975. Infectious laryngotracheitis: the virus and the immune response. Am J Vet Res 36:518-519.

28. Hitchner SB, Winterfield RW. 1960. Revaccination Procedures for Infectious Laryngotracheitis, p 291. American Association of Avian Pathologists, Inc.

29. Johnson DI, Vagnozzi A, Dorea F, Riblet SM, Mundt A, Zavala G, García M. 2010. Protection against infectious laryngotracheitis by in ovo vaccination with commercially available viral vector recombinant vaccines. Avian Dis 54:1251-1259.

30. Jordan FTW. 1981. Immunity to infectious laryngotracheitis. Avian Immunology:245–254.

31. Lee S, Markham PF, Coppo MJC, Legione AR, Markham JF, Noormohammadi AH, Browning GF, Ficorilli N, Hartley CA, Devlin JM. 2012. Attenuated vaccines can recombine to form virulent field viruses. Science (Washington) 337:188-188.

32. Legione AR, Coppo MJ, Lee SW, Noormohammadi AH, Hartley CA, Browning GF, Gilkerson JR, O'Rourke D, Devlin JM. 2012. Safety and vaccine efficacy of a glycoprotein G deficient strain of infectious laryngotracheitis virus delivered in ovo. Vaccine 30:7193-7198.

33. Mashchenko A, Riblet SM, Zavala G, Garcia M. 2013. In ovo vaccination of commercial broilers with a glycoprotein J gene-deleted strain of infectious laryngotracheitis virus. Avian Dis 57:523-531.

34. Menendez KR, García M, Spatz S, Tablante NL. 2014. Molecular epidemiology of infectious laryngotracheitis: a review. Avian Pathol 43:108-117.

35. Mundt A, Mundt E, Hogan RJ, García M. 2011. Glycoprotein J of infectious laryngotracheitis virus is required for efficient egress of infectious virions from cells. J Gen Virol 92:2586-2589.

36. Neff C, Sudler C, Hoop RK. 2008. Characterization of western European field isolates and vaccine strains of avian infectious laryngotracheitis virus by restriction fragment length polymorphism and sequence analysis. Avian Dis 52:278-283.

37. Ohta H, Ezoe S, Yamazaki K, Kawai T, Honda T. 2009. Application of aluminum hydroxide for an in ovo live Newcastle disease vaccine. Avian Dis 53:392-395.

38. Ojkic D, Swinton J, Vallieres M, Martin E, Shapiro J, Sanei B, Binnington B. 2006. Characterization of field isolates of infectious laryngotracheitis virus from Ontario. Avian Pathol 35:286-292.

39. Oldoni I, García M. 2007. Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathology: Journal Of The WVPA 36:167-176.

40. Oldoni I, Rodriguez-Avila A, Riblet SM, García M. 2008. Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Avian Dis 52:59-63.

41. Oldoni I, Rodriguez-Avila A, Riblet SM, Zavala G, García M. 2009. Pathogenicity and growth characteristics of selected infectious laryngotracheitis virus strains from the United States. Avian Pathol 38:47-53.

42. Pavlova SP, Veits J, Blohm U, Maresch C, Mettenleiter TC, Fuchs W. 2010. In vitro and in vivo characterization of glycoprotein C-deleted infectious laryngotracheitis virus. J Gen Virol 91:847-857.

43. Pavlova SP, Veits J, Mettenleiter TC, Fuchs W. 2013. Identification and functional analysis of membrane proteins gD, gE, gI, and pUS9 of Infectious laryngotracheitis virus. Avian Dis 57:416-426.

44. Portz C, Beltrão N, Furian TQ, Bianco Júnior A, Macagnan M, Griebeler J, Rosa CAVL, Colodel EM, Driemeier D, Back A, Schatzmayr OMB, Canal CW. 2008. Natural infection of turkeys by infectious laryngotracheitis virus. Veterinary Microbiology 131:57-64.

45. Ramp K, Topfstedt E, Wackerlin R, Hoper D, Ziller M, Mettenleiter TC, Grund C, Romer-Oberdorfer A. 2012. Pathogenicity and immunogenicity of different recombinant Newcastle disease virus clone 30 variants after in ovo vaccination. Avian Dis 56:208-217.

46. Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, King S, Murray L, Phelps P, Poston R, Whitfill C, Williams C. 1999. In ovo vaccination technology. Adv Vet Med 41:495-515.

47. Rispens BH, van Vloten H, Mastenbroek N, Maas HJ, Schat KA. 1972. Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis 16:108-125.

48. Rispens BH, van Vloten H, Mastenbroek N, Maas JL, Schat KA. 1972. Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease virus. Avian Dis 16:126-138.

49. Rodriguez-Avila A, Oldoni I, Riblet SM, García M. 2007. Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines. Avian Dis 51:905-911.

50. Samberg Y, Cuperstein E, Bendheim U, Aronovici I. 1971. The development of a vaccine against avian infectious laryngotracheitis. IV. Immunization of chickens with a modified laryngotracheitis vaccine in the drinking water. Avian Dis 15:413-417.

51. Sarma G, Greer W, Gildersleeve RP, Murray DL, Miles AM. 1995. Field safety and efficacy of in ovo administration of HVT + SB-1 bivalent Marek's disease vaccine in commercial broilers. Avian Dis 39:211-217.

52. Schat KA, Calnek BW. 1978. Characterization of an apparently nononcogenic Marek's disease virus. J Natl Cancer Inst 60:1075-1082.

53. Schnitzlein WM, Winans R, Ellsworth S, Tripathy DN. 1995. Generation of thymidine kinase-deficient mutants of infectious laryngotracheitis virus. Virology 209:304-314.

54. Seddon HR, Hart L. 1936. Infectivity Experiments with the Virus of Laryngotracheitis of Fowls. Australian Veterinary Journal 12:13-16.

55. Spatz SJ, Volkening JD, Keeler CL, Kutish GF, Riblet SM, Boettger CM, Clark KF, Zsak L, Afonso CL, Mundt ES, Rock DL, García M. 2012. Comparative full genome analysis of four infectious laryngotracheitis virus (Gallid herpesvirus-1) virulent isolates from the United States. Virus Genes 44:273-285.

56. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian Pathol 41:21-31.

57. Veits J, Luschow D, Kindermann K, Werner O, Teifke JP, Mettenleiter TC, Fuchs W. 2003. Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. J Gen Virol 84:3343-3352.

58. Veits J, Mettenleiter TC, Fuchs W. 2003. Five unique open reading frames of infectious laryngotracheitis virus are expressed during infection but are dispensable for virus replication in cell culture. J Gen Virol 84:1415-1425.

59. Williams CJ, Zedek AS. 2010. Comparative field evaluations of in ovo applied technology. Poult Sci 89:189-193.

60. Williams RA, Bennett M, Bradbury JM, Gaskell RM, Jones RC, Jordan FT. 1992. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. J Gen Virol 73 ( Pt 9):2415-2420.

61. Winterfield RW, So IG. 1968. Susceptibility of turkeys to infectious laryngotracheitis. Avian Dis 12:191-202.

62. Witter RL, Lee LF. 1984. Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies. Avian Pathol 13:75-92.

63. Witter RL, Nazerian K, Purchase HG, Burgoyne GH. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am J Vet Res 31:525-538.

64. Yan Z, Li S, Xie Q, Chen F, Bi Y. 2016. Characterization of field strains of infectious laryngotracheitis virus in China by restriction fragment length polymorphism and sequence analysis. J Vet Diagn Invest 28:46-49.

65. York JJ, Young JG, Fahey KJ. 1989. The appearance of viral antigen and antibody in the trachea of naive and vaccinated chickens infected with infectious laryngotracheitis virus. Avian Pathol 18:643-658.

# CHAPTER 2

# LITERATURE REVIEW

## VIRAL STRUCTURE, GENOME AND REPLICATION

Infectious laryngotracheitis virus, the causative agent of the infectious laryngotracheitis disease (ILT), is taxonomically classified as *Gallid Herpesvirus-1* (GaHV-1), belongs to the family *Herpesviridae*, subfamily *Alphaerpesvirinae*, and genus *Iltovirus*. ILTV was considered the only virus of its genus for many years, until the *Pstitacid Herpesvirus-1* (*PsHV-1*), the causative agent of a highly contagious upper respiratory and systemic disease in psittacines was also included within this genus (31, 32, 100).

Similar to Herpes Simplex Virus 1 (HSV-1), ILTV has an icosahedral viral particle with a hexagonal nucleocapsid (80-100 nm) composed of 162 elongated hollow capsomers, (29, 164). The diameter of the complete viral particle depends on the amount of incorporated tegument protein, which varies between 195-250 nm. An irregular envelope surrounds the nucleocapsid and contains viral glycoproteins on its surface (29, 133).

The ILTV genome is composed of a double stranded linear DNA with approximate size of 150–155 kb, containing 79 predicted open reading frames (ORFs) (50, 175), and consists of two regions designated Unique short ($U_S$) and Unique long ($U_L$)

flanked by two 11 kb inverted repeats. This structure allows the formation of two isomers, each of them with a different oriented $U_S$ region (71, 93).

Due to genetic similarities between ILTV and other alphaherpesvirus, the designations for its ORFs and proteins have been largely adopted from the homologous genes and proteins of *Herpes Simplex Virus-1* (*HSV-1*) genome (99), such as $U_L1$ to $U_L54$, $U_S2$ to $U_S8$ and $U_S10$, and 12 ORFs encoding homologous *HSV-1* glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gN and gM) that control viral attachment and entry, cell fusion and viral egress. However the exact function and interactions of each glycoprotein have not been fully described. When compared to *HSV-1,* ILTV genome possesses unique features, for instance it possesses the $U_L$ 3.5 protein, of unknown function, also found in most *alphaherpesvirus* (42, 97), and lacks the capsid-associated virion protein $U_L16$, which is conserved in most of *Herpesviridae* family (43). Five ORFs (A-E) of unknown function (157) and two unique genes both encoding for nuclear proteins $U_L0$ and $U_L$-1, (175). Furthermore, the translocation of $U_L47$ (tegument protein) from $U_L$ to $U_S$ region and the internal inversion of $U_L22$ (envelope glycoprotein H [gH]) to $U_L44$ (envelope glycoprotein C [gC]) genes due to a partial inversion of the $U_L$ region (148) reveal an important phylogenetic distance between *Iltovirus* and other avian herpesviruses.

Envelope glycoproteins are responsible for mediating virus entry, cell fusion and virus egress (113, 145). In addition to their roles in host range and pathogenicity, the ILTV's glycoproteins are immunogenic and responsible for stimulating humoral and cell-mediated immune responses (172-174). Twelve glycoproteins homologous to those of *HSV-1* have been identified in the ILTV genome and are designated gB, gC, gD, gE, gG,

gH, gI, gJ, gK, gL, gM and gN (148). The gB (118, 119), gC (78, 145), gN (44) and gJ (157) are characterized as functional *HSV-1* homologues and identified in infected cells and virions, while the gG, a chemokine binding protein, is secreted from infected cells, and induces the recruitment of inflammatory cells to the site of infection (36, 84). Based on antibody responses in chickens and monoclonal antibodies, the envelope glycoproteins gJ and gC have been characterized as the most immunogenic surface glycoproteins of ILTV (46, 158).

Replication of ILTV appears to be similar to that of other alphaherpesviruses. The process initiates with the attachment of viral surface glycoproteins to the cell receptor and subsequent fusion of the viral and cell membranes. Most herpesviruses utilize heparan sulfate as their primary surface receptor. In general, the major glycoproteins involved in viral attachment of herpesviruses are gB and gC, which interact with the heparan sulfate at the cell membrane surface (133), however ILTV does not interact with heparan sulfate as its gC is shorter than that of *HSV-1*, lacking the heparan binding domain (30, 79). Although the cellular receptor responsible for ILTV infection is still not known, it is believed that gC promotes initial attachment through a different mechanism than that utilized by *HSV-1*.

Following attachment, the virus initiates fusion of the envelope with the host cell plasma membrane. The nucleocapsid is released into the cytoplasm and transported to the nuclear membrane. Viral DNA is released from the nucleocapsid and migrates to the nucleus, through nuclear pores, where transcription and replication of viral DNA occurs (50). According to a recent study, ILTV infection triggers the proto-oncogene tyrosine-protein-kinase Src pathway, which results in prolonged survival of hepatocellular

carcinoma cells (LMH) and chicken embryo cells, whereas the suppression of Src enhanced viral virulence by making host cells more susceptible to ILTV-induced cell death, but limiting ILTV replication (95).

Following DNA replication, DNA-filled nucleocapsid acquire an envelope by migration through the inner lamellae of the nuclear membrane, thereafter, the nucleocapsid acquires a final envelope structure with embedded ILTV glycoproteins in a second budding event, this time at the trans-Golgi region. After the second budding, the nucleocapsid migrates through the endoplasmic reticulum and is accumulated within vacuoles in the cytoplasm. Virions are released by cell lysis or by vacuolar membrane fusion and exocytosis (54-56, 103).

*In vitro,* ILTV can replicate in a variety of cells, including chicken embryo liver cells (CEL), chicken embryo lungs, chicken embryo kidney (CEK), chicken kidney (CK) (20, 68, 79, 101). In a comparison of several cell culture systems, C. S. Hughes and R. C. Jones (68) found that chicken embryo liver (CEL) and chicken kidney (CK) cells were the preferred primary culture systems for ILTV isolation and propagation due to their sensitivity to virus isolation and higher yield in virus titers. ILTV also replicates in a continuous avian cell line derived from a chemically induced chicken liver tumor cell line named LMH (leghorn male hepatoma) (76). The LMH cell line has been instrumental in the construction of recombinant ILTV and for the study of cell-virus interactions (45). Additionally, ILTV can be propagated in the chorioallantoic membrane (CAM) of embryonated chickens eggs, usually infected at 9-11 days of embryonation. When replicating in the CAM, ILTV produces pox-like opaque plaques that result from necrosis

and proliferation of the affected cells. The plaques can be observed as early as 2 days post inoculation (PI) with embryo death occurring from 2-8 days PI (151).

Based on their temporal expression during infection and their dependence on protein synthesis or DNA replication *HSV-1* genes are identified as immediate-early (IE), early (E) and late (L) genes, (116). Immediate early gene products are expressed independently of *de novo* protein synthesis, therefore are not inhibited by cycloheximide (CHX), they function as transcription factors. Early genes are generally involved in viral DNA metabolism and replication, are dependent of *de novo* protein synthesis, and are inhibited when cells are treated with CHX. Late genes are partially or completely dependent on the replication of viral DNA and generally encode structural and other proteins involved in virion assembly. Late genes are inhibited by phosphonoacetic acid (PAA) (97, 116, 139). More recently transcription of 74 known ILTV genes in LMH infected cells was evaluated by reverse transcriptase PCR. Several of these genes are enzymes and DNA-binding proteins that regulate viral DNA replication, but most are viral structural proteins including capsid, tegument and envelope glycoproteins (97). There are evidences that, apart from a few genes ($U_L5$, $U_L11$, $U_L12$ and $U_L17$) that appear to have very low levels of expression, all other ILTV genes, including ORFs A-C and $U_L$-1 were detectable at the earliest time point of infection, with increasing expression through the progression of the infection, suggesting that transcription from most of the ILTV genes is leaky or subject to more complex patterns of regulation than those classically described for alphaherpesviruses (97).

The genome of ILTV also encodes short RNAs (approximately 22 nt long) known as microRNAs. MicroRNAs are non-coding RNAs complementary to target messenger

RNA (mRNA), that affect gene expression by binding to the untranslated region (UTR) of the target mRNA transcript, resulting in mRNA degradation or translation inhibition (161, 171). Virus-host interactions in herpesviruses are characterized by long-term survival in latent stage. This demands sophisticated methods of survival without being detected by the innate and adaptive host immune systems. A total of seven precursor and 10 mature microRNAs have been identified with the use of deep sequencing of small RNA populations from ILTV infected chicken embryo kidney (CEK) and LMH cells (124, 163). The miR-11-5p, miR-113p, mIR-12-5p, mIR-13-3p and mIR14-5p are located at the extreme 5` terminus of the genome, a region devoid of large ORFs, these microRNAs were not associated with any annotated ORFs (50, 171). One microRNA, miR-17-3p was mapped in the origin of replication (*Ori*L) located near the binding site of the origin-binding-protein $U_L9$. Four microRNA mir-16-3p, mIR-16-5p, mIR-15-3p and mIR-15-5p were mapped to the gene encoding the immediate early transcriptional activator protein ICP4. The specific roles of these microRNAs in silencing viral or host transcripts remains unknown. L. A. Waidner, et al. (162) demonstrates that ILTV`s mIR-15 and miR-16 can down regulate the transcriptional activator ICP4 by direct binding and cleavage of ICP4 mRNA. Regulation of ICP4 could impact on the balance between lytic and latent stages of infection, as example of MDV, the latency associated transcript (LAT) is antisense to ICP4 and generally shows inverse levels of expression (15, 16).

Full genome sequences for 27 strains of ILTV are available at the GenBank database, including vaccine strains and wild-type ILTV from Australia (88-90, 92), China (83), USA (19, 51, 144) and Italy (114). Full genome sequences of ILTV will further

advance our understanding of the disease epidemiology and emergence of strains and help in the identification of genes involved in virulence.

## PATHOGENESIS

Infectious laryngotracheitis virus has been detected in most countries around the world and remains a serious disease wherever susceptible high-density poultry populations exist (9, 50). Restrictions in international trade can be imposed for ILT affected areas.

Natural infections are restricted to galliform birds. It is most commonly described as an infection of chickens, although there are reports of natural infections in pheasants, peafowl and partridges (11, 28, 159). Experimental (167) and natural (117) infections were reported in turkeys. Sub-clinical infection and seroconversion have been reported in ducks infected through intratracheal and intranasal routes (170), while starlings, sparrows, crows, doves, pigeons and guinea fowl appear to be refractory to the infection, as well as non-galliform birds and mammals (12, 140).

ILTV causes an acute respiratory disease in chickens. Mild to severe forms of the disease can be seen in the field, depending on the virulence of the ILTV strain (4, 57, 96). Although it affects all ages, the most characteristics clinical signs of ILT can be observed as early as three weeks of age. Clinical signs related to severe forms of the disease include marked dyspnea with expectoration of blood-stained mucus with the presence of fibrino-hemorrhagic exudate, apathy, moderate to severe conjunctivitis, and mortality can reach 20% or more, while the clinical signs seen in mild forms include nasal discharge, conjunctivitis, sneezing, coughing and mild tracheitis with low mortality (0.1 – 2.0%) (5,

74). Decrease in feed consumption, lower egg production and reduced eggshell thickness have also been observed (125). Clinical signs usually appear within 6-14 days after natural infection, while in experimentally infected birds, clinical signs usually appear as early as two days post-infection with the peak at five days post infection (70, 74, 152).

Many factors such as host age, route of exposure, virulence of the challenge virus and initial viral load can affect the outcome of the ILTV infection (50). The first sites of replication in naturally infected chickens are the conjunctiva and the mucosa of the upper respiratory tract such as the nasal epithelium (6-8). Regardless of the infection route, the most active viral replication occurs in the epithelium of the trachea and consequently, the trachea is considered the ILTV target organ (3). Gross lesions are characterized by mucoid inflammation, degeneration and necrosis of the trachea. Diptheritic changes are common and may be seen as mucoid casts that extend the entire length of the trachea. Severe hemorrhages into the trachea lumen may result in blood casts where mucus can be mixed with blood and necrotic tissue. The cause of mortality may be the result of a decrease in feed intake and the occurrence of mucous plugs in the tracheal lumen resulting in asphyxiation. The infection curve varies with the severity of lesions, with the flock typically recovering within 10-14 days after the initial appearance of clinical signs (50, 143).

As for gross lesions, microscopic changes also vary with regard to the severity and stage of disease. Early microscopic changes in the tracheal mucosa are a result of goblet cell loss and infiltration of mucosa with inflammatory cells. Respiratory and conjunctiva epithelial cells enlarge and become edematous as the infection progresses. Multinucleated giant cells (syncytia) are also observed. Lymphocytes, histiocytes and

plasma cells migrate into the mucosa and submucosa after 2-3 days. Intranuclear inclusion bodies are found in epithelial cells only in early stages of infection (1-5 days post infection). Consequently, the affected epithelia desquamate, exposing the lamina propria and subsequently its blood vessels that may protrude to the tracheal lumen, causing hemorrhage (3, 50, 60, 122, 156). The peak of virus replication occurs in the tracheal epithelium from 2-5 days post-infection (80, 110). The virus remains in low levels in the trachea after the acute phase of replication. Other mucous membranes such as conjunctiva, respiratory sinuses, lungs, air sacs and thymus also can become infected (110, 131).

It has been largely recognized that ILTV strains related to outbreaks vary in pathogenicity and can cause high morbidity (90-100%) and variable mortality (5-70%; usually between 10-20%), while other strains, can produce mild to unapparent infection in chickens with low morbidity (5%) and very low mortality (0.1-2%) (10, 26, 111, 120, 121, 141, 149).

Considerable variation exists among ILTV tropism for the trachea and conjunctiva. N. C. Kirkpatrick, et al. (80) revealed that ILTV strains with high affinity for conjunctiva could severely affect weigh-gain, most likely due to the conjunctivitis and associated inability of the bird to locate the feed. A recent study have identified the tropism of ILTV for different organs in natural infected chickens from 60 farms, and concluded that the virus possesses great affinity for the middle portion of the trachea and conjunctiva and less affinity towards the larynx and other portions of the trachea (143).

Although ILTV strains of low and high pathogenicity are clearly recognized, currently they are considered antigenically similar based on virus neutralization (134,

142), cross-protection and immunofluorescence studies (26, 142), therefore genotyping analysis utilizing PCR-RFLP has been utilized to study epidemiological relationships between strains. However, attempts to correlate restriction length fragment polymorphism (RFLP) with strain virulence were unsuccessful (10, 17, 18, 62, 80, 81, 108, 109). More recently, genetic differences among strains have been studies by full genome sequencing (51).

