AUSTRALIAN EGG CORPORATION LIMITED

Molecular Epidemiology and Pathogenesis of Infectious Laryngotracheitis Viruses in Australia

A report for the Australian Egg Corporation Limited

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Foreword

Infectious laryngotracheitis (ILT) is a respiratory disease of chickens caused by a virus belonging to the family *Herpesviridae*. ILT has been identified in most countries around the world and remains a threat to the intensive poultry industry. The severity of ILT varies from a subclinical infection to a severe respiratory disease with a high mortality. The mild form of ILT is particularly difficult to diagnose or differentiate from other respiratory diseases. In addition, overseas investigations have shown that ILT vaccine strains can cause adverse reactions, reduced growth rate and increased FC in broilers. Hence estimation of the loss due to the disease is difficult except if a thorough epidemiological investigation is carried out. In Australia, outbreaks of mild to moderate forms of ILT have been reported in commercial layer flocks in some regional areas. In addition, sporadic outbreaks of ILT in broiler flocks has been recognised as an emerging problem as evidenced by the increasing number of ILT outbreaks in recent years particularly in the Sydney basin and in South Australia. Some of these outbreaks are suspected to be caused by Australian vaccine strains. However no documented study has investigated this possibility nor determined the relative virulence of the viral isolates. It is expected that the results of this project will address issues regarding eradication of ILTV in Australia and will be useful for designing ILT vaccination programs and/or for developing recombinant ILT vaccines. Hence the cost of control programs for ILT and losses due to the disease will ultimately be reduced.

The main objectives of this study were to determine the virulence and spread of the Australian ILTV isolates, to investigate possible genetic/virulence differences between the vaccine re-isolates and the original vaccine and to establish a genetic marker for ILTV virulence which can be developed as an epidemiological tool for investigation of ILTV infection.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report is an addition to AECL's range of research publications and forms part of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Contents

Executive Summary

Infectious laryngotracheitis (ILT) has been identified in most countries around the world and remains a threat to the intensive poultry industry. Outbreaks of mild to moderate forms of ILT are common in commercial layer flocks while sporadic outbreaks of ILT in broiler flocks have also been recognised as an emerging problem in several countries. Examination of viral isolates using Restriction Fragment Length Polymorphism of Polymerase Chain Reaction (PCR-RFLP) from individual ILTV genes has suggested that some of these outbreaks were caused by vaccine strains.

In this study, PCR-RFLP of a number of ILTV genes/genomic regions including gE, gG, TK, ICP4, ICP18.5 and open reading frame (ORF) B-TK was utilized to examine a number of historical and contemporary Australian ILTV isolates and vaccine strains. PCR-RFLP of gE using restriction endonuclease *Eae*I failed to distinguish between any of the isolates including the vaccine strains. PCR-RFLP of gG, TK and ORFB-TK using restriction endonucleases *Msp*I, *Msp*I and *Fok*I, respectively, divided all the isolates into two groups. PCR-RFLP of ICP18.5 and ICP4 using restriction endonuclease *Hae*III separated the isolates into three different groups with some field isolates only able to be distinguished from vaccine strains by PCR-RFLP of ICP18.5. A combination of groupings including gG, TK, ICP4, ICP18.5 and ORFB-TK PCR-RFLP classified the ILTV isolates under investigation into 5 different groups with most isolates distinguishable from vaccine strains.

In another study, 5 groups (16 each) of 3-week-old SPF chickens were inoculated via intratracheal route with 10^3 EID_{50} of 5 different strains of ILTv. Three further groups of chickens of the same age were inoculated similarly with the vaccine strains SA2 and A20 or with sterile phosphate buffered saline (PBS) for comparison. Four days post inoculation, clinical signs were monitored for scoring, and then eight chickens from each group were euthanised, weighed and subjected to pathological and histopathological examinations. The remaining birds in each group were monitored until 21 days post inoculation and monitored for mortality.