By definition, latency is the ability of a pathogenic virus to lie dormant in infected cells without the production of viral particles. Unapparent infection of the respiratory tract is a trait of ILTV persistence. Early studies demonstrated the establishment of a carrier state in 2% of the recovered birds for periods up to 16 months after a disease outbreak (53, 82) by migrating to the central nervous system where it can establish latent infections mainly in the trigeminal ganglion (112, 166), alongside latency in the trachea (2, 112). Periods of latency are interspersed with episodes of viral shedding (67, 69) making the control of the disease difficult. Stress can trigger viral reactivation, leading to migration of ILTV to the trachea, where production and excretion of viral particles takes place (67). Therefore, long life birds are ILTV reservoirs and contribute to the spread of the disease (10, 59, 65, 111, 132).

**VIRULENCE**

By definition, virulence is the ability of a microorganism to invade, resist to the host defense mechanism and cause injury to the host, whereas, pathogenicity is defined as the potential of a microorganism to produce disease (146, 147). Experimental evaluation of circulating ILTV strains is fundamental in order to identify changes in virulence that

can contribute to severity and spread of outbreaks and consequently influence the efficacy of vaccination programs. Several aspects of the ILTV infection have been evaluated in attempt to identify differences in virulence among strains. Tracheal pathogenicity index (TPI), mortality and viral genome copy numbers in trachea of infected chickens, restriction fragment length polymorphism (RFLP) and circulating antibody titers are some of the parameters that have been utilized to assess ILTV virulence (57, 80, 85, 91, 153, 158). Measurement of post-infection antibody titers by commercial ELISA kits were determined to be an inaccurate measurement of virulence between different ILTV strains (153). Similarly, attempts to correlate RFLP with strain virulence were unsuccessful (62, 80, 110). Two additional parameters aim to determine the virulence of ILTV strains based on evaluation of the main site of viral replication, the trachea. The first is the quantification of viral genome copy numbers in the tracheal tissues or swabs using real-time PCR (qPCR) assay. Moreover, the development of a duplex qPCR assay allowed the quantification of viral genome copy numbers based on the glycoprotein C (UL44) sequence normalized for the chicken DNA present in the sample (154). The second parameter is the tracheal pathology index (TPI), based on microscopic changes in the tracheal epithelium, such as inflammation, presence of syncytia and intranuclear inclusion bodies, mucosal thickness (edema) and hyperemia (57). Great divergences exist among studies attempting in determine differences in virulence among ILTV isolates. Studies with Australian ILTV strains have correlated the TPI with the level of virus replication as estimated by viral genome copy numbers in the trachea (80, 91). In contrast, studies have shown that TPI does not reflect the virulence of

USA strains as good as viral genome copy numbers, clinical signs and mortality rates induced by experimental ILTV infection (85, 110, 153).

The insertion of a new live vaccine strain in Australia originated two new classes of virus that thrived, becoming sources of outbreaks. According to J. Y. Lee, et al. (87), one of the characteristics that allowed these two classes of virus to emerge is their suitability to the environment and improved capacity of growth and transmission. Regarding USA strains, it is known that the CEO revertant presents higher incidence of clinical signs as compared to the TCO revertant strains post 20 consecutive passages in SPF chickens (59). This emphasizes the importance of the transmission ability of each strain in establishing permanent infections and possibly becoming source of outbreaks. Furthermore, the ability to transmit to naïve chickens must also be considered as a potential virulence factor of ILTV. Altogether, these studies highlight the importance of evaluating different aspects of ILTV infection, such as mortality rate, viral genome copy numbers, clinical signs and tracheal pathology index and transmission to better determine viral virulence.

The Code of Federal Regulations, Title 9 part 113 section 328, stipulates that at least 80% of the non-vaccinated chickens shall present clinical signs of ILT of mortality post challenge with a virulent ILTV strain for validation of safety and protection vaccine studies. Due to this requirement, the minimum infectious dose must be evaluated in order to define safety/protection parameters in evaluating vaccines against ILT. In a study by D. M. Koski, et al. (85), the USDAch strain caused clinical signs / mortality in over 80% of the chickens intratracheal inoculation of $10^{2.6}$ $EID_{50}$, whereas a virulent field isolate caused the same level of clinical signs / mortality when chickens were intratracheally

inoculated with less concentrated dilutions (between $10^{1.5}$ and $10^{1.2}$ $EID_{50}$). Intratracheal inoculation was also utilized in attempts to determine differences in virulence between the USDAch and the field isolate GA63140, by infecting chickens with $10^1$, $10^2$, $10^3$ and $10^4$ $TCID_{50}$ and observing differences in clinical signs, mortality and viral genome copy numbers in the trachea (153). I. Oldoni, et al. (110) utilized higher doses ($10^{2.5}$ $TCID_{50}$) of field strains applied via eye-drop and nasal-oral in order to determine differences in virulence among the field strains and the CEO vaccine strain applied according to the producer`s indications (approximately $10^{4.5}$ $TCID_{50}$). These studies provide evidence to support the differences in experimental outcomes based on the minimum infectious dose and route of application. It has been experimentally shown that chickens are more susceptible to ILTV virus administered by tracheal rather than conjunctival route (40), thus, this route has been widely used in experimental studies. Furthermore, the strain of choice to evaluate safety / protection induced by vaccine candidates under experimental conditions should represent the circulating field strains (85).

Viral genes have evolved to maximize viral replication by modulating expression of cellular genes and/or the host`s immune system. The chemokine binding protein, glycoprotein G (gG), common to other alphaherpesvirus such as *Equine herpesvirus I* and *Bovine herpesvirus I* and *V* (13) was identified as a virulence factor in ILTV, altering the host immune response during ILTV infection (37). In the study, the infection with a ΔgG ILTV shifts the cell populations in the trachea, inducing increased numbers of $CD4^+$ and $CD8^+$ lymphocytes combined with decreased number of mucosal B lymphocytes and concentrations of serum neutralizing antibodies, therefore, affirming the function of this protein in modulating the local immune response in the trachea, which is the main site of

viral replication. Clinically, this change was evidenced by the increased thickness in the trachea of chickens inoculated with the ΔgG. Moreover, the presence of gG creates conditions that favor virus survival in the host (37).

The manipulation of host factors that regulate the interaction between host and ILTV is known to affect viral virulence. A study has identified the host`s tyrosine-protein-kinase Src (Src) as an important proto-oncogene. Briefly, Src controls the virulence of ILTV and is phosphorylated upon ILTV infection. Function studies revealed that Src prolongs the survival of host cells by increasing the threshold of virus induced cell death, whereas the silencing of Src gene in LMH cells and chicken embryos favors apoptosis of ILTV-infected cells and decreases ILTV titers (95).

Inhibition of apoptosis in infected cells has been identified as another potential virulence factor in ILTV, however, the viral proteins and pathways involved in this process are still unknown. J. Y. Lee, et al. (87) found that the pro-apoptotic gene cholecystokinin (CCK) is down regulated in ILTV-infected lung cells, suggesting that ILTV infection inhibits apoptosis in infected cells. Utilizing immunofluorescence, V. R. Reddy, et al. (126) demonstrated that viability of ILTV-infected trachea and conjunctiva explant cells was not affected, indicating that ILTV inhibits apoptosis on infected cells, while inducing apoptosis in non-infected neighboring cells.

Although a few virulence factors of ILTV are understood, many genes have been deleted from ILTV genome, resulting in viral attenuation *in vivo*, indicating their role in virulence, but the exact mechanisms of attenuation are still unknown. For instance, an ILTV recombinant lacking $U_L47$ induced significantly less severe clinical signs of the disease, marked by moderate respiratory distress only, combined with the lack of viral re-

isolation from vaccinated SPF chickens, characterizing an attenuated recombinant virus (64). Recently, the deletion of open reading frame C (ORF C), a protein of unknown function, from the USDA challenge strain, significantly attenuated the ΔORF C ILTV for three-weeks old SPF chickens, (49). Similarly, the deletion of glycoprotein J (gJ) partially reduced the virulence of ILTV for *in ovo* vaccination of commercial broilers (98).

## IMMUNITY

A variety of responses can be generated following ILTV infection, however not all of them provide resistance to infection. The passive transfer of antibodies to offspring has been previously demonstrated (63), however no significant differences were found between chickens hatched from hyper immune and non-vaccinated parents in regard to protection against ILTV (73). Maternal antibodies to ILTV present in the egg are transferred to the progeny, but generally do not provide protection or interfere with the vaccination from 8-10-days of age (27).

ILTV neutralizing antibodies can be detected within 5-7 days, peak around 21 days post infection (73). ILTV-neutralizing antibodies may be detectable for a year or more (2). Local antibodies can be detected in tracheal secretions for approximately seven days and reach a plateau between 10-28 days post infection (2, 174). Although antibodies are produced against the virus, the humoral immune response itself is not responsible for resistance to the disease (41, 130). A poor correlation exists between antibody titers and the protection of the flock (47, 94).

Studies with bursectomized chickens have shown that the absence of antibodies did not impair the ability of vaccinated-bursectomized chickens to resist to a challenge infection with a virulent ILTV (41), and suggests that rather than the humoral arm, cell-mediated immune responses are the principal mechanism behind ILTV resistance. However, the specific pathways by which the cell-mediated immune response confers protection against ILTV is poorly understood.

The ILTV glycoprotein G (gG), is a chemokine binding protein (36). Researchers reported that SPF chickens inoculated with a gG-depleted ILTV recombinant presented higher tracheal inflammation as compared to chickens vaccinated with the parental ILTV strain. The higher tracheal inflammation correlated with significantly lower clinical signs scores and mortality post vaccination (36). Inflammation in response to infection is crucial as it influences viral replication, contributes to pathology and the subsequent adaptive immune responses, hence, the inflammatory response to ILTV infection is critical to both virus virulence and the host response (25). Specific pathogen free chickens inoculated with a ΔgG ILTV recombinant showed increased number of heterophils, CD4+ and CD8+ lymphocytes, significant decrease in B lymphocytes in tracheal mucosa at four days post inoculation, while decreased levels of serum antibodies 14 days post inoculation (37). Other study explored the use of host inflammatory factors to improve the efficiency of vaccines against ILTV. H. Y. Chen, et al. (21) demonstrated that the protective efficacy of a fowl poxvirus vector expressing the ILTV gB (FPV-ILT) was enhanced by simultaneously expressing ILTV gB and chicken interleukin 18 (IL18) (FPV-ILT/IL18). IL18 is know to stimulate T helper 1 (Th1) to secrete interferon-ϒ (138) and has been proven to have an adjuvant effect when administered with FPV vector

(104). Chicken immunized with FPV-ILT/IL18 showed enhanced T-cell proliferative response as compared to FPV-ILT immunized chickens, evidenced by increased CD4+ and CD8+ T-cell rations, and enhanced protection, as indicated by the lack of clinical signs and mortality post challenge (21). Together, this data underscores the importance of local inflammation in the development of protective cell-mediated immunity against ILTV.

## EPIDEMIOLOGY

The disease is particularly common in regions with large poultry concentrations and intensive poultry production. A trend toward growing denser poultry populations in shorter cycles, rearing different types of poultry (layers and broilers) in the same area, combined with poor biosecurity are some of the factors that have contributed to the increase of ILTV outbreaks worldwide (22, 50, 115).

The persistence of ILTV in the field is not completely understood. The virus survives for several weeks out of the host, on mechanical carriers and fomites (74, 77). Birds vaccinated with live attenuated vaccines or recovered from the disease carry the virus in latent stage (2). The virus can be reactivated spontaneously or due to stress (67), followed by direct transmission to contact chickens (66). Backyard flocks may also carry the virus and represent a source of ILTV to commercial poultry (50). Nevertheless, infected birds presenting clinical disease are the major source of virus spread as compared to clinically recovered birds (50). Transmission studies have determined that four days are necessary for the virulent virus to replicate and transmit to other birds (35), whereas live attenuated vaccine strains take 4 to 9 days to transmit to contact chickens

(24, 132). Indirect transmission of the ILTV is frequent and occurs through exposure to contaminated equipment, personnel, clothing and shoes, improperly disposed contaminated litter, manure and infected carcass (22, 38). An epidemiological study during an ILTV outbreak in 2005 in California emphasized the importance of biosecurity measures such as extended downtime between flocks combined with cleaning and disinfection together with improvements of the vaccination program were necessary (22). One potential source for spread of the virus is during the transport of infected birds to the processing plant during the movement of litter from infected houses (33, 115, 160). As expected for an air-borne disease, wind might play an important role in the spread patterns of ILTV (72, 115). Farms located within the wind stream of an infected flock are 10 times more likely to be affected by the disease (72). Seasonality plays a role on the incidence of the disease as higher numbers of cases are observed during winter (December-February), as opposed to the lower prevalence during summer and fall (115). Vertical transmission of ILTV has not been demonstrated (50).

Due to the minor antigenic changes among ILTV strains and the complexity of the serological procedures, molecular classification systems are preferred to study epidemiological relationships between strains. The methods utilized for differentiation of ILTV strains include restriction endonuclease analysis of viral DNA (58), DNA hybridization assays (86), polymerase chain reaction (PCR) combined with RFLP analysis (PCR-RFLP) (80), PCR-RFLP and PCR with gene sequencing (61, 107), and quantitative Real-Time PCR (qPCR) (154). Restriction fragment length polymorphism (RFLP) has been widely used to differentiate ILTV strains into different classes based on specific patterns on viral genes, such as ICP4, ICP18.5, gG, gM and thymidine kinase

(10, 17, 18, 81, 108, 109). Early studies differentiated ILTV strains based on RFLP of single genes, however, one of the primary concerns of this technique is that the amplification of only a small part of the genome might not be a reliable tool to differentiate between isolates/strains. Further studies used RFLP of multiple genes to genotypically differentiate strains. RFLP analysis of ORFB-TK, gM, ICP4 and gG genes separated the USA isolated in nine distinct genotypes (108). Group I includes the USDA reference strain, Group II the TCO vaccine, Group III the field isolates closely related to the TCO vaccine, Group IV includes the CEO vaccine and commercial poultry isolates genotypically identical to the CEO vaccine, Group V includes field isolates closely related to the CEO vaccine, Group VI includes commercial poultry isolates, Groups VII, VIII and IX contain backyard flock isolates (108). Similarly, the combination of RFLP of the genes gG, TK, ICP4 and ICP18.5 allowed for the genotypic classification of Australian ILTV strains related to outbreaks in five groups. RFLP sequencing of multiple genes revealed that vaccine-like strains are source of outbreaks in USA, South America, Asia, and Australia (102). RFLP analysis of the ICP4 gene has been utilized in epidemiological studies differentiates between strains involved in outbreaks in Peru and Brazil (17, 18). Recent studies utilized full genome sequence to identify genomic changes related to virulence of live attenuated vaccine strains and field isolates (51). Furthermore, the phylogenetic analysis correlates with the previous division of USA isolates in nine major groups (108), indicating that there were no early recombination events in the USA or that the conserved nature of the genes does not allow for enough bioinformatics resolution to discover recombination events (51).

By RLFP, the Australian ILTV strains were classified in six classes, with the live attenuated vaccine strains SA2 and A20 classified as class 1 and the remaining five classified as field strains (10, 81). The introduction of the European CEO Serva vaccine (class 7) in Australia, where the Australian live attenuated SA2 and A20 strains were utilized, was followed by the emergence of outbreaks of the disease due to. natural recombination events, originating the Australian Class 8 and Class 9 strains (10), each one with a distinct in vivo phenotype, and significantly increased virulence as compared to the parental strains (88). Further studies utilizing full genome sequence confirmed the existence of multiple spontaneous recombination events in the Australian field strains Vl-99 (class 2) and CSW-l (class 4), which presented the ICP4 and US4 sequences from the Serva strain and UL27 sequence similar to that of Australian SA2 strain (90). Furthermore, (90) there is evidence that recombination events occurred frequently over the ILTV evolution, highlighting the risk of new distinct ILTV strains arising in chickens simultaneously infected with multiple ILTV strains.

Until now, the full DNA sequences of 27 vaccine strains and wild-type ILTV have been identified from Australia (88-90, 92), China (83), USA (19, 51, 144) and Italy (114) and are available at GenBank database. The use of this technique is advancing epidemiological studies of ILT and will further advance our understanding of genes involved in virulence.

**CONTROL**

Infectious laryngotracheitis is a widely distributed respiratory disease of chickens, which is usually preventable by means of biosecurity and vaccination. Quarantine,

hygiene and preventing the flow of potentially contaminated personnel, feed, equipment and birds is central to the successful prevention of ILT. Disinfectants and warm temperatures when outside the host rapidly inactivate the virus, thus, contamination between successive flocks can be prevented by adequate cleanup. All carcasses, feathers, feed, water, and litter should be removed from the facilities. Additionally, buildings and equipment should be washed and sprayed with disinfectants such as sodium hypochlorite, phenolics, iodophors or quaternary ammonium compound (50). Procedures such as the installation of air scrubbers, changes in house ventilation rates, and ionization systems show to reduce dust concentrations, could reduce or eliminate infectious particles from getting in or out of farms (115). No drug has been shown to be effective in reducing the severity of lesions or minimizing the clinical signs of the disease (50). Vaccination has proven to be a satisfactory method of developing resistance to ILT in susceptible chicken populations, limiting viral spread and abbreviating de duration of a disease when applied in face of an outbreak (4, 50). Once vaccination with live attenuated vaccines can result in latently infected birds, and its use is recommended only in geographic areas where ILTV is endemic (50).

Besides biosecurity, the main method of control of ILT in USA is through vaccination with live attenuated vaccines and recombinant viral-vectored vaccines. Live attenuated vaccines are originated from outbreak-related field strains that circulated in the USA between the 50's and 60's and were attenuated by serial passages in embryonated eggs, chicken embryo origin (CEO) (135) or in embryonic tissue culture origin (TCO) (52). TCO vaccines are licensed for eye-drop application only, whereas CEO vaccines can induce protective efficacy when applied through eye-drop and mass vaccination

routes such as coarse spray and drinking water. Some available live-attenuated vaccines provide different grades of protection when applied by different routes, particularly when applied by coarse spray or drinking water (47, 65). Successful vaccination with live attenuated vaccines via drinking water requires contact of virus with nasal epithelial cells as a result of the aspiration of the virus through the external nares and choanae. This contact might be poor in chickens vaccinated through drinking water, resulting in poorly vaccinated birds (131). In the same way, spray vaccination can result in low coverage, deep penetration in the lungs due to small droplets size, or excessive dose (23, 123). Eye-drop vaccination has been demonstrated to be the optimal delivery method to ensure uniform and effective protection (47). Vaccination with live attenuated vaccines has been proven to be a satisfactory method for developing resistance to the disease in susceptible chicken populations. A study comparing the protection efficacy of both live attenuated ILTV strains administered via eye-drop route, concluded that the protection of the CEO vaccine is significantly higher than that of the TCO vaccine, based on clinical signs scores and reduction of challenge virus (155). Moreover, both vaccine strains replicate in the eye conjunctiva and the trachea of infected chickens, however the CEO strain replicates and spreads faster and reaching higher viral genome load than the TCO strain (132). Although protective, the use of this type of vaccine is only recommended in geographic areas where the disease is enzootic because live attenuated vaccine strains can establish life-long latent infections and contribute to the spread of the disease and persistence of the virus in the field (50).

The advance of recombinant DNA technology allowed for the production of recombinant vaccines utilizing the turkey herpesvirus (HVT) and fowl poxvirus (FPV) as

vectors to express exogenous viral proteins. Commercially available in the USA are the HVT vector containing the ILTV glycoproteins D and I (Innovax® ILT) (70); the HVT vector expressing the ILTV glycoprotein B (Vectormune® HVT-ILT) (39); and the FPV vector containing the ILTV glycoprotein B and Unique Long 32 (Vectormune® FP-ILT) (70, 150). The hallmark of these products is their safety for the chickens, including *in ovo* vaccination and lack of transmission and reversion to virulence.

Recombinant vaccines are most commonly applied *in ovo* and to a lesser extent via subcutaneous or wing-web vaccination post-hatch. S. Davison, et al. (34) reported that FPV-ILT provides adequate protection against ILTV in layers vaccinated at seven weeks of age via wing-web and challenged at 20 weeks of age, however, only partial protection, as compared to that of CEO vaccine, was achieved in commercial broilers vaccinated *in ovo* with HVT-ILT and FPV-ILT vaccines (70). Significant differences were also observed between the protection efficiency induced by the two different viral vectors. A. Vagnozzi, et al. (155) reported that HVT-ILT was more effective than FPV-ILT when delivered *in ovo* or subcutaneously (one-day of age), mitigating the disease and reducing levels of viral challenge virus in the trachea. Nonetheless, studies with FPV and HVT vectored vaccines have indicated that the development of protective immunity may be delayed compared to immunity induced by live attenuated vaccines (155).

In the USA, most broiler breeders and commercial layers are vaccinated twice against ILTV either with TCO vaccine via eye-drop route, with CEO vaccine via drinking water, eye-drop or coarse spray, or with recombinant viral vector vaccines (FPV-LT and HVT-LT) via *in ovo*, subcutaneous or wing-web application; whereas commercial broilers are vaccinated only in the face of an outbreak with recombinant vaccines *in ovo*

or with CEO vaccine via drinking water at 12 or 16-days of age.

*In ovo* vaccination is a highly effective mass vaccination method due to the uniform coverage, fast delivery, reduction in bird handling, and lower costs when compared to other field vaccination methods (127). This mass vaccination method has facilitated the delivery of recombinant vaccines, becoming a standard field procedure for immunization of broilers and broiler breeders in the United States against Marek's disease (MD) (48, 106, 165). Embryo vaccination against MD including the serotypes 3 (MDV or HVT) (169), a combination of serotypes 2 (SB1) and 3 (14, 137, 168), and an attenuated serotype 1 (CVI-988) (128, 129) is considered safe. MD vaccine for *in ovo* administration have been commercially available in the US since 1992 (136). Other vaccines commonly applied *in ovo* are infectious bursal disease (IBDV) and Fowlpox disease (FPV).

In the future, he use of respiratory viruses as vectors is likely to facilitate mass application of viral-vectored vaccines, in a similar way to commercial live attenuated vaccines (1). For instance, two NDV LaSota recombinants expressing ILTV gB of gD have been generated and shown to induce up to 90% protection against clinical signs of ILT and reduce challenge virus loads in the trachea. Moreover, the NDV-gB strain induced protection level similar to that of the ILTV CEO vaccine (75).

As a vaccine adjuvant and an immune-modulatory molecule, the interleukin 18 (IL-18) has been shown to regulate the immune response toward a Th1 type (105). Furthermore, a recombinant FPV vector expressing the ILTV gB and the chicken IL-18 was generated and applied via wing-web in SPF chickens and shown to induce high protection efficacy (100%) post-challenge on preliminary studies. The study suggests that

the higher level of protection with this recombinant virus is due to the higher T-lymphocyte proliferation (CD4+ and CD8+) known to be significantly involved on the protective efficacy against ILTV (105).

## REFERENCES


1. Armour NK, García M. 2014. Current and Future Applications of Viral-Vectored Recombinant Vaccines in Poultry. The Poultry Informed Professional:1-9.
2. Bagust TJ. 1986. Laryngotracheitis (Gallid-1) herpesvirus infection in the chicken. 4. Latency establishment by wild and vaccine strains of ILT virus. Avian Pathol 15:581-595.
3. Bagust TJ, Calnek BW, Fahey KJ. 1986. Gallid-1 herpesvirus infection in the chicken. 3. Reinvestigation of the pathogenesis of infectious laryngotracheitis in acute and early post-acute respiratory disease. Avian Dis 30:179-190.
4. Bagust TJ, Johnson MA. 1995. Avian infectious laryngotracheitis: virus-host interactions in relation to prospects for eradication. Avian Pathol 24:373-391.
5. Bagust TJ, Jones RC, Guy JS. 2000. Avian infectious laryngotracheitis. Rev Sci Tech 19:483-492.
6. Beach JR. 1931. A Filtrable Virus, the Cause of Infectious Laryngotracheitis of Chickens. J Exp Med 54:809-816.
7. Beach JR. 1930. The Virus of Laryngotracheitis of Fowls. Science 72:633-634.
8. Beaudette F. 1937. Infectious laryngotracheitis. Poultry Science 16:103-105.
9. Biggs PM. 1982. The world of poultry disease. Avian Pathol 11:281-300.
10. Blacker HP, Kirkpatrick NC, Rubite A, O'Rourke D, Noormohammadi AH. 2011. Epidemiology of recent outbreaks of infectious laryngotracheitis in poultry in Australia. Aust Vet J 89:89-94.
11. Brandly CA. 1936. Studies on the Egg-Propagated Viruses of Infectious Laryngotracheitis and Fowl-Pox. Journal of the American Veterinary Medical Association 88:587-599.

12. Brandly CA, Bushnell LD. 1934. A Report of Some Investigations of Infectious Laryngotracheitis. Poultry Science 13:212-217.

13. Bryant NA, Davis-Poynter N, Vanderplasschen A, Alcami A. 2003. Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. EMBO Journal 22:833-846.

14. Calnek BW, Schat KA, Peckham MC, Fabricant J. 1983. Field trials with a bivalent vaccine (HVT and SB-1) against Marek's disease. Avian Dis 27:844-849.

15. Cantello JL, Anderson AS, Morgan RW. 1994. Identification of latency-associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. J Virol 68:6280-6290.

16. Cantello JL, Parcells MS, Anderson AS, Morgan RW. 1997. Marek's disease virus latency-associated transcripts belong to a family of spliced RNAs that are antisense to the ICP4 homolog gene. J Virol 71:1353-1361.

17. Chacon JL, Ferreira AJ. 2009. Differentiation of field isolates and vaccine strains of infectious laryngotracheitis virus by DNA sequencing. Vaccine 27:6731-6738.

18. Chacon JL, Nunez LF, Vejarano MP, Parra SH, Astolfi-Ferreira CS, Ferreira AJ. 2015. Persistence and spreading of field and vaccine strains of infectious laryngotracheitis virus (ILTV) in vaccinated and unvaccinated geographic regions, in Brazil. Trop Anim Health Prod 47:1101-1108.

19. Chandra YG, Lee J, Kong BW. 2012. Genome sequence comparison of two United States live attenuated vaccines of infectious laryngotracheitis virus (ILTV). Virus Genes 44:470-474.

20. Chang PW, Yates VJ, Dardiri AH, Fry DE. 1960. Some Observations of the Propagation of Infectious Laryngotracheitis Virus in Tissue Culture, p 384. American Association of Avian Pathologists, Inc.

21. Chen HY, Cui P, Cui BA, Li HP, Jiao XQ, Zheng LL, Cheng G, Chao AJ. 2011. Immune responses of chickens inoculated with a recombinant fowlpox vaccine coexpressing glycoprotein B of infectious laryngotracheitis virus and chicken IL-18. FEMS Immunol Med Microbiol 63:289-295.

22. Chin RP, García M, Corsiglia C, Riblet SM, Crespo R, Shivaprasad HL, Rodriguez-Avila A, Woolcock PR, Franca M. 2009. Intervention strategies for laryngotracheitis: impact of extended downtime and enhanced biosecurity auditing. Avian Dis 53:574-577.

23. Clarke JK, Robertson GM, Purcell DA. 1980. Spray vaccination of chickens using infectious laryngotracheitis virus. Aust Vet J 56:424-428.

24. Coppo MJ, Devlin JM, Noormohammadi AH. 2012. Comparison of the replication and transmissibility of two infectious laryngotracheitis virus chicken embryo origin vaccines delivered via drinking water. Avian Pathol 41:195-202.

25. Coppo MJ, Hartley CA, Devlin JM. 2013. Immune responses to infectious laryngotracheitis virus. Dev Comp Immunol 41:454-462.

26. Cover MS, Benton WJ. 1958. The Biological Variation of the Infectious Laryngotracheitis Virus, p 375. Cornell Veterinarian, Inc.

27. Cover MS, Benton WJ, Krauss WC. 1960. The Effect of Parental Immunity and Age on the Response to Infectious Laryngotracheitis Vaccination, p 467. American Association of Avian Pathologists, Inc.

28. Crawshaw GJ, Boycott BR. 1982. Infectious laryngotracheitis in peafowl and pheasants. Avian Dis 26:397-401.

29. Cruickshank JG, Berry DM, Hay B. 1963. The fine structure of infectious laryngotracheitis virus. Virology 20:376-378.

30. David HK, James WH, Calvin LK, Jr. 1994. Identification and Characterization of the Infectious Laryngotracheitis Virus Glycoprotein C Gene. Virology 203:336.

31. Davison AJ, Eberle R, Ehlers B, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2009. The order Herpesvirales. Arch Virol 154:171-177.

32. Davison AJ, Eberle R, Hayward GS, McGeoch DJ, Minson AC, Pellett PE, Roizman B, Studdert MJ, Thiry E. 2005. Herpesviridae, p 193-212. *In* Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (ed), Virus taxonomy: eighth report of the International Committee on Taxonomy of Viruses. Elsevier Academic Press, San Diego.

33. Davison S. Vaccinal Laryngotracheitis - Overview in the United States, p 580-618. *In* (ed),

34. Davison S, Gingerich EN, Casavant S, Eckroade RJ. 2006. Evaluation of the efficacy of a live fowlpox-vectored infectious laryngotracheitis/avian encephalomyelitis vaccine against ILT viral challenge. Avian Diseases 50:50-54.

35. Davison S, Smith G, Eckroade RJ. 1989. Laryngotracheitis in chickens: the length of the preinfectious and infectious periods. Avian Dis 33:18-23.

36. Devlin JM, Mahmoudian A, Noormohammadi AH, Gilkerson JR, Browning GF, Hartley CA, Kirkpatrick NC. 2006. Glycoprotein G is a virulence factor in infectious laryngotracheitis virus [electronic resource]. Journal of general virology 87:2839-2847.

37. Devlin JM, Viejo-Borbolla A, Browning GF, Noormohammadi AH, Gilkerson JR, Alcami A, Hartley CA. 2010. Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus. Vaccine 28:1325-1332.

38. Dufour-Zavala L. 2008. Epizootiology of infectious laryngotracheitis and presentation of an industry control program. Avian Dis 52:1-7.

39. Esaki M, Noland L, Eddins T, Godoy A, Saeki S, Saitoh S, Yasuda A, Dorsey KM. 2013. Safety and Efficacy of a Turkey Herpesvirus Vector Laryngotracheitis Vaccine for Chickens. Avian Diseases 57:192-198.

40. Fahey KJ, Bagust TJ, York JJ. 1983. Laryngotracheitis herpesvirus infection in the chicken: the role of humoral antibody in immunity to a graded challenge infection. Avian Pathol 12:505-514.

41. Fahey KJ, York JJ. 1990. The role of mucosal antibody in immunity to infectious laryngotracheitis virus in chickens. J Gen Virol 71 ( Pt 10):2401-2405.

42. Fuchs W, Mettenleiter TC. 1996. DNA sequence and transcriptional analysis of the UL1 to UL5 gene cluster of infectious laryngotracheitis virus. Journal of General Virology 77:2221-2229.

43. Fuchs W, Mettenleiter TC. 1999. DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL 10 gene product as a nonglycosylated and nonessential virion protein. Journal of General Virology 80:2173-2182.

44. Fuchs W, Mettenleiter TC. 2005. The nonessential UL49.5 gene of infectious laryngotracheitis virus encodes an O-glycosylated protein which forms a complex with the non-glycosylated UL10 gene product. Virus Res 112:108-114.

45. Fuchs W, Veits J, Helferich D, Granzow H, Teifke JP, Mettenleiter TC. 2007. Molecular biology of avian infectious laryngotracheitis virus. Vet Res 38:261-279.

46. Fuchs W, Wiesner D, Veits J, Teifke JP, Mettenleiter TC. 2005. In vitro and in vivo relevance of infectious laryngotracheitis virus gJ proteins that are expressed from spliced and nonspliced mRNAs. J Virol 79:705-716.

47. Fulton RM, Schrader DL, Will M. 2000. Effect of route of vaccination on the prevention of infectious laryngotracheitis in commercial egg-laying chickens. Avian Dis 44:8-16.

48. Gagic M, St Hill CA, Sharma JM. 1999. In ovo vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis 43:293-301.

49. García M, Cheng Y, Spatz SJ, Riblet SM, Schneiders GH, Volkening J. 2016 Submitted. Attenuation and Protection Efficacy of Open Reading Frame C (ORF C) Gene Deleted Strain of The Alphaherpesvirus Infectious Laryngotracheitis Virus (ILTV)

50. García M, Spatz S, Guy JS. 2013. Laryngotracheitis, p 161-179. *In* Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair V (ed), Diseases of Poultry, 13th ed. Blackwell Publishing, Ames, Iowa.

51. García M, Volkening J, Riblet SM, Spatz S. 2013. Genomic sequence analysis of the United States infectious laryngotracheitis vaccine strains chicken embryo origin (CEO) and tissue culture origin (TCO). Virology 440:64-74.

52. Gelenczei EF, Marty EW. 1964. Studies on a Tissue-Culture-Modified Infectious Laryngotracheitis Virus, p 105. American Association of Avian Pathologists, Inc.

53. Gibbs CS. 1933. The Massachusetts Plan for the Eradication and Control of Infectious Laryngotraeheitis. Journal of the American Veterinary Medical Association 83:214-217.

54. Granzow H, Klupp BG, Fuchs W, Veits J, Osterrieder N, Mettenleiter TC. 2001. Egress of alphaherpesviruses: comparative ultrastructural study. J Virol 75:3675-3684.

55. Guo P, Scholz E, Maloney B, Welniak E. 1994. Construction of recombinant avian infectious laryngotracheitis virus expressing the beta-galactosidase gene and DNA sequencing of the insertion region. Virology 202:771-781.

56. Guo P, Scholz E, Turek J, Nodgreen R, Maloney B. 1993. Assembly pathway of avian infectious laryngotracheitis virus. Am J Vet Res 54:2031-2039.

57. Guy JS, Barnes HJ, Morgan LM. 1990. Virulence of Infectious Laryngotracheitis Viruses: Comparison of Modified-Live Vaccine Viruses and North Carolina Field Isolates, p 106. American Association of Avian Pathologists, Inc.

58. Guy JS, Barnes HJ, Munger LL, Rose L. 1989. Restriction endonuclease analysis of infectious laryngotracheitis viruses: comparison of modified-live vaccine viruses and North Carolina field isolates. Avian Dis 33:316-323.

59. Guy JS, Barnes HJ, Smith L. 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis 35:348-355.

60. Guy JS, Barnes HJ, Smith LG. 1992. Rapid diagnosis of infectious laryngotracheitis using a monoclonal antibody-based immunoperoxidase procedure. Avian Pathol 21:77-86.

61. Han MG, Kim SJ. 2001. Analysis of Korean strains of infectious laryngotracheitis virus by nucleotide sequences and restriction fragment length polymorphism. Vet Microbiol 83:321-331.

62. Han MG, Kim SJ. 2001. Comparison of virulence and restriction endonuclease cleavage patterns of infectious laryngotracheitis viruses isolated in Korea. Avian Pathol 30:337-344.

63. Hayles LB, Hamilton D, Newby WC. 1976. Transfer of parental immunity to infectious laryngotracheitis in chicks. Can J Comp Med 40:218-219.

64. Helferich D, Veits J, Teifke JP, Mettenleiter TC, Fuchs W. 2007. The UL47 gene of avian infectious laryngotracheitis virus is not essential for in vitro replication but is relevant for virulence in chickens. J Gen Virol 88:732-742.

65. Hilbink FW, Oei HL, Roozelaar DJv. 1987. Virulence of five live vaccines against avian infectious laryngotracheitis and their immunogenicity and spread after eyedrop or spray application. Veterinary Quarterly 9:215-225.

66. Hughes CS, Gaskell RM, Bradbury JM, Jordan FT, Jones RC. 1991. Survey of field outbreaks of avian infectious laryngotracheitis in England and Wales. Vet Rec 129:258-260.

67. Hughes CS, Gaskell RM, Jones RC, Bradbury JM, Jordan FT. 1989. Effects of certain stress factors on the re-excretion of infectious laryngotracheitis virus from latently infected carrier birds. Res Vet Sci 46:274-276.

68. Hughes CS, Jones RC. 1988. Comparison of cultural methods for primary isolation of infectious laryngotracheitis virus from field material. Avian Pathology 17:295-303.

69. Hughes CS, Jones RC, Gaskell RM, Jordan FT, Bradbury JM. 1987. Demonstration in live chickens of the carrier state in infectious laryngotracheitis. Res Vet Sci 42:407-410.

70. Johnson DI, Vagnozzi A, Dorea F, Riblet SM, Mundt A, Zavala G, García M. 2010. Protection against infectious laryngotracheitis by in ovo vaccination with commercially available viral vector recombinant vaccines. Avian Dis 54:1251-1259.

71. Johnson MA, Prideaux CT, Kongsuwan K, Sheppard M, Fahey KJ. 1991. Gallid herpesvirus 1 (infectious laryngotracheitis virus): cloning and physical maps of the SA-2 strain. Archives of Virology 119:181-198.

72. Johnson YJ, Gedamu N, Colby MM, Myint MS, Steele SE, Salem M, N.L.Tablante. 2005. Wind-Borne Transmission of Infectious Laryngotracheitis Between Commercial Poultry Operations. International Journal of Poultry Science:263.

73. Jordan FTW. 1981. Immunity to infectious laryngotracheitis. Avian Immunology:245–254.

74. Jordan FTW. 1966. A Review of the Literature on Infectious Laryngotracheitis (ILT), p 1. American Association of Avian Pathologists, Inc.

75. Kanabagatte Basavarajappa M, Kumar S, Khattar SK, Gebreluul GT, Paldurai A, Samal SK. 2014. A recombinant Newcastle disease virus (NDV) expressing infectious laryngotracheitis virus (ILTV) surface glycoprotein D protects against highly virulent ILTV and NDV challenges in chickens. Vaccine 32:3555-3563.

76. Kawaguchi T, Nomura K, Hirayama Y, Kitagawa T. 1987. Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. Cancer Res 47:4460-4464.

77. Kingsbury FW, Jungherr EL. 1958. Indirect Transmission of Infectious Laryngotracheitis in Chickens, p 54. Cornell Veterinarian, Inc.

78. Kingsley DH, Hazel JW, Keeler CL, Jr. 1994. Identification and characterization of the infectious laryngotracheitis virus glycoprotein C gene. Virology 203:336-343.

79. Kingsley DH, Keeler CL, Jr. 1999. Infectious laryngotracheitis virus, an alpha herpesvirus that does not interact with cell surface heparan sulfate. Virology 256:213-219.

80. Kirkpatrick NC, Mahmoudian A, Colson CA, Devlin JM, Noormohammadi AH. 2006. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. Avian Pathol 35:449-453.

81. Kirkpatrick NC, Mahmoudian A, O'Rourke D, Noormohammadi AH. 2006. Differentiation of infectious laryngotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. Avian Dis 50:28-34.

82. Komarov A, Beaudette FR. 1932. Carriers of Infectious Bronchitis. Poultry Science 11:335.

83. Kong C, Zhao Y, Cui X, Zhang X, Cui H, Xue M, Wang Y. 2013. Complete genome sequence of the first Chinese virulent infectious laryngotracheitis virus. PLoS One 8:e70154.

84. Kongsuwan K, Johnson MA, Prideaux CT, Sheppard M. 1993. Identification of an infectious laryngotracheitis virus gene encoding an immunogenic protein with a predicted M(r) of 32 kilodaltons. Virus Res 29:125-140.

85. Koski DM, Predgen AS, Trampel DW, Conrad SK, Narwold DR, Hermann JR. 2015. Comparison of the pathogenicity of the USDA challenge virus strain to a field strain of infectious laryngotracheitis virus. Biologicals 43:232-237.

86. Kotiw M, Sheppard M, May JT, Wilks CR. 1986. Differentiation between virulent and avirulent strains of infectious laryngotracheitis virus by DNA:DNA hybridization using a cloned DNA marker. Vet Microbiol 11:319-330.

87. Lee JY, Bottje WG, Kong BW. 2012. Genome-wide host responses against infectious laryngotracheitis virus vaccine infection in chicken embryo lung cells. BMC Genomics 13:(24 A-(24 A.

88. Lee S, Markham PF, Coppo MJC, Legione AR, Markham JF, Noormohammadi AH, Browning GF, Ficorilli N, Hartley CA, Devlin JM. 2012. Attenuated vaccines can recombine to form virulent field viruses. Science (Washington) 337:188-188.

89. Lee SW, Devlin JM, Markham JF, Noormohammadi AH, Browning GF, Ficorilli NP, Hartley CA, Markham PF. 2011. Comparative analysis of the complete genome sequences of two Australian origin live attenuated vaccines of infectious laryngotracheitis virus. Vaccine 29:9583-9587.

90. Lee SW, Devlin JM, Markham JF, Noormohammadi AH, Browning GF, Ficorilli NP, Hartley CA, Markham PF. 2013. Phylogenetic and molecular epidemiological studies

reveal evidence of multiple past recombination events between infectious laryngotracheitis viruses. PLoS One 8:e55121.

91. Lee SW, Hartley CA, Coppo MJ, Vaz PK, Legione AR, Quinteros JA, Noormohammadi AH, Markham PF, Browning GF, Devlin JM. 2015. Growth kinetics and transmission potential of existing and emerging field strains of infectious laryngotracheitis virus. PLoS One 10:e0120282.

92. Lee SW, Markham PF, Markham JF, Petermann I, Noormohammadi AH, Browning GF, Ficorilli NP, Hartley CA, Devlin JM. 2011. First complete genome sequence of infectious laryngotracheitis virus. BMC Genomics 12:197.

93. Leib D, Bradbury J, Hart C, McCarthy K. 1987. Genome isomerism in two alphaherpesviruses: Herpesvirus saimiri-1 (Herpesvirus tamarinus) and avian infectious laryngotracheitis virus. Archives of Virology 93:287.

94. Leong VY, Glisson JR, Resurreccion RS, Cheng IH. 1994. Infectious laryngotracheitis virus in commercial hens: a serological study based on enzyme-linked immunosorbent assay. Avian Dis 38:304-307.

95. Li H, Wang F, Han Z, Gao Q, Li H, Shao Y, Sun N, Liu S. 2015. Genome-Wide Gene Expression Analysis Identifies the Proto-oncogene Tyrosine-Protein Kinase Src as a Crucial Virulence Determinant of Infectious Laryngotracheitis Virus in Chicken Cells. J Virol 90:9-21.

96. Linares JA, Bickford AA, Cooper GL, Charlton BR, Woolcock PR. 1994. An outbreak of infectious laryngotracheitis in California broilers. Avian Dis 38:188-192.

97. Mahmoudian A, Markham PF, Noormohammadi AH, Browning GF. 2012. Kinetics of transcription of infectious laryngotracheitis virus genes. Comparative Immunology, Microbiology and Infectious Diseases 35:103-115.

98. Mashchenko A, Riblet SM, Zavala G, García M. 2013. In Ovo Vaccination of Commercial Broilers with a Glycoprotein J Gene-Deleted Strain of Infectious Laryngotracheitis Virus. Avian Diseases 57:523-531.

99. McGeoch DJ, Dalrymple MA, Davison AJ, Dolan A, Frame MC, McNab D, Perry LJ, Scott JE, Taylor P. 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. The Journal Of General Virology 69 ( Pt 7):1531-1574.

100. McGeoch DJ, Dolan A, Ralph AC. 2000. Toward a comprehensive phylogeny for mammalian and avian herpesviruses. Journal of virology 74:10401-10406.

101. McNulty MS, Allan GM, McCracken RM. 1985. Infectious laryngotracheitis in Ireland. Irish Veterinary Journal 39:124-125.

102. Menendez KR, García M, Spatz S, Tablante NL. 2014. Molecular epidemiology of infectious laryngotracheitis: a review. Avian Pathol 43:108-117.

103. Mettenleiter TC. 2002. Herpesvirus assembly and egress. J Virol 76:1537-1547.

104. Mingxiao M, Ningyi J, Zhenguo W, Ruilin W, Dongliang F, Min Z, Gefen Y, Chang L, Leili J, Kuoshi J, Yingjiu Z. 2006. Construction and immunogenicity of recombinant fowlpox vaccines coexpressing HA of AIV H5N1 and chicken IL18. Vaccine 24:4304-4311.