All groups inoculated with ILTv strains showed moderate to severe clinical symptoms 4 days after inoculation while birds inoculated with sterile PBS did not reveal any clinical symptoms. A scoring system was developed to compare the effects of ILTv strains on breathing, demeanour and on conjunctiva of the birds. Comparison of the scores for breathing revealed that strain Q1-96 caused only minimal breathing symptoms with a median of scores that was not significantly lower to that of the group inoculated with sterile PBS, but was significantly different to those of the groups inoculated with other ILTv strains. Comparison of scores for conjunctiva revealed that the strain Q1-96 caused severe photophobia and conjunctivitis with a median of scores that was significantly higher than those of all other groups except for the group inoculated with the strain N3-04. All ILTv strains caused a significant reduction in weight gain when compared to that of the sterile PBS. The ILTv strain Q1-96 caused an average weigh loss of 14% that was significantly higher than those of other ILTv strains. The ILTv strains S2-04 and Q1-96 induced only minor microscopic tracheal lesions that were not significantly different to that of the group inoculated with sterile PBS while all the other ILTv strains, including the vaccine strains A20 and SA2 induced moderate to severe microscopic tracheal lesions that were significantly higher than that of the group inoculated with sterile media. Median scores for microscopic tracheal lesions were well correlated with the number of viral genome detected in trachea.

Results from these studies reveal that to achieve reliable identification of strains of ILTV, the examination of multiple gene regions will be required, and that most of the recent ILT outbreaks in Australia are not being caused by vaccine strains. These results also reveal that there is considerable variation among ILTv strains in their tropism for trachea or conjunctiva with the ILTv strains with high affinity for conjunctiva can severely affect weigh gain. The ILTv numbers and microscopic lesions in trachea are not reliable indicators of virulence since they are not necessarily correlated with

mortality rate in ILT. Finally, it is suggested that perhaps factors other than the variation in RFLP patterns of genes reported in previous studies may be involved in virulence of the ILTv strains.

Diferentiation of Infectious Laryngotracheitis Virus Isolates by Restriction Fragment Length Polymorphic Analysis of Polymerase Chain Reaction Products Amplified From Multiple Genes

Introduction

Infectious laryngotracheitis (ILT) has been identified in most countries around the world and remains a threat to the intensive chicken industry. It is caused by an alphaherpesvirus, involved in respiratory disease and reduced egg production, and can predispose the birds to other respiratory pathogens (Guy & Bagust, 2003). Outbreaks of mild to moderate forms of ILT are not uncommon in commercial layer flocks worldwide, while sporadic outbreaks of ILT in broiler flocks has also been recognised in recent years as an emerging problem in several countries including Australia (Critchley, 2004; Wells, 2004). Field evidence indicates that many of the ILT outbreaks may have been due to lack of uniform flock immunity and the transmission of vaccine strain from vaccinated to unvaccinated birds (Andreasen *et al.*, 1989; Churchill, 1965; Hilbink *et al.*, 1987; Samberg *et al.*, 1971). Additionally, it is speculated that some live attenuated vaccine strains of ILTV may occasionally revert to parental-type virulence, causing sporadic ILT outbreaks (Guy *et al.*, 1991). Two live attenuated vaccines (SA2 and A20, Fort Dodge, Australia, Pty. Ltd.) are used to immunize chickens against ILT in Australia. The vaccine strain SA2 was originated from an Australian field isolate by attenuation through sequential passages in chicken embryo (Purcell & Surman, 1974a) while strain A20 originated from SA2 through further passages in chicken embryonic cell culture to lessen residual virulence (Bagust, T. J. 2005, pers. comm.). While the A20 vaccine is considered relatively safe and can be used in very young chickens, the SA2 vaccine is less attenuated and is only recommended for use in adult birds or as a booster vaccine after A20 administration.