105. Nakanishi K, Yoshimoto T, Tsutsui H, Okamura H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. Annu Rev Immunol 19:423-474.

106. Ohta H, Ezoe S, Yamazaki K, Kawai T, Honda T. 2009. Application of aluminum hydroxide for an in ovo live Newcastle disease vaccine. Avian Dis 53:392-395.

107. Ojkic D, Swinton J, Vallieres M, Martin E, Shapiro J, Sanei B, Binnington B. 2006. Characterization of field isolates of infectious laryngotracheitis virus from Ontario. Avian Pathol 35:286-292.

108. Oldoni I, García M. 2007. Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathology: Journal Of The WVPA 36:167-176.

109. Oldoni I, Rodriguez-Avila A, Riblet SM, García M. 2008. Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). Avian Dis 52:59-63.

110. Oldoni I, Rodriguez-Avila A, Riblet SM, Zavala G, García M. 2009. Pathogenicity and growth characteristics of selected infectious laryngotracheitis virus strains from the United States. Avian Pathol 38:47-53.

111. Parra SH, Nunez LF, Astolfi-Ferreira CS, Ferreira AJ. 2015. Persistence of the tissue culture origin vaccine for infectious laryngotracheitis virus in commercial chicken flocks in Brazil. Poult Sci 94:2608-2615.

112. Parra SHS, Nunez LFN, Astolfi-Ferreira CS, Ferreira JP. 2015. Occurrence of Infectious Laryngotracheitis Virus (Iltv) in 2009-2013 in the State of Sao Paulo - Brazil. Brazilian Journal of Poultry Science 17:117-120.

113. Pereira L. 1994. Function of glycoprotein B homologues of the family herpesviridae. Infect Agents Dis 3:9-28.

114. Piccirillo A, Lavezzo E, Niero G, Moreno A, Massi P, Franchin E, Toppo S, Salata C, Palu G. 2016. Full Genome Sequence-Based Comparative Study of Wild-Type and Vaccine Strains of Infectious Laryngotracheitis Virus from Italy. PLoS One 11:e0149529.

115. Pitesky M, Chin RP, Carnaccini S, Senties-Cue CG, Charlton B, Woolcock PR, Shivaprasad HL. 2014. Spatial and temporal epidemiology of infectious laryngotracheitis in central California: 2000-2012. Avian Dis 58:558-565.

116. Pizer LI, Tedder DG, Betz JL, Wilcox KW, Beard P. 1986. Regulation of transcription in vitro from herpes simplex virus genes. J Virol 60:950-959.

117. Portz C, Beltrão N, Furian TQ, Bianco Júnior A, Macagnan M, Griebeler J, Rosa CAVL, Colodel EM, Driemeier D, Back A, Schatzmayr OMB, Canal CW. 2008. Natural infection of turkeys by infectious laryngotracheitis virus. Veterinary Microbiology 131:57-64.

118. Poulsen DJ, Burton CR, O'Brian JJ, Rabin SJ, Keeler CL, Jr. 1991. Identification of the infectious laryngotracheitis virus glycoprotein gB gene by the polymerase chain reaction. Virus Genes 5:335-347.

119. Poulsen DJ, Keeler CL. 1997. Characterization of the assembly and processing of infectious laryngotracheitis virus glycoprotein B. Journal of General Virology 78:2945.

120. Pulsford MF. 1963. Infectious laryngotracheitis of poultry: Part I. Virus variation, immunology and vaccination. Veterinary Bulletin 33:415-420.

121. Pulsford MF, Stokes J. 1953. INFECTIOUS LARYNGOTRACHEITIS IN SOUTH AUSTRALIA: A N ote on its O ccurrence and a S urvey of the D istribution of A ntibodies A ctive A gainst the V irus. Australian Veterinary Journal 29:8.

122. Purcell DA. 1971. The ultrastructural changes produced by infectious laryngotracheitis virus in tracheal epithelium of the fowl. Res Vet Sci 12:455-458.

123. Purcell DA, Surman PG. 1974. Letter: Aerosol administration of the SA-2 vaccine strain of infectious laryngotracheitis virus. Aust Vet J 50:419-420.

124. Rachamadugu R, Lee JY, Wooming A, Kong BW. 2009. Identification and expression analysis of infectious laryngotracheitis virus encoding microRNAs. Virus Genes 39:301-308.

125. Raggi LG, Brownell JR, Stewart GF. 1961. Effects of Infectious Laryngotracheitis Virus on Egg Production and Quality. Poultry Science 40:134.

126. Reddy VR, Steukers L, Li Y, Fuchs W, Vanderplasschen A, Nauwynck HJ. 2014. Replication characteristics of infectious laryngotracheitis virus in the respiratory and conjunctival mucosa. Avian Pathol 43:450-457.

127. Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, King S, Murray L, Phelps P, Poston R, Whitfill C, Williams C. 1999. In ovo vaccination technology. Adv Vet Med 41:495-515.

128. Rispens BH, van Vloten H, Mastenbroek N, Maas HJ, Schat KA. 1972. Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis 16:108-125.

129. Rispens BH, van Vloten H, Mastenbroek N, Maas JL, Schat KA. 1972. Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease virus. Avian Dis 16:126-138.

130. Robertson GM. 1977. The role of bursa-dependent responses in immunity to infectious laryngotracheitis. Res Vet Sci 22:281-284.

131. Robertson GM, Egerton JR. 1981. Replication of infectious laryngotracheitis virus in chickens following vaccination. Aust Vet J 57:119-123.

132. Rodriguez-Avila A, Oldoni I, Riblet SM, García M. 2007. Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines. Avian Dis 51:905-911.

133. Roizman B, Pellett PE. 2001. The family Herpesviridae: A brief introduction, p 2381–2397. *In* Knipe DM, Howley PM (ed), In: Fields Virology. Lippincott Williams & Wilkins, Philadelphia.

134. Russell RG, Turner AJ. 1983. Characterization of infectious laryngotracheitis viruses, antigenic comparison by kinetics of neutralization and immunization studies. Can J Comp Med 47:163-171.

135. Samberg Y, Cuperstein E, Bendheim U, Aronovici I. 1971. The development of a vaccine against avian infectious laryngotracheitis. IV. Immunization of chickens with a modified laryngotracheitis vaccine in the drinking water. Avian Dis 15:413-417.

136. Sarma G, Greer W, Gildersleeve RP, Murray DL, Miles AM. 1995. Field safety and efficacy of in ovo administration of HVT + SB-1 bivalent Marek's disease vaccine in commercial broilers. Avian Dis 39:211-217.

137. Schat KA, Calnek BW. 1978. Characterization of an apparently nononcogenic Marek's disease virus. J Natl Cancer Inst 60:1075-1082.

138. Schneider K, Puehler F, Baeuerle D, Elvers S, Staeheli P, Kaspers B, Weining KC. 2000. cDNA cloning of biologically active chicken interleukin-18. J Interferon Cytokine Res 20:879-883.

139. Seal BS, Whetstone CA, Zamb TJ, Bello LJ, Lawrence WC. 1992. Relationship of bovine herpesvirus 1 immediate-early, early, and late gene expression to host cellular gene transcription. Virology 188:152-159.

140. Seddon HR, Hart L. 1936. Infectivity Experiments with the Virus of Laryngotracheitis of Fowls. Australian Veterinary Journal 12:13-16.

141. Sellers HS, García M, Glisson JR, Brown TP, Sander JS, Guy JS. 2004. Mild infectious laryngotracheitis in broilers in the southeast. Avian Dis 48:430-436.

142. Shibley GP, Luginbuhl RE, Helmboldt CF. 1962. A Study of Infectious Laryngotracheitis Virus. I. Comparison of Serologic and Immunogenic Properties, p 59. American Association of Avian Pathologists, Inc.

143. Sivaseelan S, Rajan T, Malmarugan S, Balasubramaniam GA, Madheswaran R. 2014. Tissue Tropism and Pathobiology of Infectious Laryngotracheitis Virus in Natural Cases of Chickens. 69:197-202.

144. Spatz SJ, Volkening JD, Keeler CL, Kutish GF, Riblet SM, Boettger CM, Clark KF, Zsak L, Afonso CL, Mundt ES, Rock DL, García M. 2012. Comparative full genome analysis of four infectious laryngotracheitis virus (Gallid herpesvirus-1) virulent isolates from the United States. Virus Genes 44:273-285.

145. Spear PG. 1993. Entry of alphaherpesviruses into cells. Seminars in virology 4:167-180.

146. Steinhaus EA, Martignoni ME. An abridged glossary of terms used in invertebrate pathology [2d ed. Portland, Or.] 1970.

147. Tanada Y, Kaya HK. 1993. Insect pathology. San Diego : Academic Press, c1993.

148. Thureen DR, Keeler CL, Jr. 2006. Psittacid herpesvirus 1 and infectious laryngotracheitis virus: comparative genome sequence analysis of two avian alphaherpesviruses. Journal of Virology 80:7863-7872.

149. Timurkaan N, Yilmaz F, Bulut H, Ozer H, Bolat Y. 2003. Pathological and immunohistochemical findings in broilers inoculated with a low virulent strain of infectious laryngotracheitis virus. J Vet Sci 4:175-180.

150. Tong GZ, Zhang SJ, Wang L, Qiu HJ, Wang YF, Wang M. 2001. Protection of chickens from infectious laryngotracheitis with a recombinant fowlpox virus expressing glycoprotein B of infectious laryngotracheitis virus. Avian Pathol 30:143-148.

151. Tripathy DN, Garcia M. 2008. Laryngotracheitis, p 94-98. *In* Dufour-Zavala L, Swayne DE, Glisson JR, Pearson JE, Reed WM, Jackwood MW, Woolcock PR (ed), A laboratory manual for the isolation and identification of avian pathogens, 5th ed. American Association of Avian Pathologists, Madison, Wisconsin.

152. Vagnozzi A, García M, Riblet SM, Zavala G. 2010. Protection induced by infectious laryngotracheitis virus vaccines alone and combined with Newcastle disease virus and/or infectious bronchitis virus vaccines. Avian Dis 54:1210-1219.

153. Vagnozzi A, Riblet SM, Williams SM, Zavala G, García M. 2015. Infection of Broilers with Two Virulent Strains of Infectious Laryngotracheitis Virus: Criteria for Evaluation of Experimental Infections. Avian Diseases 59:394.

154. Vagnozzi A, Riblet SM, Zavala G, García M. 2012. Optimization of a duplex real-time PCR method for relative quantitation of infectious laryngotracheitis virus. Avian Dis 56:406-410.

155. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian Pathol 41:21-31.

156. Vanderkop MA. 1993. Infectious laryngotracheitis in commercial broiler chickens. Can Vet J 34:185.

157. Veits J, Köllner B, Teifke JP, Granzow H, Mettenleiter TC, Fuchs W. 2003. Isolation and characterization of monoclonal antibodies against structural proteins of Infectious laryngotracheitis virus, p 330. American Association of Avian Pathologists, Inc.

158. Veits J, Luschow D, Kindermann K, Werner O, Teifke JP, Mettenleiter TC, Fuchs W. 2003. Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT)

virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. J Gen Virol 84:3343-3352.

159. Vivo LM, Masdeu V. 1980. Infectious laryngotracheitis in a peacock (Pavo cristatus) under natural conditions in Cuba. / Presencia de la laringotraqueitis infecciosa aviar en el pavo real (Pavo cristatus) en las condiciones naturales en Cuba. Revista Avicultura, Cuba 24:205-210.

160. Volkova V, Thornton D, Hubbard SA, Magee D, Cummings T, Luna L, Watson J, Wills R. 2012. Factors Associated with Introduction of Infectious Laryngotracheitis Virus on Broiler Farms During a Localized Outbreak, p 521. American Association of Avian Pathologists.

161. Wahid F, Shehzad A, Khan T, Kim YY. 2010. MicroRNAs: Synthesis, mechanism, function, and recent clinical trials. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1803:1231-1243.

162. Waidner LA, Burnside J, Anderson AS, Bernberg EL, German MA, Meyers BC, Green PJ, Morgan RW. 2011. A microRNA of infectious laryngotracheitis virus can downregulate and direct cleavage of ICP4 mRNA. Virology 411:25-31.

163. Waidner LA, Morgan RW, Anderson AS, Bernberg EL, Kamboj S, Garcia M, Riblet SM, Ouyang M, Isaacs GK, Markis M, Meyers BC, Green PJ, Burnside J. 2009. MicroRNAs of Gallid and Meleagrid herpesviruses show generally conserved genomic locations and are virus-specific. Virology 388:128-136.

164. Watrach AM, Hanson LE, Watrach MA. 1963. The structure of infectious laryngotracheitis virus. Virology 21:601-608.

165. Williams CJ, Zedek AS. 2010. Comparative field evaluations of in ovo applied technology. Poult Sci 89:189-193.

166. Williams RA, Bennett M, Bradbury JM, Gaskell RM, Jones RC, Jordan FT. 1992. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. J Gen Virol 73 ( Pt 9):2415-2420.

167. Winterfield RW, So IG. 1968. Susceptibility of turkeys to infectious laryngotracheitis. Avian Dis 12:191-202.

168. Witter RL, Lee LF. 1984. Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies. Avian Pathol 13:75-92.

169. Witter RL, Nazerian K, Purchase HG, Burgoyne GH. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am J Vet Res 31:525-538.

170. Yamada S, Matsuo K, Fukuda T, Uchinuno Y. 1980. Susceptibility of ducks to the virus of infectious laryngotracheitis. Avian Dis 24:930-938.

171. Yao Y, Nair V. 2014. Role of virus-encoded microRNAs in Avian viral diseases. Viruses 6:1379-1394.

172. York JJ, Fahey KJ. 1990. Humoral and cell-mediated immune responses to the glycoproteins of infectious laryngotracheitis herpesvirus. Arch Virol 115:289-297.

173. York JJ, Sonza S, Fahey KJ. 1987. Immunogenic glycoproteins of infectious laryngotracheitis herpesvirus. Virology 161:340-347.

174. York JJ, Young JG, Fahey KJ. 1989. The appearance of viral antigen and antibody in the trachea of naive and vaccinated chickens infected with infectious laryngotracheitis virus. Avian Pathol 18:643-658.

175. Ziemann K, Mettenleiter TC, Fuchs W. 1998. Infectious laryngotracheitis herpesvirus expresses a related pair of unique nuclear proteins which are encoded by split genes located at the right end of the UL genome region. J Virol 72:6867-6874.

# CHAPTER 3

# ATTENUATION AND PROTECTION EFFICACY OF A RECOMBINANT ΔORF C INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) WHEN DELIVERED *IN OVO* [1]

[1] Gustavo H. Schneiders, Sylva M. Riblet, Maricarmen García. To be submitted to *Avian Diseases*

**Key words**

*In ovo vaccination, Maternal antibodies (MAb), Attenuation, Protection efficacy*

**Abbreviations**

ΔORF C: recombinant ILTV depleted of open reading frame C gene

HC: Hatch contacts

MDV: Marek`s disease virus

PC: Post hatch contacts

SPF: Specific pathogen free

CK: Chicken kidney cells

CSS: Clinical signs scores

Maternal antibodies: MAbs

**ABSTRACT**

A recombinant Infectious laryngotracheitis virus (ILTV) depleted of the open reading frame C (ORF C) gene was previously developed and showed significant levels of attenuation post intratracheal/ocular and eye-drop delivery and induced satisfactory protection against Infectious laryngotracheitis (ILT). *In ovo* vaccination is a highly effective method for mass immunization of poultry, providing increased coverage, fast delivery and lower costs as compared to other field vaccination methods. In the USA, *in ovo* vaccination with Marek`s disease virus (MDV) is a standard procedure, capable of inducing early and effective immune response against Marek's disease. The objectives of this study were to evaluate the attenuation and protection efficacy of the ΔORF C recombinant virus when delivered *in ovo* in the absence or presence of ILTV maternally derived antibodies (MAb). *In ovo* delivery of ΔORF C recombinant virus did not affect hatchability or weight-gain, and transmission of the virus to contact chickens was low. However, 5 to 19 % mortality was observed in specific pathogen free (SPF) chickens vaccinated with 3.5 ($log_{10}$) and 3.8 ($log_{10}$) $TCID_{50}$ of ΔORF C per embryo, respectively. While in commercial layers, in the presence of MAbs, no mortalities were observed. *In ovo* vaccination with ΔORF C ILTV induced satisfactory protection after challenge, particularly in the absence of maternal antibodies, as SPF chickens vaccinated with 3.8 ($log_{10}$) $TCID_{50}$ dose showed significant reduction in clinical signs of the disease and tracheal virus load after challenge, while reduction of challenge virus load in commercial layers was not as significant. These results indicate that the ΔORF C recombinant virus is still not properly attenuated for *in ovo* vaccination and the protection efficacy of ΔORF C recombinant virus was affected by maternally derived antibodies.

## INTRODUCTION

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens that results in severe production losses to the poultry industry (4, 9). The etiological agent of the disease is *Gallid Herpesvirus-I* (*GaHV-I*) but is also commonly referred as Infectious laryngotracheitis virus (ILTV). Currently, the main method of controlling the disease is through vaccination with live attenuated vaccines applied via eye-drop drinking water and spray, or with recombinant viral vector vaccines administered *in ovo*.

Live attenuated vaccines commercialized in the United States are originated from virulent field strains of ILTV that circulated during the late 50's to early 60's in the country (19), and were attenuated by serial passages either in embryonated eggs (chicken embryo origin [CEO]) or in embryonic chicken tissue cultures (tissue culture origin [TCO]) (14, 32). Although effective in preventing and reducing mortality, viral shedding and clinical signs of the disease, these vaccines, in particular CEO, are composed of mixed viral subpopulations (12, 13), and the selection of virulent vaccine subpopulations aided in part by poor mass vaccination coverage has contributed to an increase in vaccine strain virulence and to the persistence of vaccine derived outbreaks (3, 13, 25).

Widely used for immunization of broilers and broiler breeders in the United States, *in ovo* vaccination is a highly effective mass immunization method with increased coverage, fast delivery, reduction in bird handling, lower costs as compared to other field vaccination methods (28), and early induction of protective immunity (16). *In ovo* vaccination is considered a standard procedure for immunization against Marek's disease (MD) (10, 23, 42). Embryo vaccination against MD with MDV serotypes 1 (CV1-988,

Rispens), 2 (SB-1), and serotype 3 (HVT) (44), or combination of serotypes 1, 2, and 3 in bivalent (HVT-SB1) and trivalent vaccines (5, 29, 30, 34, 43) have been considered safe and are commercially available in the US since 1992 (33). Other vaccines commonly applied *in ovo* are infectious bursal disease (IBD) and Fowlpox disease (FP) (2, 15).

The advance of recombinant DNA technology allowed for the production of recombinant vaccines utilizing the turkey herpesvirus (HVT) as a vector to express exogenous viral proteins. HVT vectored vaccines expressing proteins from infectious bursal disease virus (IBDV), Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV) and avian influenza virus (AIV); and fowl poxvirus (FPV) expressing proteins of NDV, ILTV and AIV (1) are commercially available in the USA. The hallmark of these viral vector vaccines is their lack of transmission and reversion to virulence. Notably, for ILTV, FPV and HVT vectored vaccines the development of protective immunity may be delayed as compared to that induced by live attenuated vaccines (37).

In an effort to improve control of ILTV, attention has turned towards developing more stable ILTV live attenuated vaccines by deletion of genes associated with virulence. The deletion of the gene coding for glycoprotein G (gG), a viral chemokine binding protein, produced an attenuated recombinant virus suitable for vaccination of three-weeks old SPF chickens. The authors have shown that the lack of gG shifts the immune response away from the humoral branch, enhancing cell-mediated immunity against ILTV, eliciting protection comparable to that of live attenuated vaccines (6, 8). In the same way, another recombinant ILTV depleted of glycoprotein J gene was considered poorly attenuated for *in ovo* vaccination of commercial broilers, inducing high mortality

of chickens during the first weeks of age (22). Recently, a recombinant ILTV depleted of open reading frame C (ORF C) gene was developed and shown to be significantly attenuated for intratracheal/ocular and eye-drop delivery in SPF chickens, yet eliciting high level of protection post challenge, similar to that of the TCO vaccine (11). The objective of the present study was to evaluate the attenuation and protection efficacy of the ΔORF C recombinant virus when administered *in ovo* in the absence and presence of ILTV maternally derived antibodies.

## MATERIALS AND METHODS

**Virus strains.** The ΔORF C recombinant virus (11) was passed in chicken kidney cells (CK) and the fifth passage was used to vaccinate chicken embryos *in ovo*. The MDV bivalent commercial vaccine Cevac HVT-SB1® (Ceva Animal Health, Lenexa, Kansas, USA) was prepared according to manufacturer`s recommendations. The challenge virus strain used in this experiment was the virulent GA 63140, genotype V field isolate (36). Both ILT viruses were propagated and titrated in chicken kidney (CK) cells prepared from 3 to 4 weeks old chickens as previously described (31). Titers were calculated as the 50% tissue culture infective dose ($TCID_{50}$) using the method of Reed and Meunch (27).

**Experimental design.** A standard practice of the poultry industry is to administer MDV vaccines *in ovo*. In this study in order to mimic this practice, the ILTV ΔORF C recombinant virus was co-administered with a full dose of the bivalent MDV vaccine. Embryos that were not vaccinated with ILTV received a full dose of MDV bivalent vaccine by itself (Table 3.1). A total of 500 SPF (Valo BioMedia North America Inc.,

Adel, Iowa, USA) and 330 commercial layers (Hy-line North America Inc., Mansfield, Georgia, USA) embryos were incubated at 99.5˚F and 55% humidity. At 18 days of embryonation eggs were candled and non-viable embryos were eliminated. At 19.5 days of embryonation embryos were inoculated with 200 µL of ILTV/MDV or only MDV. Vaccine was delivered into the amniotic cavity using a 23-gauge 1-inch needle. A total of 100 SPF embryos were inoculated with ΔORF C recombinant ILTV, 50 with a 3.5 ($log_{10}$) $TCID_{50}$ dose (MAb-Vx3.5) and 50 with a 3.8 ($log_{10}$) $TCID_{50}$ dose (MAb-Vx3.8) combined with a full dose of MDV vaccine. To evaluate the effect of maternally derived antibodies on *in ovo* vaccination with ΔORF C recombinant virus, eggs from commercial layer breeders expected to have high levels of ILTV maternal antibodies, as they were vaccinated twice with live attenuated CEO vaccine, were utilized. A total of 50 commercial layer embryos were vaccinated with ΔORF C recombinant ILTV, 25 with a 3.5 ($log_{10}$) $TCID_{50}$ dose (MAb+Vx3.5) and 25 with a 3.8 ($log_{10}$) $TCID_{50}$ dose (MAb+Vx3.8) combined with a full dose of MDV vaccine. At hatch, 18 SPF chickens vaccinated only with MDV and 180 non-vaccinated commercial layer chickens were bled to determine the level of ILTV maternally derived antibodies.

A total of 250 SPF embryos were vaccinated only with one full dose of MDV vaccine. 100 of the MDV vaccinated SPF embryos were hatched separated from the ILTV vaccinated groups and identified as non-vaccinated for ILTV (NVx). The transmission of ΔORF C recombinant virus was evaluated by introducing MDV vaccinated embryos before hatch (hatch contacts) and chickens post hatch (post hatch contacts) together with ILTV/MDV vaccinated embryos and chickens, respectively. At weeks 1, 2, and 3 of age tracheal swabs were collected from hatch contact and post hatch

contact chickens to detect and quantify the presence of ILTV genomes by real-time PCR (qPCR). A total of 150 MDV vaccinated SPF embryos, identified as hatch contacts (HC), were divided in four groups. Fifty hatch contacts were placed with each MAb-Vx group and 25 hatch contacts were placed with each MAb+Vx group of embryos. Chickens hatched in baskets containing 25 ILTV/MDV vaccinated embryos in one side and 25 hatch contact embryos in the other side, divided by a 0.5 cm plastic mesh. Hatch contact chickens were housed separately from ILTV vaccinated chickens.

In order to assess transmission of ΔORF C recombinant virus post vaccination, a total of 100 MDV vaccinated SPF embryos, identified as post hatch contacts (pHC) hatched in a separate incubator. These were divided, in groups containing 9 to 12 chickens, and housed with MAb-Vx3.5, MAb-Vx3.8, MAb+Vx3.5, and MAb+Vx3.8 groups of chickens. The number of eggs that were vaccinated per group and groups of chickens are listed in Table 3.1.

At 28-days of age chickens from the NVx, MAb-Vx3.5, MAb-Vx3.8, MAb+Vx3.5, and MAb+Vx3.8 groups were inoculated with the virulent GA 63140 isolate at 3.5 ($log_{10}$) $TCID_{50}$ /chicken. The challenge virus was administered in a total volume of 200 µl per chicken, 100 µL were applied intratracheally and 100 µL via eye-drop (50 µL in each eye). A group of NVx chickens was inoculated with 200 µL of cell culture media (100 µL intratracheally and 50 µL in each eye).

**Evaluation post vaccination.** Hatchability, cumulative mortality, clinical signs, weight-gain and transmission to hatch contacts and post hatch contacts were evaluated post vaccination. Clinical signs of conjunctivitis, respiratory distress and apathy were

evaluated at week 1, 2, and 3 of age and categorized as negative, mild, moderate and severe. Mortality was recorded from hatch until three weeks of age. Lungs and tracheas were collected from dead chickens and analyzed through qPCR to quantify ILTV genome load and correlate it with the cause of death. The percentage body weight gained until three weeks of age was calculated using the following formula: [(Final weight – Initial weight) / Initial weight]. Tracheal swabs were collected from hatch contacts (HC) and post hatch contacts (pHC) at 7, 14 and 21 days of age and analyzed by qPCR to quantify ILTV genome load and evaluate transmission to contact birds.

**Evaluation post challenge.** Clinical sings and mortality were scored from day 3 to 6 post challenge (PC) as previously described by A. Vagnozzi, et al. (37). Briefly, each chicken received a score for each clinical sign category, in which a score of normal = 0, mild =1, moderate = 2 and severe = 3, dead chickens received a total score of 6. The sum of clinical signs for each bird was averaged and a mean clinical signs score (CSS) was assigned at each time point for each group of chickens. Tracheal swabs were collected from all chickens at five days post challenge and analyzed by qPCR to quantify challenge virus genome load. All chickens were weighed before challenge at 28-days of age and at 35-days of age (seven-days post-challenge) to calculate the average percentage body weight gained per group of chicken after challenge.

**DNA extraction.** Tracheal swabs were placed in 2 mL sterile tubes containing PBS solution + 1% antibiotic-antimycotic (penicillin + streptomycin+ amphotericin B) + 2% newborn calf serum and stored at -80˚C until processing for DNA extraction. DNA was

extracted using MegaZorb DNA mini-prep kit® (Promega, Madison, Wisconsin, USA), according to manufacturer's recommendations with some modifications. Briefly, 70 µL swab suspension was resuspended with 7 µL of proteinase K and 50 µL of lysis buffer at 56˚C for 10 minutes in a 96-well plate. 20 µL of magnetic beads were added along with 125 µL of binding buffer to each well, following incubation for 10 minutes at room temperature. The supernatant was removed and the beads were washed twice with washing buffer. Finally, the DNA was eluted from the beads with 100 µL of elution buffer.

**Quantitative real-time PCR for ILTV.**

ILTV genome load were determined by qPCR in tracheas and lungs collected from dead birds post hatch until three weeks of age, in tracheal swabs collected from hatch and post hatch contacts at 1, 2, and 3 weeks post hatch, and tracheal swabs collected at five days post challenge. A duplex real-time PCR assay normalized for α-collagen chicken DNA was utilized as previously described by A. Vagnozzi, et al. (36). Briefly, the viral genome load for each sample was normalized to the amount of host collagen DNA. The relative amount of viral DNA was expressed as $Log_{10}^{2-\Delta\Delta Ct}$ (21).

**Serology.** Commercial layers used in the study are originated from CEO vaccinated breeders, so the presence of ILTV maternal antibodies was expected. To determine the levels of ILTV maternal antibodies, serum samples were collected at one day of age from non-vaccinated commercial layers and MDV-vaccinated SPF chickens. Antibodies were detected with an in-house developed recombinant glycoprotein B (gB) based ELISA

previously described (17). Briefly, recombinant glycoprotein B expressed by bacuolavirus was purified from culture supernatants. The gB ELISA was shown not to cross-react with polyclonal sera raised against other diseases of poultry. Reactivity of sera was expressed as sample to positive ratio (SP Value) per plate. The SP values were calculated as corrected optical density (OD) of the unknown sample using the following formula [(OD unknown sample - average OD of known negative sample) / average OD of known positive sample]. Samples that received an SP value greater than 0.11 were considered positive for glycoprotein B antibodies.

**Statistical analysis.** Data were entered into Microsoft Office Excel 2011 and analyzed via Prism 6.0 software (GraphPad Software Inc., La Jolla, CA). Mann-Whitney test was applied to analyze statistical differences between maternal antibodies level in SPF and commercial layers. Kruskal-wallis test was used to determine statistical differences in weight-gain post vaccination and post challenge and differences in clinical signs scores at five days post challenge among all the groups. Kruskal-wallis test was used to determine statistical differences in viral genome load post-challenge between each vaccinated group and the NVxCh group. All statistical analyses were performed at the 5% level of significance. Percentage hatchability and cumulative mortality were calculated in Microsoft Excel and were not statistically analyzed.

## RESULTS

**Serology.** The mean SP value obtained for SPF chickens ($N$=18) was -0.12. On the other hand, the mean SP value for commercial layers ($N$=180) was 0.35. Differences in SP

values were significant between the two groups (Mann-Whitney test, $p<0.0001$) (Figure 3.1). These results indicate that contrary to SPFs, commercial layers were positive for ILTV gB antibodies at one day of age. Therefore as expected commercial layers were positive for maternally derived ILTV antibodies.

**Attenuation of recombinant ΔORF C virus.** Hatchability, mortality, clinical signs, weight-gain, and transmission to contacts during weeks 1 to 3 of age were evaluated. The hatchability of NVx and vaccinated MAb- groups of chickens ranged from 88% to 90%. Whereas the hatchability for vaccinated MAb+ groups of chickens ranged from 72% to 76% (Table 3.2).

Cumulative mortality until three weeks of age reached 1.5% to 2.9% for NVx chickens, whereas mortality for MAb-Vx3.5 and MAb-Vx3.8 groups of chickens reached 5.7% and 19.4%, respectively and no mortalities were recorded for MAb+ groups of chickens (Table 3.2).

ILTV genome load was quantified in trachea and lungs collected from one, two and seven mortalities within the NVx, MAb-Vx3.5 and MAb-Vx3.8 groups of chickens, respectively. No ILTV DNA was detected in samples from the NVx chicken. While in samples from MAb-Vx3.5 and MAb-Vx3.8 groups of chickens ILTV genomes were detected in all tracheas, and in eight of the nine lungs analyzed, viral genome load in these tissues ranged from 3.9 to 8.1 (data not shown).

Clinical signs scores were evaluated for all groups of chickens from hatch to three weeks of age. Differing from NVx chickens, the MAb-Vx3.5 group of chickens presented low incidence of moderate respiratory distress (4.0%), whereas the MAb-Vx3.8 group of

chickens in addition to low incidence of moderate respiratory distress (2.%), presented mild conjunctivitis (1.1%). The MAb+Vx3.8 group presented low incidence of mild conjunctivitis (3.7%). The MAb+Vx3.5, HC and pHC groups of chickens did not present discernible clinical signs of ILT (data not shown).

The average weigh-gain for each group from hatch to three-weeks of age is shown in Table 3.2. The NVx group of chickens gained an average of 441.4%, whereas MAb-Vx3.5 and MAb-Vx3.8 groups an average of 455.4% and 454.6%, respectively. Percentage weight gained for MAb- vaccinated groups of chickens was statistically similar to the NVx group of chickens ($p$=≥0.42). Mab+Vx3.8 chickens achieved 436.2% increase in weight-gain, statistically similar to NVx chickens ($p$>0.99), while MAb+Vx3.5 chickens reached only 390.3% increase in weight-gain, which was significantly lower compared to the NVx group of chickens ($p$=0.013). Chickens from the HC and pHC groups gained 435.1% and 422.6% weight, which was statistically similar to NVx chickens ($p$≥0.82).

Tracheal swabs were collected at 1, 2, and 3 weeks of from hatch contacts (HC) and post hatch contacts (pHC) and analyzed by qPCR to quantify ILTV genome load and evaluate transmission to contact birds. The proportion of tracheal swabs positive for the presence of ILTV DNA is shown in Table 3.2. None of the tracheal swabs collected from the NVx group ($N$=46) had detectable levels of viral genome load, whereas ILTV genome load was detected in 3.7% ($n$=12/326) of the tracheal swabs collected from HC chickens and in 1.6% ($n$=38/192) of the tracheal swabs collected from pHC chickens.

**Protection efficacy induced by the recombinant ΔORF C virus.** To evaluate the protection efficacy induced by the vaccination with ΔORF C *in ovo*, at 28-days of age, MAb-Vx35Ch, MAb-Vx38Ch, MAb+Vx3.5Ch, MAb+Vx3.8Ch and NVxCh groups of chickens were challenged with the virulent ILTV strain GA 63140 and monitored for clinical signs of ILT from 3 to 6 days post challenge (PC). As shown in Figure 3.2A, the peak of clinical signs was identified at five days post challenge particularly noticeable for the NVxCh group of chickens. Although no mortality was observed within the NVxCh group of chickens moderate to severe respiratory distress, moderate apathy and conjunctivitis were observed. The mean clinical signs at five days post challenge for each group of chickens are shown in Figure 3.2B. Clinical signs scores were significantly lower for the vaccinated groups (MAb-Vx3.5Ch, MAb-Vx3.8Ch, MAb+Vx3.5Ch, and MAb+Vx3.8Ch) as compared to clinical signs scores for the NVxCh group of chickens ($p \leq 0.015$). Clinical signs scored for vaccinated groups of chickens were significantly higher than those scored for the NVxNCh group ($p \leq 0.004$), however no significant differences in clinical signs scores were observed among vaccinated groups of chickens ($p \geq 0.87$).

The post-challenge weight-gain between 28 and 35-days of age (7 days PC) was calculated. The percentage mean weight-gain post challenge for each group is shown in Figure 3.2C. The highest weight-gain was noted for NVxNCh chickens (31.78%), statistically higher ($p<0.0001$) than the weight gained by the NVxCh group (3.7%). Percentage mean weight gained for MAb-Vx3.8Ch was 25.5%, statistically similar to NVxNCh ($p=0.46$) and significantly higher than NVxCh ($p<0.0001$). While the MAb-Vx3.5Ch group achieved 12.5% increase in weight-gain, significantly lower than weight

gained by NVxNCh group of chickens ($p$<0.0001) and similar to the weight gained by NVxCh group of chickens ($p$=0.41). Average weight-gained by the MAb+Vx3.8Ch was 21.72%, statistically similar to NVxNCh group of chickens ($p$=0.09) and significantly higher than NVxCh group of chickens ($p$<0.0001). Percentage mean weight gained for the MAb+Vx3.5Ch chickens was 12.44%, which was statistically similar to weight gained by the NVxCh ($p$=0.9) group of chickens and significantly lower than the NVxNCh group of chickens ($p$<0.0001) (Figure 3.2C).

Quantification of challenge virus load in trachea at five days post challenge is expressed as viral genome load ($\log_{10}{}^{2\text{-}\Delta\Delta Ct}$) (Figure 3.2D). There was no detection of ILTV DNA in tracheal swabs collected from NVxNCh chickens. Mean viral genome load detected in tracheal swabs from the NVxCh group of chickens was 4.47. The mean challenge viral genome load from MAb-Vx3.5Ch and MAb-Vx3.8Ch groups of chickens was 2.95 and 2.40, respectively, which was significantly lower than viral load detected from NVxCh group of chickens ($p$≤0.02). The mean challenge virus genome load for MAb+Vx3.5Ch and MAb+Vx3.8Ch groups of chickens was 3.26 and 3.85, respectively, statistically similar to the mean genome viral load detected for the NVxCh group ($p$≥0.57).

## DISCUSSION

The objective of the present study was to evaluate attenuation and protection efficacy of the ΔORF C recombinant virus when administered *in ovo* in MAb- and MAb+ chickens. Placement of vaccines into the various areas within the egg may enhance or limit the embryo`s response to the virus. The highest protection efficacy of bivalent

Marek`s disease vaccine (HVT-SB1) was found when the inoculum was applied into the amniotic fluid (40). In commercial practice, the broiler embryos are vaccinated between 17 and 19 days of embryonation (16, 39), the embryo stage that ensures greater rate of deposition of inoculum in the amniotic fluid (41). Due to the lower embryo development of leghorn type birds as compared to broiler chickens (38), in this study, embryos were vaccinated at 19.5 days of embryonation, which, in our experience, correspond to the optimal development stage under our incubation conditions (data not shown).

Attenuation of the recombinant ΔORF C virus was evaluated by assessing hatchability, mortality, clinical signs, weight-gain and transmission to contact chickens post vaccination. In agreement with previous studies where *in ovo* vaccination with ILTV recombinants ΔgJ (22) and ΔgG (20) was performed in broilers and SPFs, no effect on hatchability was observed for either SPF and commercial layers in this study. Overall, hatchability values from MAb- groups were similar to that of non-vaccinated chickens. The lower hatchability in MAb+ chickens was most likely due to incubation irregularities rather than effects of the recombinant virus. Similar to what was observed when ΔgJ ILTV recombinant was administered *in ovo* in commercial broilers (22), moderate to high levels of ILTV DNA were detected in trachea and lung from chickens presenting severe respiratory distress post hatch, between 1 and 14-days of age, that resulted in elevated mortalities. In contrast, no mortalities were observed in MAb+ chickens when the recombinant ΔORF C virus was administered. The absence of mortality was most likely associated with interaction of maternally derived antibodies with the replication of the recombinant ΔORF C virus by a not yet known mechanism.

Based on the lack of ILT clinical signs and low tracheal pathology the recombinant ΔORF C virus appeared to be attenuated for three week old SPFs chickens when administered via the intratracheal or eye-drop routes (11). In this study, the increased mortality and the incidence of mild to moderate clinical signs of the disease 1 to 3 weeks post hatch in SPFs chickens indicates that recombinant ΔORF C virus still retains residual virulence for *in ovo* vaccination. *In ovo* vaccination of SPF chickens with recombinant ILT virus deficient in the viral chemokine binding protein (ΔgG) gene has also shown mortalities induced during the first week post hatch due to increase replication of the recombinant virus in trachea and yolk sac with viral load ranging from 4.0 to 8.7 ($log_{10}$).

The Newcastle disease lentogenic vaccine strain clone 30 was genetically modified by introducing genetic changes that resulted in attenuated strains as indicated by reduced intracerebral pathogenicity indexes. When these recombinant NDV clone 30 viruses were administered to SPFs embryos at 18 days of embryonation, it resulted in overall high rates of infection observed at one day post-hatch and 20 to 60% mortality up to 21-days of age (26). Considered together post-hatch mortalities induced after *in ovo* administration with attenuated NDV clone 30 recombinants and ILTV recombinants ΔgJ (22), ΔgG (20) and ΔORF C were associated with an increased replication of the respective recombinant virus, suggesting that the capacity of the embryo to respond to vaccination was overwhelmed by viral replication. In a recent study inoculation of 18 day old SPFs embryos with cpG, the ligand that stimulates Toll like receptors 21 and 9, resulted in reduction of ILTV replication in lungs and trachea of chickens four and seven

days post-hatch (35). Currently, no live attenuated vaccines against respiratory disease of poultry for *in ovo* administration are commercially available.

Regardless of mortality induced by the recombinant ΔORF C ILT virus no effect on weight-gain was observed for MAb-Vx3.5, MAb-Vx3.8 or MAb+Vx3.8 groups of chickens. The MAb+Vx3.5 group of chickens showed decrease weight-gain, however the lack of clinical signs and mortality in this group of chickens is not related to *in ovo* vaccination with ΔORF C ILT virus.

The low proportion of tracheal swabs from hatch contacts and post hatch contacts where ILTV genome load was detected indicates a low rate of transmission of the ΔORF C ILT virus from vaccinated to non-vaccinated chickens. The lack of transmission is a desired characteristic for ILTV vaccines, as it will reduce the persistence of vaccine strains in the field. The high rate of transmission of the CEO vaccine (31), in particular, contributes to the emergence of vaccine-derived strains that are responsible for outbreaks of the disease while circulating in the field (7, 18, 24). Further studies are necessary to compare the transmission of ΔORF C and the commercial counterparts under the same experimental conditions.

The protection efficacy of recombinant ΔORF C virus was assessed after challenge at 28-days of age by assessment of clinical signs, weight-gain, and challenge virus load. In this study protection of vaccinated groups of chickens was defined as reduction in clinical signs and challenge virus genome load combined with increased weigh-gain post challenge. All vaccinated groups showed a significant reduction in clinical signs, MAb – and MAb+ groups of chickens that received a 3.8 ($log_{10}$) dose of recombinant ΔORF C virus showed significant increase in weight-gain, however, only

MAb- groups of chickens showed a significant reduction of virus load post-challenge. Based on decreased clinical signs, reduction of challenge virus load, and increased weight-gain after challenge, only the MAb-Vx3.8Ch group of chickens was considered fully protected against ILT, while MAb+ Vx3.8Ch was partially protected because vaccination did not significantly decrease challenge virus replication in the trachea. In ovo vaccination of SPFs chickens with recombinant ΔgG ILT virus did not decrease challenge virus replication in the trachea, indicating that protection efficacy of recombinant ΔgG ILT virus was partial (Legion et al., 2012).

The reduction of challenge virus genome load in trachea after challenge was contingent on the presence of maternally derived ILTV antibodies at vaccination. The presence of ILTV maternally derived antibodies most likely interacted with the replication of recombinant ΔORF C virus and consequently interfered with protection efficacy in a portion of the vaccinated chickens.

In conclusion, *in ovo* vaccination with the recombinant ΔORF C virus has no effect on hatchability and low transmission to contact chickens, however induce considerable mortality in MAb- chickens in contrast to MAb+ chickens. Therefore, this recombinant virus was sufficiently attenuated for *in ovo* vaccination of commercial layers but not for SPF chickens. Based on clinical signs, weight-gain and viral genome load in trachea post challenge, *in ovo* administration of ΔORF C has the potential to protect chickens against ILT disease. The possible interaction between maternal antibodies and ΔORF C reduces ILT-derived mortality in the first weeks of age, however diminishing the protection efficacy post challenge. Further studies are necessary to determine the

optimal dose to be applied *in ovo* in combination with adjuvants that could increase the immune response of the embryo as a way to improve protection efficacy.