ILTV strains may vary in their virulence in chickens, chicken embryo and cell culture. However, ILTV strains are antigenically homogenous and can not be differentiated by virus neutralisation, immunofluorescence or cross protection tests (Guy & Bagust, 2003). Differentiation of ILTV strains of varying virulence, particularly wild-type and modified live vaccine viruses is an important practical problem. While restriction fragment analysis of viral genomic DNA has been used for epidemiological investigations for over a decade (Guy *et al.*, 1989), extraction and purification of the viral genomic DNA requires propagation of the virus and is time consuming. In the past few years, restriction fragment length polymorphism (RFLP) analyses of the viral genes TK, gC, gX, ICP4, gE and gG have been described for epidemiological investigations but resulted in varying degrees of success (Chang *et al.*, 1997; Garcia & Riblet, 2001; Graham *et al.*, 2000; Han & Kim, 2001a). The major criticism of PCR-RFLP of individual genes used by these workers is that only a very small region of the viral genome (approximately $1/30^{th}$ or less) is examined, and that the lack of difference between two isolates/strains in such a relatively small region of the genome cannot necessarily be interpreted as evidence for identical strains.

Objectives

The main purpose of the present study was to develop a rapid method for reliable identification of ILTV strains in epidemiological investigations of ILT whilst also evaluating the possible role of vaccine strains in outbreaks of ILT.

Methodology

Viruses

The commercial ILTV vaccine strains SA2 and A20 (Fort Dodge) and several recent ILTV isolates were used in this study (Table 1). All field isolates were isolated from the upper respiratory tract of infected birds during outbreaks of ILT in Australia. Field isolates V1-99, V2-02, V3-02, Q1-95, Q1-96, Q1-00 and Q1-01 were isolated by the Australian government diagnostic laboratories (Department of Primary Industries, Victoria/Queensland). The field strain CSW was isolated from the upper respiratory tract of infected birds (Fahey *et al.*, 1983). The field isolates V1-03, V1-04, N1-03, N1-04, N2-04, N3-04, S1-04, S2-04, S3-04, and S4-04 were isolated within our laboratory during the course of this study. All virus isolates were propagated on the chorioallantoic membrane (CAM) of 10 day old chicken embryos using method standard techniques (Tripathy & Hanson, 1980).

Table 1. Details of the ILTV isolates used in this study.

Isolate/strain	Year of isolation	Origin
SA2	C1966	Vaccine
A20	1983	Vaccine[B]
CSW	1970	Layer
V1-99	1999	Layer
V2-02	2002	Backyard
V3-02	2002	Backyard
V1-03	2003	Broiler Breeder
V1-04	2004	Backyard
N1-03	2004	Broiler
N1-04	2004	Broiler
N2-04	2004	Broiler
N3-04	2004	Broiler
S1-04	2004	Broiler
S2-04	2004	Layer
S3-04	2004	Layer
S4-04	2004	Unknown
Q1-95	1995	Unknown
Q1-96	1996	Unknown
Q1-00	2000	Layer
Q1-01	2001	Backyard

[B] A20 vaccine derived from clone - purified strain SA2 by Dr T. J. Bagust.

Extraction of viral DNA

Pocks excised from infected CAMs were homogenised in 1 ml PBS, centrifuged at 1000 X *g* for 5 min and the supernatant was removed and used for DNA extraction. DNA was extracted from CAM homogenate supernatant, commercial vaccines or directly from swabs taken from infected trachea using a method described previously (Sykes *et al.*, 1997). Briefly, tracheal swabs, or a 50-µl volume of reconstituted commercial vaccines or of the CAM homogenate supernatant, were placed into 400 µl of RLT lysis buffer (Qiagen) containing 4 µl of 2-ß mercaptoethanol (1.12 g/ml) and incubated at 4°C overnight. A total of 15 µl of Qiaex®II matrix (Qiagen) and 300 µl of 100% ethanol were added

to the mixture and the matrix was resuspended by vortexing. This suspension was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA). Columns were centrifuged for 30 sec at 10,000 X *g* in a microcentrifuge and the flow-through discarded. The matrix was washed once with 600 µl buffer RW1 (Qiagen) and twice with 500 µl buffer RPE (Qiagen). For each wash, columns were centrifuged for 30 sec at 10,000 X *g*. The columns were centrifuged for 90 sec at 18,000 X *g* to dry the matrix. The matrix was overlaid with 30 µl distilled water. After incubation at room temperature for 1 min, columns were centrifuged for 1 min at 10,000 X *g*. The supernatant containing extracted DNA were used immediately or stored at –20°C prior to use in the PCR.