Table 3.1 Groups vaccinated *in ovo* with MDV or MDV and ILTV at 19.5-days of embryonation and challenged as 28-days of age

| Maternal antibodies | *In ovo* vaccine | Groups | *N* | Challenge | Groups post Ch | *N* |
|---|---|---|---|---|---|---|
| MAb negative[a] | MDV[c] | Non-vaccinated (NVx) | 50 | No | NVxNCh | 38 |
| | MDV | Hatch Contacts (HC) | 150 | No | - | - |
| | MDV | Post-hatch Contacts (pHC) | 100 | No | - | - |
| | MDV | Non-vaccinated (NVx) | 50 | Yes | NVxCh | 33 |
| | MDV [c] +ILTV 3.5 ($log_{10}$) [d] | MAb-Vx3.5 | 50 | Yes | MAb-Vx3.5Ch | 31 |
| | MDV+ILTV 3.8 ($log_{10}$) | MAb-Vx3.8 | 50 | Yes | MAb-Vx3.8Ch | 29 |
| MAb positive[b] | MDV+ILTV 3.5 ($log_{10}$) | MAb+Vx3.5 | 25 | Yes | MAb+Vx3.5Ch | 18 |
| | MDV+ILTV 3.8 ($log_{10}$) | MAb+Vx3.8 | 25 | Yes | MAb+Vx3.8Ch | 18 |

[a] Specific pathogen free (SPF); [b] Commercial layers; [c] Full dose bivalent vaccine Cevac HVT& SB-1® (Ceva Animal Health); [d] ILTV ΔORF C recombinant virus dose per embryo

Table 3.2. Attenuation of ΔORF C virus post *in ovo* vaccination

| Group ID | Hatchability (%) | Cumulative mortality (%) [a] | Weight-gain (%) [b] | qPCR ILTV [c] |
|---|---|---|---|---|
| NVx | 90 | 2.85 | 441.4 | 0% (0/46) |
| HC | - | 1.52 | 435.1 | 3.7% (12/326) |
| pHC | - | 1.52 | 422.6 | 1.6% (3/192) |
| MAb-Vx3.5 | 88 | 5.72 | 455.4 | - |
| MAb-Vx3.8 | 90 | 19.44 | 454.6 | - |
| MAb+Vx3.5 | 76 | 0 | 390.3* | - |
| MAb+Vx3.8 | 72 | 0 | 436.2 | - |

[a] cumulative mortality from 1-21-days of age; [b] weight-gain from 1-21-days of age; [c] samples positive for ILTV detection, tracheal swabs collected from 1-3 weeks of age; * significantly lower as compared to the NVx group.

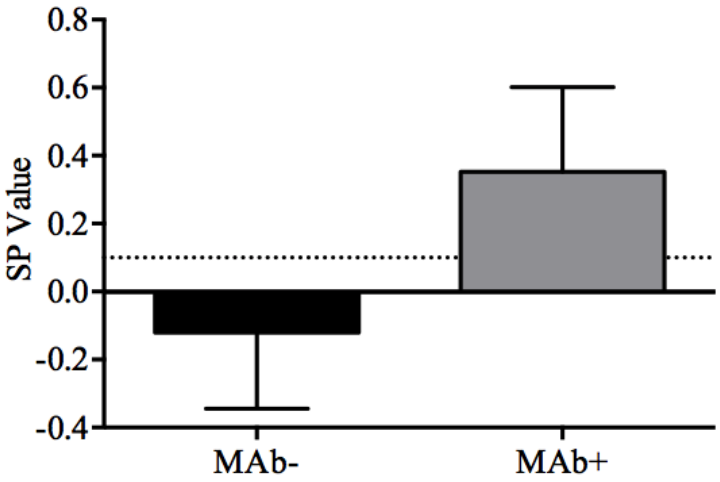


**Figure 3.1.** Maternal antibodies (MAb) against ILTV in non-vaccinated Specific Pathogen Free (MAb-) and commercial layers (MAb+) at 1-day-of-age. The presence of MAb was analyzed by an in-house developed glycoprotein B (gB) based ELISA utilizing serum from one-day-old chickens. Reactivity of sera was expressed as sample to positive ratio (SP Value).

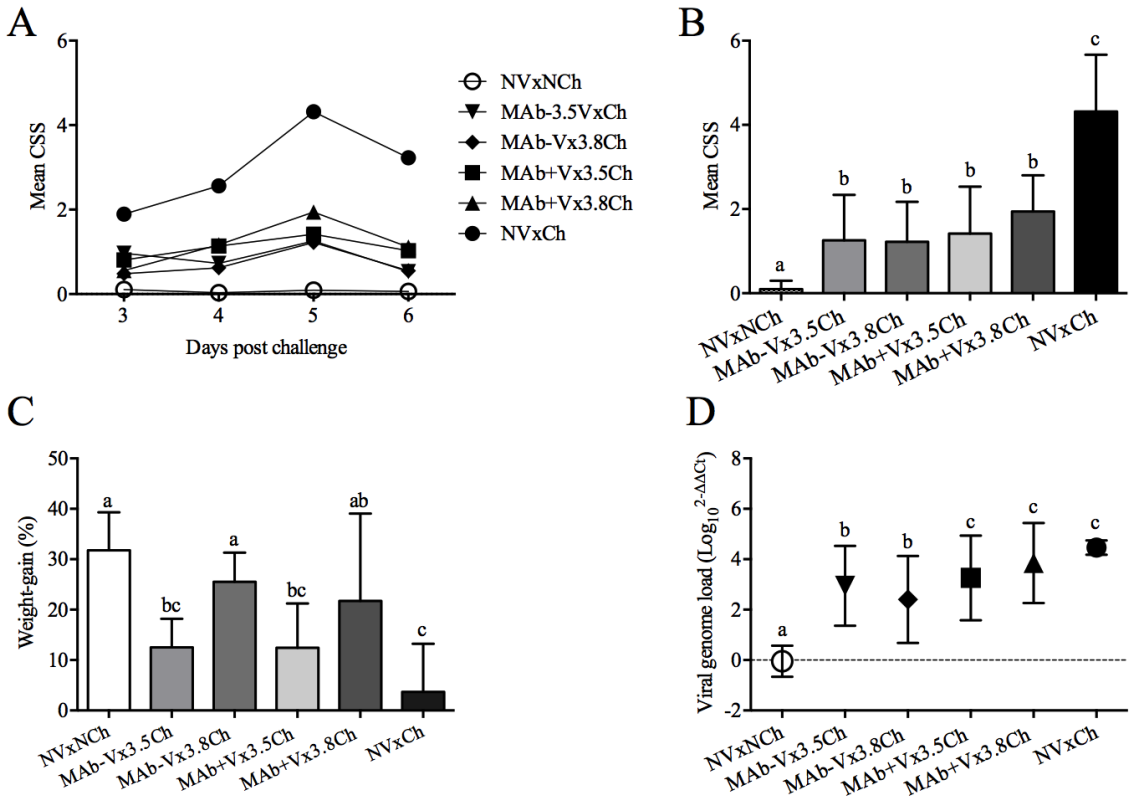


**Figure 3.2.** Protection efficacy elicited by inoculation of ΔORFC *in ovo*. Chickens inoculated with ΔORF C and one group of non-vaccinated chickens (NVxCh) were challenged with the virulent ILTV strain GA 63140. One group of chickens remained unchallenged (NVxNCh). Peak of clinical signs (A), mean clinical signs scores per group at five days post challenge (B), mean weight-gain at five days post challenge (C), viral genome load in the trachea at five days post challenge (D). Statistical differences among groups are expressed by different letter superscripts.

## REFERENCES


1. Armour NK, García M. 2014. Current and Future Applications of Viral-Vectored Recombinant Vaccines in Poultry. The Poultry Informed Professional:1-9.
2. Avakian A, Klein D, Whitfill C, Tripathy D, Singbeil B, Poston R, Grosse D. 1999. Safety and efficacy of fowl and pigeon pox vaccines administered in ovo to SPF and broiler embryos. Western Poultry Disease Conference Proceedings of Western Poultry Disease Conference:56-60.
3. Avila AR. 2007. Replication, transmission, and protection of live-attenuated infectious laryngotracheitis virus (ILTV) vaccines. [electronic resource]. 2007.
4. Bagust TJ, Jones RC, Guy JS. 2000. Avian infectious laryngotracheitis. Rev Sci Tech 19:483-492.
5. Calnek BW, Schat KA, Peckham MC, Fabricant J. 1983. Field trials with a bivalent vaccine (HVT and SB-1) against Marek's disease. Avian Dis 27:844-849.
6. Coppo MJ, Noormohammadi AH, Hartley CA, Gilkerson JR, Browning GF, Devlin JM. 2011. Comparative in vivo safety and efficacy of a glycoprotein G-deficient candidate vaccine strain of infectious laryngotracheitis virus delivered via eye drop. Avian Pathol 40:411-417.
7. Davison S, Dufour-Zavala L, Garcı´a M, Ghori H, Hoerr F, Hopkins B, Smith J, Waldrip D. Proceedings of the 109th Annual Meeting of the United States Animal Health Association, p 580–603. *In* (ed),
8. Devlin JM, Viejo-Borbolla A, Browning GF, Noormohammadi AH, Gilkerson JR, Alcami A, Hartley CA. 2010. Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus. Vaccine 28:1325-1332.

9. Dufour-Zavala L. 2008. Epizootiology of infectious laryngotracheitis and presentation of an industry control program. Avian Dis 52:1-7.

10. Gagic M, St Hill CA, Sharma JM. 1999. In ovo vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis 43:293-301.

11. García M, Cheng Y, Spatz SJ, Riblet SM, Schneiders GH, Volkening J. 2016 Submitted. Attenuation and Protection Efficacy of Open Reading Frame C (ORF C) Gene Deleted Strain of The Alphaherpesvirus Infectious Laryngotracheitis Virus (ILTV)

12. García M, Riblet SM. 2001. Characterization of Infectious Laryngotracheitis Virus Isolates: Demonstration of Viral Subpopulations within Vaccine Preparations, p 558. American Association of Avian Pathologists, Inc.

13. García M, Volkening J, Riblet SM, Spatz S. 2013. Genomic sequence analysis of the United States infectious laryngotracheitis vaccine strains chicken embryo origin (CEO) and tissue culture origin (TCO). Virology 440:64-74.

14. Gelenczei EF, Marty EW. 1965. Strain Stability and Immunologic Characteristics of a Tissue-Culture-Modified Infectious Laryngotracheitis Virus. Avian Dis 9:44-56.

15. Giambrone JJ, Dormitorio T, Brown T. 2001. Safety and Efficacy of in ovo Administration of Infectious Bursal Disease Viral Vaccines, p 144. American Association of Avian Pathologists, Inc.

16. Gimeno IM, Faiz NM, Cortes AL, Barbosa T, Villalobos T, Pandiri AR. 2015. In Ovo Vaccination with Turkey Herpesvirus Hastens Maturation of Chicken Embryo Immune Responses in Specific-Pathogen-Free Chickens. Avian Dis 59:375-383.

17. Godoy A, Icard A, Martinez M, Mashchenko A, García M, El-Attrache J. 2013. Detection of Infectious Laryngotracheitis Virus Antibodies by Glycoprotein-Specific ELISAs in Chickens Vaccinated with Viral Vector Vaccines. Avian Diseases 57:432-436.

18. Guy JS, Barnes HJ, Smith L. 1991. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis 35:348-355.

19. Guy JS, García M. 2008. Laryngotracheitis, p 137-152. *In* Saif YM (ed), Diseases of Poultry, 12th ed. Blackwell Publishing, Ames, Iowa.

20. Legione AR, Coppo MJ, Lee SW, Noormohammadi AH, Hartley CA, Browning GF, Gilkerson JR, O'Rourke D, Devlin JM. 2012. Safety and vaccine efficacy of a glycoprotein G deficient strain of infectious laryngotracheitis virus delivered in ovo. Vaccine 30:7193-7198.

21. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

22. Mashchenko A, Riblet SM, Zavala G, García M. 2013. In Ovo Vaccination of Commercial Broilers with a Glycoprotein J Gene-Deleted Strain of Infectious Laryngotracheitis Virus. Avian Diseases 57:523-531.

23. Ohta H, Ezoe S, Yamazaki K, Kawai T, Honda T. 2009. Application of aluminum hydroxide for an in ovo live Newcastle disease vaccine. Avian Dis 53:392-395.

24. Oldoni I, García M. 2007. Characterization of infectious laryngotracheitis virus isolates from the US by polymerase chain reaction and restriction fragment length polymorphism of multiple genome regions. Avian Pathology: Journal Of The WVPA 36:167-176.

25. Oldoni I, Rodriguez-Avila A, Riblet SM, Zavala G, García M. 2009. Pathogenicity and growth characteristics of selected infectious laryngotracheitis virus strains from the United States. Avian Pathol 38:47-53.

26. Ramp K, Topfstedt E, Wackerlin R, Hoper D, Ziller M, Mettenleiter TC, Grund C, Romer-Oberdorfer A. 2012. Pathogenicity and immunogenicity of different

recombinant Newcastle disease virus clone 30 variants after in ovo vaccination. Avian Dis 56:208-217.

27. Reed LJ, Muench H. 1938. A Simple Method of Estimating Fifty Per Cent Endpoints. American Journal of Hygiene 27:493-497.

28. Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, King S, Murray L, Phelps P, Poston R, Whitfill C, Williams C. 1999. In ovo vaccination technology. Adv Vet Med 41:495-515.

29. Rispens BH, van Vloten H, Mastenbroek N, Maas HJ, Schat KA. 1972. Control of Marek's disease in the Netherlands. I. Isolation of an avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis 16:108-125.

30. Rispens BH, van Vloten H, Mastenbroek N, Maas JL, Schat KA. 1972. Control of Marek's disease in the Netherlands. II. Field trials on vaccination with an avirulent strain (CVI 988) of Marek's disease virus. Avian Dis 16:126-138.

31. Rodriguez-Avila A, Oldoni I, Riblet SM, García M. 2007. Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines. Avian Dis 51:905-911.

32. Samberg Y, Aronovici I. 1969. The development of a vaccine against avian infectious laryngotracheitis. I. Modification of a layngotracheitís virus. Refuah Veterinarith 26:54-59.

33. Sarma G, Greer W, Gildersleeve RP, Murray DL, Miles AM. 1995. Field safety and efficacy of in ovo administration of HVT + SB-1 bivalent Marek's disease vaccine in commercial broilers. Avian Dis 39:211-217.

34. Schat KA, Calnek BW. 1978. Characterization of an apparently nononcogenic Marek's disease virus. J Natl Cancer Inst 60:1075-1082.

35. Thapa S, Cader MS, Murugananthan K, Nagy E, Sharif S, Czub M, Abdul-Careem MF. 2015. In ovo delivery of CpG DNA reduces avian infectious laryngotracheitis virus induced mortality and morbidity. Viruses 7:1832-1852.

36. Vagnozzi A, García M, Riblet SM, Zavala G. 2010. Protection induced by infectious laryngotracheitis virus vaccines alone and combined with Newcastle disease virus and/or infectious bronchitis virus vaccines. Avian Dis 54:1210-1219.

37. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian Pathol 41:21-31.

38. Villalobos T. 2014. Optimal timing for in ovo vaccination - understanding embryo development. International Hatchery Practice 28:15-15.

39. Wakenell PS, Bryan T, Schaeffer J, Avakian A, Williams C, Whitfill C. 2002. Effect of in ovo vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia. Avian Dis 46:274-280.

40. Williams C. 2007. In ovo vaccination for disease prevention, vol 15, p 7-9. Positive Action Publications Ltd, Driffield; UK.

41. Williams CJ, Hopkins BA. 2011. Field evaluation of the accuracy of vaccine deposition by two different commercially available in ovo injection systems. Poult Sci 90:223-226.

42. Williams CJ, Zedek AS. 2010. Comparative field evaluations of in ovo applied technology. Poult Sci 89:189-193.

43. Witter RL, Lee LF. 1984. Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies. Avian Pathol 13:75-92.

44. Witter RL, Nazerian K, Purchase HG, Burgoyne GH. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am J Vet Res 31:525-538.

# CHAPTER 4

# PROTECTION EFFICACY OF RECOMBINANT ΔORF C INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) WHEN DELIVERED *IN OVO*, SPRAY AND BY NASAL-ORAL IN THE PRESENCE OF MATERNALLY DERIVED ILTV ANTIBODIES [1]

[1] Gustavo H. Schneiders, Sylva M. Riblet, Maricarmen García**.** To be submitted to *Avian Diseases*

**Key-words**

*Gallid Herpesvirus, Open reading frame C, In ovo vaccination, Immunity, Virulence, Protection efficacy, Prime-boost strategy*

**Abbreviations**

ΔORF C: ILTV virus lacking the open reading frame C gene

CSS: Clinical signs scores

*GaHV-1*: *Gallid Herpesvirus 1*

ILT: Infectious laryngotracheitis

ILTV: Infectious laryngotracheitis virus

MDV: Marek`s disease virus

ORF C: Open reading frame C

SPF: Specific pathogen free

MAb: Maternal antibodies

**ABSTRACT**

A recombinant Infectious laryngotracheitis virus (ILTV) depleted of the open reading frame C (ORF C) gene was previously developed and evaluated for *in ovo* vaccination. Although the recombinant ΔORF C virus induced considerable mortality in specific pathogen free (SPF) chickens it also elicited a strong protection against ILT after *in ovo* vaccination. In contrast, *in ovo* vaccination with recombinant ΔORF C virus in commercial layers was safe, while the protection efficacy was hampered due to increased levels of maternally derived ILTV antibodies. The objective of this study was to evaluate the protection efficacy of the recombinant ΔORF C virus when administered singly via *in ovo*, spray, or nasal-oral routes, and when administered *in ovo* followed by either spray or nasal-oral at eight-days of age in commercial layers. *In ovo* delivery of ΔORF C recombinant virus did not affect hatchability or weight post vaccination. Chickens vaccinated with ΔORF C recombinant virus presented up to 6.5% mortality while non-vaccinated embryos presented up to 2.2% mortality by eight-days of age. Optimal protection was obtained when *in ovo* vaccination with ΔORF C ILTV was combined with nasal-oral revaccination. Chickens vaccinated singly *in ovo* or *in ovo* followed by spray were partially protected as they showed clinical signs of the disease and failed to gain weight as compared to non-vaccinated chickens. Chickens vaccinated at eight-days of age singly via spray or nasal-oral were not protected. Overall the recombinant ΔORF C ILTV induced protection against ILTV when administered *in ovo* in the presence of maternally derived antibodies. Furthermore priming by *in ovo* immunization with ΔORF C was essential to elicit a strong protective response to ILTV challenge.

## INTRODUCTION

Infectious laryngotracheitis (ITL) is a highly contagious respiratory disease of chickens that results in severe production losses to the poultry industry (4, 9). The etiological agent of the disease is *Gallid Herpesvirus-1* (*GaHV-1*), commonly referred to as Infectious laryngotracheitis virus (ILTV). Currently, the main method of controlling the disease is through vaccination. In United States, most broiler breeders and commercial layers are vaccinated twice against ILTV either by eye-drop route with a live vaccine attenuated by consecutive passages in tissue culture (TCO) (16); via drinking water or coarse spray or eye-drop with a live vaccine attenuated in embryos (CEO) (11, 33). Commercial broilers are vaccinated only in the face of outbreaks, *in ovo* or subcutaneously with the recombinants turkey herpesvirus (HVT) and fowl poxvirus (FPV) vectored vaccines expressing the ILTV glycoproteins D and I (HVT-LT) and glycoprotein B (HVT-LT) (10, 39), ILTV glycoprotein B and Unique Long 32 (FPV-LT) (2, 39) or with CEO vaccine via drinking water between 10 to 16-days of age.

Mucosal vaccination is a non-invasive alternative for vaccination in chickens as it is capable of eliciting strong local and systemic immune responses in mucosal-associated lymphoid tissues (MALT) (21). Eye-drop, coarse spray and drinking water remain the traditional methods used for delivery of ILTV vaccines into the bird`s mucosa. These vaccination methods have been experimentally tested in chickens from 1 to 28-days of age (5, 14). In particular with the CEO vaccine, severe clinical signs were detected in birds vaccinated via course spray at one-day of age (5), while mild vaccine reactions were observed when the vaccine was administered via spray, drinking water and eye-drop between 14 to 28-days of age. In previous studies all CEO vaccinated chickens were

satisfactorily protected after challenge (5, 14). Contrary to earlier studies where it was proposed that underdevelopment of the immune system renders young birds non-responsive to ILTV vaccination (1, 7, 15, 16), J. K. Clarke, et al. (5) found that despite the harsh reaction induced by ILTV vaccination at one day of age, chickens were protected nine days after vaccination, suggesting that early post-hatch vaccination induced a fast protective immune response.

*In ovo* vaccination is a highly effective mass immunization method with increased coverage, fast delivery, reduction in bird handling and lower costs as compared to other field vaccination methods (31). Live Marek`s disease virus (MDV) serotypes 1 (CV1988, Rispens), 2 (SB-1), and serotype 3 (Turkey herpesvirus - HVT), Fowl poxvirus (FPV) and Infectious bursal disease virus (IBDV) vaccines are commonly administered *in ovo* (3, 12, 17, 28, 43, 44).

Early studies showed that embryo vaccination at 18-days of age with HVT resulted in better protection at hatch than subcutaneous vaccination at one day of age followed by challenge at three-days of age. Furthermore MDV maternally derived antibodies did not inhibit the response to *in ovo* vaccination, despite their reduction in the protection efficacy of HVT (36).

Although the exact mechanisms by which *in ovo* vaccination works are not fully understood, early studies with HVT vaccination at 17 and 18 days of embryonation promptly showed vaccine replication in the lungs (37) as well as a high interferon response (35). The uptake of *in ovo* vaccines applied at the amniotic and intra-embryonic routes was localized to the intestinal and respiratory tracts indicating the ingestion of the vaccine by the embryo (23). In a recent study I. M. Gimeno, et al. (19) reports that in ovo

vaccination with MDV serotypes 1, 2 and 3 resulted in the expansion and activation of splenic T cell populations. In particular vaccination with HVT induced a significant activation of T leukocytes and enhanced the immune responses against unrelated antigens, suggesting that HVT vaccination accelerates the maturation of the immune system. This effect may explain the efficacy of *in ovo* vaccination with HVT to prevent Marek's disease. Experimentally, *in ovo* vaccination of chickens against MDV followed by revaccination with homologous and heterologous serotypes at one day of age by the subcutaneous route resulted in higher protection against early challenge (two-days of age) (18). The most prevalent practice in the industry is *in ovo* vaccination against MD and other diseases controlled by HVT recombinant viral vector vaccines. Other less frequently vaccines applied *in ovo* are Infectious bursal disease (IBD) and fowl pox (FP) disease (3, 17), the use of live attenuated vaccines against respiratory viruses is still limited for commercial in ovo vaccination.

In an effort to enable *in ovo* vaccination with ILTV, attenuation of the virus by deletion of genes associated with virulence has been performed. The deletion of the gene coding for glycoprotein G (gG), a viral chemokine binding protein, produced an attenuated recombinant virus suitable for vaccination of SPF chickens *in ovo* and at three-weeks of age. The authors have shown that the lack of gG shifts the immune response away from the humoral branch, enhancing cell-mediated immunity against ILTV, eliciting protection comparable to that of live attenuated vaccines (6, 8, 25). Another recombinant ILTV depleted of glycoprotein J gene was considered poorly attenuated for *in ovo* vaccination of commercial broilers, as it induced high mortality during the first weeks of age (27). Recently, a recombinant ILTV depleted of open reading frame C

(ORF C) gene induced protection similar to that of the TCO vaccine when delivered via eye drop in three-week old SPF chickens (13). *In ovo* vaccination with ΔORF C resulted in increased mortality post-hatch while inducing satisfactory protection of SPFs chickens. In contrast *in ovo* vaccination of commercial layers, bearing maternally derived antibodies, showed absence of mortality post-hatch accompanied of diminished protection post-challenge (34).

Vaccination of commercial layers with live attenuated ILT vaccines at six weeks of age followed by vaccination via spray at 10 weeks of age considerably improved the protection as compared to chickens that were not re-vaccinated (22), indicating that a second immunization against ILTV is necessary for longer living birds.

Based on our previous studies, the *in ovo* vaccination with recombinant ΔORF C virus is capable of inducing protection in the absence or presence of maternally derived antibodies suggesting that *in ovo* vaccination primes an immune response against ILTV. The objective of this study was to evaluate if a second immunization with ΔORF C virus enhances the protection elicited by *in ovo* vaccination. The protection efficacy of ΔORF C virus was evaluated in commercial layers when administered via *in ovo*, spray, or nasal-oral routes, and *in ovo* followed by either spray or nasal-oral revaccination at eight-days of age in the presence of maternally derived antibodies.

## MATERIALS AND METHODS

**Virus strains.** The ΔORF C recombinant virus (13) was passaged in chicken kidney cells (CK) and the fifth passage was used to vaccinate chicken embryos *in ovo*. The MDV bivalent commercial vaccine Cevac HVT& SB-1® (Ceva Animal Health, Lenexa,

Kansas, USA) was prepared according to manufacturer`s recommendations. The challenge virus strain used in this experiment was the virulent GA 63140, genotype V field isolate (38). Both ILT viruses were propagated and titrated in chicken kidney (CK) cells prepared from 3 to 4-weeks old chickens as previously described (32). Titers were calculated as the 50% tissue culture infective dose ($TCID_{50}$) (30).

***In ovo* vaccination.** Before vaccination, each set of eggs was placed inside a class II type A2 safety cabinet, sprayed with BioSentry® 904 Disinfectant (1:249 dilution) (HACCO, Inc., Randolph, Wisconsin, USA), air dried for 2 to 3 minutes, and the external shell punched by introduction of 2 mm 18-gauge needle. Two hundred µL of virus suspension was delivered in the amniotic cavity of each embryo using a 23-gauge one-inch needle.

**Spray vaccination.** A total of 1 liter of ΔORF C suspension containing 4.5 ($log_{10}$) $TCID_{50}$/mL was prepared by diluting the cell free virus stock in tissue culture distilled water. Approximately 200 µL of virus at dose of 3.8 ($log_{10}$) $TCID_{50}$ per chicken was sprayed per group of chickens. After drying out, the chickens were separated according to their respective groups.

**Nasal-oral vaccination.** The ΔORF C virus was diluted in cell culture media (DMEM + 2% calf serum) at a concentration of 4.5 ($log_{10}$) $TCID_{50}$/mL, and administered in a total volume of 200 µL per chicken (3.8 ($log_{10}$) $TCID_{50}$/chicken), 100 µL were applied orally and 100 µL via nasal (50 µL in each nostril).

**Experimental design.** A standard practice of the poultry industry is to administer MDV vaccine *in ovo*. In this study in order to mimic this practice, the ILTV ΔORF C recombinant virus was administered combined with a full dose of the bivalent MDV vaccine. Embryos that were not vaccinated with ILTV received a full dose of MDV bivalent vaccine by itself (Table 4.1). A total of 600 commercial layer eggs (Hy-line North America Inc., Mansfield, Georgia, USA) were incubated at 99.5˚F and 55% humidity. At 18 days of embryonation the eggs were candled and non-viable embryos were eliminated. At 19.5 days of embryonation 582 embryos were vaccinated with 200 μL of ILTV/MDV or MDV vaccine, delivered into the amniotic fluid. A total of 240 embryos were inoculated with a full dose of MDV vaccine together with recombinant ΔORF C virus, 120 with a 3.8 ($log_{10}$) $TCID_{50}$ dose (Vx3.8) and the remaining 120 with a 4.1 ($log_{10}$) $TCID_{50}$ dose (Vx4.1). A total of 342 embryos were *in ovo* vaccinated solely with a full dose of MDV vaccine and identified as non-vaccinated for ILTV *in ovo* (NVx). Each group of embryos was hatched in a separate incubator and identified with neck tags at hatch.

In order to evaluate the effects of a second vaccination with ΔORF C, at eight-days of age, the Vx3.8 group of chickens was divided in three subgroups: the first (*n*=36) did not receive any second vaccination (Vx3.8); the second (*n*=34) was revaccinated via spray (Vx3.8+S); and the third (*n*=33) was revaccinated via nasal-oral (Vx3.8+N). Similarly, the Vx4.1 group of chickens was divided in three subgroups: the first (*n*=37) did not receive any second vaccination (Vx4.1); the second (*n*=35) was revaccinated via spray (Vx4.1+S); and the third (*n*=29) was revaccinated via nasal-oral (Vx4.1+N).

In order to assess the protection of ΔORF C virus when administered singly by spray or nasal-oral post-hatch a total of 139 chickens not previously vaccinated in ovo with ILTV (NVx) were vaccinated either via spray (Vx (S) *n*=69) or via nasal-oral (Vx (N) *n*=70) (Table 1) with ΔORF C virus at the dose mention above.

At 28-days of age, chickens from NVx, Vx (S), Vx (N), Vx3.8, Vx3.8+S, Vx3.8+N, Vx 4.1, Vx4.1+S and Vx4.1+N were challenged with the virulent ILTV strain GA 63140 at 3.0 ($log_{10}$) $TCID_{50}$/chicken (Table 4.1). The challenge virus was administered in a total volume of 200 µL per chicken, 100 µL were applied intratracheally and 100 µL via eye-drop (50 µL in each eye). A group of NVx chickens (NXvNCh) was inoculated with 200 µL of cell culture media (100 µL intratracheally and 50 µL in each eye).

**Evaluation post vaccination.** Hatchability, cumulative mortality and weight were used as parameters to assess the impact of the recombinant ΔORF C virus post vaccination. Cumulative mortality was recorded from hatch until eight-days of age. All chickens were weighed at 28-days of age.

**Evaluation post challenge.** Clinical sings and mortality were scored from 3 to 7 days post challenge (PC) as previously described by A. Vagnozzi, et al. (39). Briefly, each chicken received a score for each category of clinical sign, in which normal condition received a score of 0, mild a score of 1, moderate a score of 2 and severe a score of 3, dead chickens received a total score of 6. The sum of clinical signs for each bird was averaged and a mean clinical signs score (CSS) was assigned at each time point for each

group of chickens. Mean clinical signs scores at the peak of clinical signs were compared among groups of chickens. Tracheal swabs were collected from all chickens at five days post challenge and analyzed by qPCR to quantify challenge virus genome load. All chickens were weighed before challenge at 28-days of age and at seven days post-challenge (35-days of age) to calculate the average percentage body weight gained per group of chickens after challenge. The percentage body weight gained per chickens was calculated using the following formula: [(Final weight – Initial weight) / Initial weight]. The percentage reduction in tracheal viral genome load for vaccinated groups of chickens was calculated as described below.

**DNA extraction.** Tracheal swabs were placed in 2 mL sterile tubes containing PBS solution + 1% antibiotic-antimycotic (penicillin + streptomycin+ amphotericin B) + 2% newborn calf serum and stored at -80˚C until processing for DNA extraction. DNA was extracted using MegaZorb DNA mini-prep kit® (Promega, Madison, Wisconsin, USA), according to manufacturer's recommendations with some modifications. Briefly, 70 µL swab suspension was resuspended with 7 µL of proteinase K and 50 µL of lysis buffer at 56˚C for 10 minutes in a 96-well plate. 20 µL of magnetic beads were added along with 125 µL of binding buffer to each well, following incubation for 10 minutes at room temperature. The supernatant was removed and the beads were washed twice with washing buffer. Finally, the DNA was eluted from the beads with 100 µL of elution buffer.

**Quantitative real-time PCR for ILTV.** Viral genome load were determined by qPCR in tracheal swabs collected at five days post challenge. A duplex real-time PCR assay normalized for α-collagen chicken DNA was utilized as previously described by A. Vagnozzi, et al. (38). Briefly, the viral genome load for each sample was normalized to the amount of host collagen DNA. The relative amount of viral DNA was per sample was expressed as $\log_{10}^{2-\Delta\Delta Ct}$ (26). Percentage reduction in tracheal viral genome load for vaccinated groups was calculated using the following formula: [(mean $\log_{10}^{2-\Delta\Delta Ct}$ from NVx-Ch – mean $\log_{10}^{2-\Delta\Delta Ct}$ from the group) / mean $\log_{10}^{2-\Delta\Delta Ct}$ from NVx-Ch].

**Serology.** Commercial layers used in the study originated from CEO vaccinated breeders, so the presence of ILTV maternal antibodies was expected. To determine the levels of ILTV maternal antibodies, serum samples were collected at 1 and 4-days of age from 51 to 55 chickens originated from the same company. Antibodies were detected with an in-house developed recombinant glycoprotein B (gB) based ELISA previously described by (20). Briefly, recombinant glycoprotein B expressed by bacuolavirus was purified from culture supernatants. The gB ELISA was shown not to cross-react with polyclonal sera raised against other diseases of poultry. Reactivity of sera was expressed as sample to positive ratio (SP Value) per plate. The SP values were calculated as a corrected optical density (OD) of the unknown sample. Briefly, OD unknown sample minus average OD of known negative divided by the average OD of known positive sample. Samples that received an SP value greater than 0.11 were considered positive for glycoprotein B antibodies.

**Statistical analysis.** Data were entered into Microsoft Office Excel 2011 and analyzed via Prism 6.0 software (GraphPad Software Inc., La Jolla, CA). Kruskal-wallis test was used to determine statistical differences in weight post vaccination, weight-gain post challenge, clinical signs scores at five days post challenge and, viral genome load post challenge among all the groups. All statistical analyses were performed at the 5% level of significance. Antibody levels, percentage hatchability and cumulative mortality were calculated in Microsoft Excel and were not statistically analyzed.

## RESULTS

**Serology.** The mean SP value obtained for commercial layer chickens at 1 day of age ($N$=55) was 0.22, and at four-days of age ($N$=51) was 0.24 (Figure 4.1). These results indicate that as previously tested the source of commercial layer embryos (Hy-line North America) utilized in this experiment carries maternally derived ILTV antibodies.

**Hatchability, mortality and weight post-vaccination.** The hatchability of NVx and vaccinated groups of chickens ranged from 89% to 92% (Table 4.2). Cumulative mortality until eight-days of age was notably higher in Vx3.8 and Vx4.1 groups, reaching 6.4% and 6.5%, respectively, whereas the NVx group reached only 2.2% cumulative mortality (Table 4.2). Average weight at 28-days of age ranged from 274.0g to 299.8g. Weights of ILTV vaccinated groups of chickens were statistically no different ($P\geq0.08$; $n\geq29$) to the NVx group of chickens (Table 4.2).

**Protection efficacy.** To evaluate protection efficacy induced by the *in ovo* vaccination with ΔORF C followed by revaccination via nasal-oral or spray post-hatch, at 28-days of age, chickens vaccinated with ΔORF C and one group of non-vaccinated chickens (NVxCh) where challenged with a virulent ILTV GA 63140 and monitored for clinical signs of ILT from 3 to 7 days post challenge (PC). As shown in figure 4.2A, the peak of clinical signs was identified at five days PC, particularly noticeable for the NVxCh group of chickens. The mean clinical signs at five days post challenge for each group of chickens is shown in figure 4.2B. Clinical signs scored for Vx(S), Vx(N), Vx3.8 and Vx3.8+S groups of chickens were statistically similar to the clinical signs of the NVxCh group ($P \geq 0.07$). Clinical signs scored for Vx3.8+N, Vx4.1, Vx4.1+S and Vx4.1+N were statistically lower as compared to the NVxCh group ($P < 0.0001$). However, still statistically higher ($P \leq 0.002$) as compared to NVxNCh group of chickens (Figure 4.2B)

The weight-gain between 28 and 35-days of age (7 days PC) was calculated. The percentage mean weight-gain post challenge for each group is shown in Figure 4.2C. The highest weight-gain was noted for NVx-NCh chickens (35.2%), statistically higher ($P < 0.0001$) than the weight-gain of the NVx-Ch group (21.5%). The mean weight-gain recorded for Vx3.8 (31.8%), Vx3.8+S (32.6%), Vx3.8+N (36.1%) and Vx4.1+N (31.7%) groups were statistically similar to the weight gained by the NVx-NCh group ($P \geq 0.10$). Interestingly, Vx4.1 and Vx4.1+S gained 31.1% and 29.6% weight, respectively, which were significantly lower to weight gained by the NVx-NCh group of chickens ($P \leq 0.02$). Mean weight-gain for the groups Vx(S) and Vx(N) was 24.8% and 24.5%, respectively, and significantly lower compared to NVx-NCh chickens ($P < 0.0001$).

Mean challenge virus genome load in tracheal swabs collected at five days post challenge are presented in figure 4.2D. Mean challenge virus genome load from Vx(S) and Vx(N) groups was 3.14 and 2.82, respectively, similar to genome load (3.02) detected for NVxCh group ($P>0.99$). Mean challenge virus genome load detected for Vx3.8, Vx3.8+S, Vx3.8+N, Vx4.1, Vx4.1+S and Vx4.1+N groups of chickens ranged from 0.38 to 1.75, and were significantly lower as compared to the NVxCh group ($P\leq0.0013$). Mean challenge viral loads from Vx4.1+S and Vx4.1+N groups of chickens were 0.38 and 0.68, respectively, significantly lower than those detected for NVx-Ch group of chickens ($P<0.0001$) (Figure 4.2D). Challenge virus load percentage reduction of Vx (S) and Vx (N) groups of chickens was 0 and 7%. Vaccinated groups Vx3.8, Vx3.8+S, Vx3.8+N and Vx4.1 reached 48%, 48%, 62% and 67% reduction of challenge virus load, respectively, while Vx4.1+N and Vx4.1+S reached 77% and 87% reduction in challenge virus genome load (Figure 4.2D).

**DISCUSSION**

We have previously shown that *in ovo* vaccination with the recombinant ΔORF C induced partial protection in commercial layers. The diminished protective efficacy was attributed to the interaction of maternally derived antibodies with the recombinant virus, (34). The objective of this study was to evaluate if a second immunization with ΔORF C virus enhances the protection elicited by *in ovo* vaccination in commercial layers. The protection efficacy of ΔORF C virus was evaluated when administered via *in ovo*, spray, or nasal-oral routes, and *in ovo* followed by either spray or nasal-oral revaccination at eight-days of age in the presence of maternally derived antibodies.

*In ovo* deposition of MDV vaccines into the amniotic fluid or embryo body enhances the embryo`s response and consequently improves performance of vaccination (41, 42). The optimal time for *in ovo* vaccination that ensures greater rate of deposition of inoculum in the amniotic fluid is between 17.5 and 19 days of embryonation (42). Due to the lower embryo development of leghorn type birds as compared to broiler chickens (40), in this study chicken embryos were vaccinated at 19.5 days of embryonation. Performing *in ovo* vaccination of commercial layers manually at 19.5 days of embryonation we obtained a deposition of 81.5% in the amniotic fluid and 12.5% in the embryo body (data not shown).

*In ovo* vaccination with the recombinant ILTV had no effect on the hatchability, in agreement with previous studies in commercial broilers, layers and SPF chickens (27, 34).

*In ovo* vaccination with ILTV and NDV recombinant viruses induced high mortality in SPFs chickens, that was associated with increased viral replication (29, 34). However we have observed that mortality induced by *in ovo* vaccination of commercial layers with ΔORF C virus can vary. In two consecutive experiments utilizing the same source of commercial layers vaccinated *in ovo* at 19.5-days of embryonation, with dosages ranging from 3.5 to 4.1 ($log_{10}$) mortality varied from 0% to 6.5%. The presence of ILTV maternally derived antibodies was confirmed by an in-house developed recombinant glycoprotein B (gB) ELISA. The mean SP value for anti-gB antibodies obtained from one-day old chickens within the group that did not presented mortality was 0.35 (+/- 0.25 SD) (34). While in this study, where mortality reached up to 6.5%, the mean SP values for anti-gB antibodies was 0.22 (+/- 0.34 SD). Although we cannot

determine if there are significant differences in maternal antibodies levels between the two experiments, these results may hint that mortality induced by *in ovo* vaccination with ΔORF C virus is related to the level of maternally derived antibodies. In contrast to *in ovo* vaccination, revaccination with ΔORF C virus at eight-days of age via spray and nasal-oral route did not further increase the mortality rate within the vaccinated groups. This was expected as previous work has shown that recombinant ΔORF C virus is highly attenuated as indicated by the low level of replication in trachea after mucosal vaccination via eye-drop (13).

Weight-gain has been previously utilized as a parameter to characterize the virulence of ILTV strains (24, 34). Although this study analyzes weight at 28-days of age, our data corroborates previous studies indicating that *in ovo* vaccination with the recombinant ΔORF C virus has no effect on weight-gain (25, 34).

In this study a fully protected group of vaccinated chickens was defined by a reduction in clinical signs and challenge virus genome load, and increased weigh-gain post challenge. Based on the established parameters, the Vx3.8+N and Vx4.1+N groups of chickens were fully protected against ILT. While Vx4.1 and Vx4.1+S were partially protected based on the lower weight gained; and Vx3.8 and Vx3.8+S were partially protected based on failure to reduce clinical signs. Groups Vx(S) and Vx(N) were considered not protected based on failure to reduce clinical signs and tracheal challenge virus load, and low weight gain. Among the parameters evaluated after challenge the reduction of challenge virus genome load ranging from 48% to 62% in groups vaccinated *in ovo* with 3.8 ($log_{10}$) and 67% to 87% in groups vaccinated *in ovo* with 4.1 ($log_{10}$) indicated that both the *in ovo* priming dose and revaccination greatly enhanced protection

efficacy. A. Vagnozzi, et al. (38) and A. Vagnozzi, et al. (39) reported that commercial broilers vaccinated with the commercial CEO vaccine via eye-drop at 14-days of age had reduction in tracheal challenge virus load comparable to that of non-vaccinated non-challenged chickens. Although the objective of this study was not to compare the protection induced by ΔORF C virus to the commercial counterparts, the reduction in challenge virus load post *in ovo* vaccination of commercial layers is a strong indicator of the potential of this recombinant virus in reducing challenge virus load as well as the CEO vaccine, however with the advantage of a mass vaccination method.

In agreement with our previous study, these results demonstrate that recombinant ΔORF C virus is capable of eliciting a protective immune response to challenge after *in ovo* vaccination. Furthermore, we suggested an interaction of maternally derived antibodies with the ΔORF C virus, consequently interfering with the protection efficacy induced by ILTV (34). In this study the protection efficacy induced by ΔORF C in commercial layers indicates that the interaction of the virus with maternally derived antibodies can be overcome and elicit protective response to challenge.

The vaccination with the recombinant ΔORF C induced satisfactory protection post eye-drop vaccination in three-weeks old SPF chickens (13). In contrast, we observed that commercial layer chickens were not protected when vaccinated at eight-days of age via spray and nasal-oral routes. A possible reason for these differences in protection is most likely linked due to the route of administration, suggesting that the ocular mucosa is more responsive to ΔORF C infection than the nasal-oral mucosa. When the virus enters the host via the ocular route, it will first come in contact with the conjunctiva associated lymphoid tissues (CALT) and the peri-ocular Harderian gland, tissues rich in lymphoid

cells (21) and essential sites where immune responses are generated to the express of virus uptake and processing by immune cells (13). Although eye-drop is an accurate mucosal delivery method for poultry vaccination, it is a labor-intensive procedure. Future studies should improve the use of post-hatch mass vaccination methods by optimizing the dose of ΔORF C and the diluent, and the spray technique.

In conclusion, the possible interaction between maternally derived antibodies against ILTV and *in ovo* vaccination with ΔORF C can be overcome by increased doses of the virus *in ovo* and revaccination. Based on clinical signs, weight-gain and viral genome load in trachea post challenge, *in ovo* administration of ΔORF C has the potential to protect chickens bearing maternally derived antibodies against ILTV. *In ovo* priming dose and revaccination greatly enhanced protection efficacy, however, the administration of ΔORF C solely via spray or nasal-oral routes did not induce protection post-challenge highlighting the better suitability of the ΔORF C virus for *in ovo* vaccination. Future studies remain necessary to optimize the protection efficacy induced by vaccination with ΔORF C against ILT and to satisfy the safety considerations according to the Title 9 of the Code of Federal Regulations, in SPF chickens.

Table 4.1. Groups vaccinated *in ovo* with MDV or MDV+ILTV at 19.5-days of embryonation, revaccinated at eight-days of age and challenged at 28-days of age

| *In ovo* vaccine | Post-hatch vaccine [c] | Group post vaccination | *N* [d] | Challenge (Ch) | Groups post Ch | *N* Ch |
|---|---|---|---|---|---|---|
| MDV [a] | - | Non-vaccinated (NVx) | 65 | No | NVxNCh | 65 |
| MDV | - | Non-vaccinated (NVx) | 64 | Yes | NVxCh | 64 |
| MDV | Spray | Vx(S) | 69 | Yes | Vx(S)Ch | 69 |
| MDV | Nasal-oral | Vx(N) | 70 | Yes | Vx(N)Ch | 70 |
| MDV [a] +ILTV 3.8 ($log_{10}$) [b] | - | Vx3.8 | 36 | Yes | Vx3.8Ch | 36 |
| MDV+ILTV 3.8 ($log_{10}$) | Spray | Vx3.8+S | 34 | Yes | Vx3.8+SpCh | 34 |
| MDV+ILTV 3.8 ($log_{10}$) | Nasal-oral | Vx3.8+N | 33 | Yes | Vx3.8+InCh | 33 |
| MDV+ILTV 4.1 ($log_{10}$) | - | Vx4.1 | 37 | Yes | Vx4.1Ch | 37 |
| MDV+ILTV 4.1 ($log_{10}$) | Spray | Vx4.1+S | 35 | Yes | Vx4.1+SpCh | 35 |
| MDV+ILTV 4.1 ($log_{10}$) | Nasal-oral | Vx4.1+N | 29 | Yes | Vx4.1+InCh | 29 |

[a] Full dose bivalent vaccine Cevac HVT& SB-1® (Ceva Animal Health); [b] ILTV ΔORF C recombinant virus dose per embryo; [c] Post-hatch vaccination with BΔORFC at 3.8 ($log_{10}$) $TCID_{50}$/embryo via spray or nasal-oral application at eight-days of age; [d] Number of chickens in each group at eight-days of age.

Table 4.2. Hatchability, mortality and weight post *in ovo* vaccination with MDV or MDV+ILTV

| Group | Hatchability (%) | Cumulative mortality (%)[a] | Weight (g) [b] |
|---|---|---|---|
| NVx | 89% | 2.2% | 295.2 (± 33.9) |
| Vx(S) | | - | 284.8 (± 31.2) |
| Vx(N) | | - | 289.5 (± 34.7) |
| Vx3.8 | 92% | 6.4% | 274.0 (± 34.1) |
| Vx3.8+S | | - | 278.2 (± 33.9) |
| Vx3.8+N | | - | 289.8 (± 38.4) |
| Vx4.1 | 92% | 6.5% | 286.4 (± 29.6) |
| Vx4.1+S | | - | 299.8 (± 33.6) |
| Vx4.1+N | | - | 289.1 (± 37.0) |

[a] Cumulative mortality from 1 to 8-days of age; [b] Weight at 28-days of age.

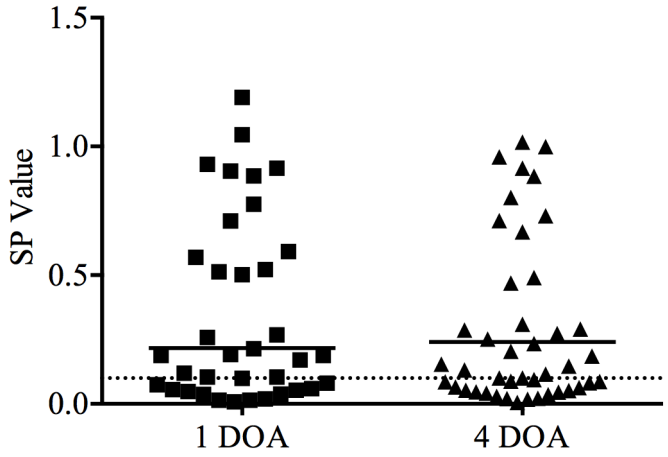


Figure 4.3.Maternal antibodies against ILTV in non-vaccinated commercial layers at 1 and 4-days of age (DOA). The presence of maternal antibodies was analyzed by an in-house developed glycoprotein B (gB) based ELISA utilizing serum from 1 and 4 days old chickens. Reactivity of sera was expressed as sample to positive ratio (SP Value). Dashed line represents the SP cut off value (0.11).

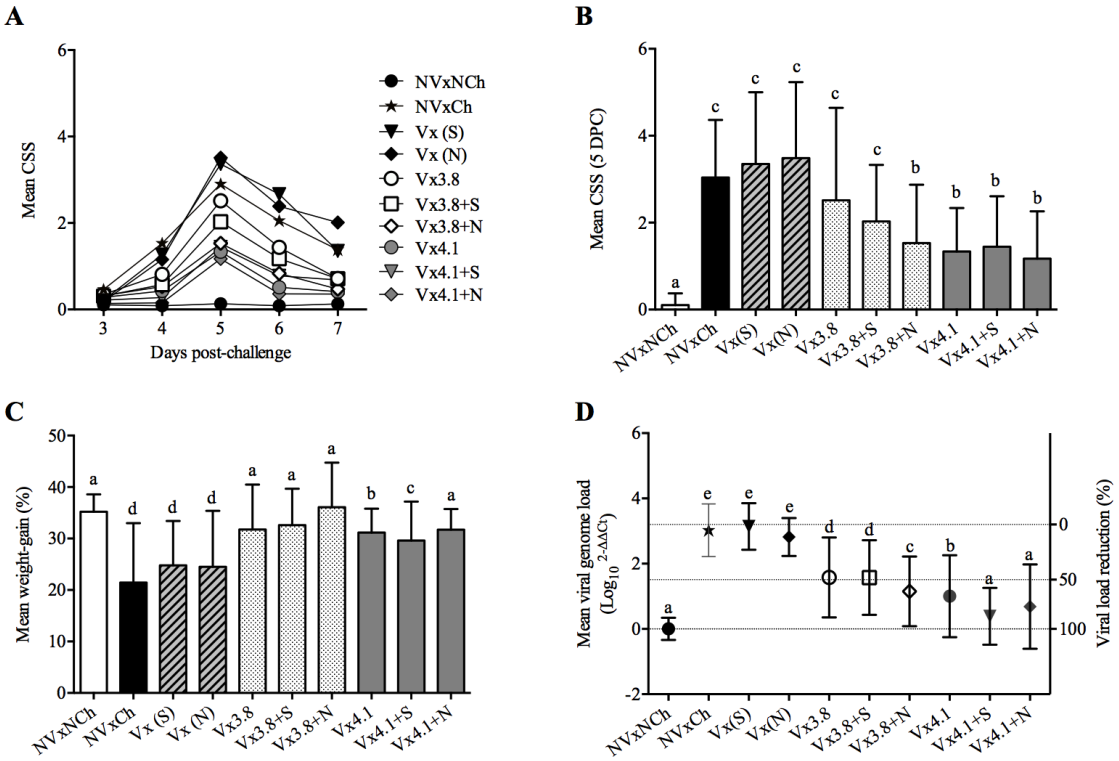


**Figure 4.4.** Protection efficacy elicited by inoculation of ΔORF C *in ovo*. Chickens inoculated with ΔORF C and one group of non-vaccinated chickens (NVxCh) were challenged with the virulent ILTV strain GA 63140. One group of chickens remained unchallenged (NVxNCh). Peak of clinical signs (A); Mean clinical signs scores per group at five days post challenge (B); Mean weight-gain at five days post challenge (C); Mean viral genome load in the trachea and percentage reduction in viral load at five days post challenge (D). Different superscripts letters express statistical differences among the groups.

## REFERENCES


1. Alls AA, Ipson JR, Vaughan WD. 1969. Studies on an ocular infectious laryngotracheitis vaccine. Avian Dis 13:36-45.
2. Armour NK, García M. 2014. Current and Future Applications of Viral-Vectored Recombinant Vaccines in Poultry. The Poultry Informed Professional:1-9.
3. Avakian A, Klein D, Whitfill C, Tripathy D, Singbeil B, Poston R, Grosse D. 1999. Safety and efficacy of fowl and pigeon pox vaccines administered in ovo to SPF and broiler embryos. Western Poultry Disease Conference Proceedings of Western Poultry Disease Conference:56-60.
4. Bagust TJ, Jones RC, Guy JS. 2000. Avian infectious laryngotracheitis. Rev Sci Tech 19:483-492.
5. Clarke JK, Robertson GM, Purcell DA. 1980. Spray vaccination of chickens using infectious laryngotracheitis virus. Aust Vet J 56:424-428.
6. Coppo MJ, Noormohammadi AH, Hartley CA, Gilkerson JR, Browning GF, Devlin JM. 2011. Comparative in vivo safety and efficacy of a glycoprotein G-deficient candidate vaccine strain of infectious laryngotracheitis virus delivered via eye drop. Avian Pathol 40:411-417.
7. Cover MS, Benton WJ, Krauss WC. 1960. The Effect of Parental Immunity and Age on the Response to Infectious Laryngotracheitis Vaccination, p 467. American Association of Avian Pathologists, Inc.
8. Devlin JM, Viejo-Borbolla A, Browning GF, Noormohammadi AH, Gilkerson JR, Alcami A, Hartley CA. 2010. Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus. Vaccine 28:1325-1332.

9. Dufour-Zavala L. 2008. Epizootiology of infectious laryngotracheitis and presentation of an industry control program. Avian Dis 52:1-7.

10. Esaki M, Noland L, Eddins T, Godoy A, Saeki S, Saitoh S, Yasuda A, Dorsey KM. 2013. Safety and Efficacy of a Turkey Herpesvirus Vector Laryngotracheitis Vaccine for Chickens. Avian Diseases 57:192-198.

11. Fulton RM, Schrader DL, Will M. 2000. Effect of route of vaccination on the prevention of infectious laryngotracheitis in commercial egg-laying chickens. Avian Dis 44:8-16.

12. Gagic M, St Hill CA, Sharma JM. 1999. In ovo vaccination of specific-pathogen-free chickens with vaccines containing multiple agents. Avian Dis 43:293-301.

13. García M, Cheng Y, Spatz SJ, Riblet SM, Schneiders GH, Volkening J. 2016 Submitted. Attenuation and Protection Efficacy of Open Reading Frame C (ORF C) Gene Deleted Strain of The Alphaherpesvirus Infectious Laryngotracheitis Virus (ILTV)

14. García M, Spatz S, Guy JS. 2013. Laryngotracheitis, p 161-179. *In* Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair V (ed), Diseases of Poultry, 13th ed. Blackwell Publishing, Ames, Iowa.

15. Gelenczei EF, Marty EW. 1965. Strain Stability and Immunologic Characteristics of a Tissue-Culture-Modified Infectious Laryngotracheitis Virus. Avian Dis 9:44-56.

16. Gelenczei EF, Marty EW. 1964. Studies on a Tissue-Culture-Modified Infectious Laryngotracheitis Virus, p 105. American Association of Avian Pathologists, Inc.

17. Giambrone JJ, Dormitorio T, Brown T. 2001. Safety and Efficacy of in ovo Administration of Infectious Bursal Disease Viral Vaccines, p 144. American Association of Avian Pathologists, Inc.

18. Gimeno IM, Cortes AL, Witter RL, Pandiri AR. 2012. Optimization of the protocols for double vaccination against Marek's disease by using commercially available

vaccines: evaluation of protection, vaccine replication, and activation of T cells. Avian Dis 56:295-305.

19. Gimeno IM, Faiz NM, Cortes AL, Barbosa T, Villalobos T, Pandiri AR. 2015. In Ovo Vaccination with Turkey Herpesvirus Hastens Maturation of Chicken Embryo Immune Responses in Specific-Pathogen-Free Chickens. Avian Dis 59:375-383.

20. Godoy A, Icard A, Martinez M, Mashchenko A, García M, El-Attrache J. 2013. Detection of Infectious Laryngotracheitis Virus Antibodies by Glycoprotein-Specific ELISAs in Chickens Vaccinated with Viral Vector Vaccines. Avian Diseases 57:432-436.

21. Härtle S, Kaspers B. 2014. Chapter 14 - The Avian Respiratory Immune System A2 - Kaiser, Karel A. SchatBernd KaspersPete, p 251-263, Avian Immunology (Second Edition) doi:http://dx.doi.org/10.1016/B978-0-12-396965-1.00014-5. Academic Press, Boston.

22. Hitchner SB, Winterfield RW. 1960. Revaccination Procedures for Infectious Laryngotracheitis, p 291. American Association of Avian Pathologists, Inc.

23. Jochemsen P, Jeurissen SH. 2002. The localization and uptake of in ovo injected soluble and particulate substances in the chicken. Poult Sci 81:1811-1817.

24. Kirkpatrick NC, Mahmoudian A, Colson CA, Devlin JM, Noormohammadi AH. 2006. Relationship between mortality, clinical signs and tracheal pathology in infectious laryngotracheitis. Avian Pathol 35:449-453.

25. Legione AR, Coppo MJ, Lee SW, Noormohammadi AH, Hartley CA, Browning GF, Gilkerson JR, O'Rourke D, Devlin JM. 2012. Safety and vaccine efficacy of a glycoprotein G deficient strain of infectious laryngotracheitis virus delivered in ovo. Vaccine 30:7193-7198.

26. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

27. Mashchenko A, Riblet SM, Zavala G, García M. 2013. In Ovo Vaccination of Commercial Broilers with a Glycoprotein J Gene-Deleted Strain of Infectious Laryngotracheitis Virus. Avian Diseases 57:523-531.

28. Ohta H, Ezoe S, Yamazaki K, Kawai T, Honda T. 2009. Application of aluminum hydroxide for an in ovo live Newcastle disease vaccine. Avian Dis 53:392-395.

29. Ramp K, Topfstedt E, Wackerlin R, Hoper D, Ziller M, Mettenleiter TC, Grund C, Romer-Oberdorfer A. 2012. Pathogenicity and immunogenicity of different recombinant Newcastle disease virus clone 30 variants after in ovo vaccination. Avian Dis 56:208-217.

30. Reed LJ, Muench H. 1938. A Simple Method of Estimating Fifty Per Cent Endpoints. American Journal of Hygiene 27:493-497.

31. Ricks CA, Avakian A, Bryan T, Gildersleeve R, Haddad E, Ilich R, King S, Murray L, Phelps P, Poston R, Whitfill C, Williams C. 1999. In ovo vaccination technology. Adv Vet Med 41:495-515.

32. Rodriguez-Avila A, Oldoni I, Riblet SM, García M. 2007. Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines. Avian Dis 51:905-911.

33. Samberg Y, Cuperstein E, Bendheim U, Aronovici I. 1971. The development of a vaccine against avian infectious laryngotracheitis. IV. Immunization of chickens with a modified laryngotracheitis vaccine in the drinking water. Avian Dis 15:413-417.

34. Schneiders GH, Riblet SM, García M. 2016. Potential of an infectious laryngotracheitis (ILT) recombinant virus depleted of open reading frame (ORF) C gene as a live attenuated vaccine for in ovo usage

35. Sharma JM. 1989. In situ production of interferon in tissues of chickens exposed as embryos to turkey herpesvirus and Marek's disease virus. Am J Vet Res 50:882-886.

36. Sharma JM, Burmester BR. 1982. Resistance of Marek's Disease at Hatching in Chickens Vaccinated as Embryos with the Turkey Herpesvirus, p 134. American Association of Avian Pathologists, Inc.

37. Sharma JM, Lee LF, Wakenell PS. 1984. Comparative viral, immunologic, and pathologic responses of chickens inoculated with herpesvirus of turkeys as embryos or at hatch. Am J Vet Res 45:1619-1623.

38. Vagnozzi A, García M, Riblet SM, Zavala G. 2010. Protection induced by infectious laryngotracheitis virus vaccines alone and combined with Newcastle disease virus and/or infectious bronchitis virus vaccines. Avian Dis 54:1210-1219.

39. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian Pathol 41:21-31.

40. Villalobos T. 2014. Optimal timing for in ovo vaccination - understanding embryo development. International Hatchery Practice 28:15-15.

41. Wakenell PS, Bryan T, Schaeffer J, Avakian A, Williams C, Whitfill C. 2002. Effect of in ovo vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia. Avian Dis 46:274-280.

42. Williams C. 2007. In ovo vaccination for disease prevention, vol 15, p 7-9. Positive Action Publications Ltd, Driffield; UK.

43. Williams CJ, Zedek AS. 2010. Comparative field evaluations of in ovo applied technology. Poult Sci 89:189-193.

44. Witter RL, Nazerian K, Purchase HG, Burgoyne GH. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am J Vet Res 31:525-538.

# CHAPTER 5

# DISCUSSION

The avian infectious laryngotracheitis virus (ILTV) continues to cause outbreaks of the disease worldwide. While live attenuated vaccines can offer a good protection, the vaccine strains can also produce latent infections as well as clinical disease after consecutive passages in chickens in the field. Therefore, lapses in biosecurity and improper vaccine administration contribute to the spread of ILTV and subsequent emergence of outbreaks. In an effort to improve control of ILTV, attention has turned towards developing more stable ILTV live attenuated vaccines by deletion of genes associated with viral virulence. Recently, a recombinant ILTV depleted of open reading frame C (ORF C) gene was developed and shown to be significantly attenuated for intratracheal/ocular and eye-drop delivery in SPF chickens, yet eliciting high level of protection post challenge, similar to that of the TCO vaccine (1). This thesis assesses the possibility of mass vaccination of chickens with recombinant ΔORF C virus, via *in ovo* route.

The objective of the first study (Chapter 3) was to evaluate the attenuation and protection efficacy of recombinant ΔORF C virus for *in ovo* vaccination of chickens in the absence and presence of maternally derived antibodies. Attenuation was assessed based on hatchability, cumulative mortality, clinical signs, weight-gain and transmission post-vaccination. Overall, *in ovo* vaccination with ΔORFC did not influence hatchability and weight-gain, showed low levels of transmission to naïve contact chickens and induced low levels of clinical signs post-vaccination. No mortalities post-vaccination were recorded for chickens bearing maternally derived antibodies, however, the virus still induces high mortality levels during the first three weeks of age in the absence of maternally derived antibodies. Protection efficacy was assessed on

the basis of prevention or reduction of clinical signs, and reduction of tracheal viral load without negative effects in weight-gain post-challenge. *In ovo* vaccination with ΔORF C was capable of inducing complete protection in chickens impaired of maternally derived antibodies, however only partial protection in chickens bearing maternally derived antibodies. Therefore, maternally derived antibodies against ILTV most likely interacted with the replication of recombinant ΔORF C virus post-vaccination and consequently interfered with protection efficacy in a portion of the vaccinated chickens.

The objective of the second study (Chapter 4) was to evaluate the potential of the recombinant ΔORF C virus to elicit protection against ILT in chickens bearing maternally derived antibodies when administered singly via *in ovo*, spray, or nasal-oral, and when vaccinated *in ovo* and revaccianted by either spray or nasal-oral application at eight-days post-hatch. Hatchability, cumulative mortality and weight were assessed post-vaccination. Overall, the results of this study confirm that ΔORF C had no effects in hatchability and weight post *in ovo* vaccination, however chickens with maternally derive antibodies vaccinated *in ovo* with recombinant ΔORF C virus exhibited up to 6.5% mortality during the first week of age while no ILT vaccinated chickens presented up to 2.2% mortality during the first week of age. No mortalities were observed after revaccination by oral-nasal or spray administration. Chickens vaccinated *in ovo* and revaccinated via nasal-oral route were completely protected against ILT, whereas chickens vaccinated solely *in ovo* or vaccinated *in ovo* and revaccinated via spray were partially protected. The *in ovo* priming dose and the route of revaccination greatly enhanced the protection efficacy of the virus while the sole vaccination via spray or nasal-oral routes at eight days of age was not protective. As shown by our laboratory the ΔORF C virus induced a solid protective response in three week-old SPF chickens when applied via the ocular mucosal route (1). Therefore the failure of ΔORF C virus to protect eight day-old chickens may be associated with the administration route. It appears that the nasal-oral route was not as effective as the ocular route in eliciting an adequate immune response, but further studies remain necessary to confirm this hypothesis.

A highlight of this study (Chapter 3) was the significant reduction of challenge virus load observed in trachea of chickens vaccinated *in ovo* with the ΔORF C virus in the presence of maternally derived antibodies. Although our objective was not to compare the recombinant ILTV with the commercial counterparts, the challenge virus reduction achieved by *in ovo* with the ΔORF C virus in the presence of maternally derived antibodies was comparable to that reported for the live attenuated CEO vaccine when administered via eye-drop to 14-day old chickens (4, 5). Two other gene-deleted ILTV recombinants have been tested for their suitability for *in ovo* vaccination of chickens. Whereas one of them, depleted of the glycoprotein J (gJ) still induced high mortality of commercial broilers (3), the other, depleted of glycoprotein G (gG) failed to reduce tracheal virus load post-challenge (2).

In conclusion, *in ovo* vaccination with the recombinant ΔORF C was capable of eliciting a protective response to challenge in the presence or absence of maternally derived antibodies. Although ILTV maternally derived antibodies weakened the protective efficacy of *in ovo* vaccination *in ovo* priming dose and revaccination greatly enhanced protection efficacy and outdid interference of maternally derived antibodies. To our knowledge, this study is the first one to demonstrate the interference of maternal antibodies with *in ovo* vaccination against ILTV. The administration of ΔORF C solely via spray or intranasally did not induce protection post-challenge highlighting the suitability of the ΔORF C virus for *in ovo* vaccination. Due to the considerable mortality, especially during the first week of age of SPF chickens, the ΔORF C virus in its present form does not satisfy the USDA safety considerations.

## REFERENCES

1. García M, Cheng Y, Spatz SJ, Riblet SM, Schneiders GH, Volkening J. 2016 Submitted. Attenuation and Protection Efficacy of Open Reading Frame C (ORF C) Gene Deleted Strain of The Alphaherpesvirus Infectious Laryngotracheitis Virus (ILTV)
2. Legione AR, Coppo MJ, Lee SW, Noormohammadi AH, Hartley CA, Browning GF, Gilkerson JR, O'Rourke D, Devlin JM. 2012. Safety and vaccine efficacy of a glycoprotein G deficient strain of infectious laryngotracheitis virus delivered in ovo. Vaccine 30:7193-7198.
3. Mashchenko A, Riblet SM, Zavala G, García M. 2013. In Ovo Vaccination of Commercial Broilers with a Glycoprotein J Gene-Deleted Strain of Infectious Laryngotracheitis Virus. Avian Diseases 57:523-531.
4. Vagnozzi A, García M, Riblet SM, Zavala G. 2010. Protection induced by infectious laryngotracheitis virus vaccines alone and combined with Newcastle disease virus and/or infectious bronchitis virus vaccines. Avian Dis 54:1210-1219.
5. Vagnozzi A, Zavala G, Riblet SM, Mundt A, García M. 2012. Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens. Avian Pathol 41:21-31.