Polymerase chain reactions

On the basis of the available GenBank sequences for ILTV genes gE and gG (U2883), TK (D00565) ICP4 (L32139), ICP18.5 (AF168792) and open reading frame (ORF) B-TK (Y14300), or previously published reports (Chang *et al.*, 1997; Garcia & Riblet, 2001; Graham *et al.*, 2000; Han & Kim, 2001a), oligonucleotide primers were designed and used to amplify several different genes or regions of the ILTV genome. Table 2 describes the oligonucleotide primers used in this study and their target sequences.

Table 2. Oligonucleotide primers used in this study.

Name	Target genes	Sequence (5' - 3')	Approximate size (kbp) of the expected PCR product
gE forward	gE	GGCTGACCAGGATAGTGAAC	1.87
gE reverse		GGTAAGATTTCCCGATTTCTC	
gG forward	gG	CCTTCTCGTGCCGATTCAATATG	1.48
gG reverse		AACCACACCTGATGCTTTTGTAC	
TK forward	TK	CTGGGCTAAATCATCCAAGACATCA	2.24
TK reverse		GCTCTCTCGAGTAAGAATGAGTACA	
ICP4 forward	ICP4	AAACCTGTAGAGACAGTACCGTGAC	4.98
ICP4 reverse		ATTACTACGTGACCTACATTGAGCC	
ICP18.5 forward	ICP18.5	TCGCTTGCAAGGTCTTCTGATGG	5.89
ICP18.5 reverse		AGAAGATGTTAATTCACACGGACAC	
ORFB-TK forward	ORFB-TK[A]	TCTGCGATCTTCGCAGTGGTCAG	4.68
ORFB-TK reverse		TGACGAGGAGAGCGAACTTTAATCC	

[A] Contains ORFB, ORFC, ORFD, ORFE, gH and TK genes.

For amplification of gE, gG and TK, PCR was performed using *Taq* DNA polymerase (Promega). Briefly, a 50-µl reaction mixture contained 200 µM each dATP, dCTP, dGTP, and dTTP, 1 mM $MgCl_2$, 250 µM each primer, 1.25 U of *Taq* DNA polymerase (Promega), 5 µl of 10X *Taq* DNA polymerase buffer, and 2 µl of extracted DNA. The reaction mixture was incubated at 94°C for 3 min, then subjected to 35 cycles of 94°C for 15 sec, 60°C for 45 sec and 72°C for 60 sec, and finally incubated at 72°C for 3 min.

For amplification of ICP4, ICP18.5 and ORFB-TK, PCR was performed using Platinum *Taq* DNA polymerase high fidelity (Invitrogen). Briefly, a 50-µl reaction mixture contained 200 µM each dATP, dCTP, dGTP, and dTTP, 2 mM $MgSO_4$, 250 µM each primer, 1 U of Platinum *Taq* DNA Polymerase high fidelity (Invitrogen), 5 µl of 10X Platinum *Taq* DNA polymerase buffer, and 2 µl of

extracted DNA. The reaction mixture was incubated at 94°C for 1 min, then subjected to 35 cycles of 94°C for 1 min, 68°C for 7 min, and finally incubated at 68°C for 10 min.

In all series of PCR, a control tube containing distilled H_2O, instead of extracted DNA, was included as negative control. All amplification reactions were performed in a thermal cycler (iCycler™, Biorad Laboratories Pty Ltd, Australia). The PCR products were separated by electrophoresis in 0.8% agarose gels, stained with ethidium bromide and exposed to ultraviolet light for visualisation.

RFLP and silver staining

10-µl volumes of PCR products were digested separately with the restriction endonucleases *Eae*I, *Msp*I, *Hae*III or *Fok*I at 37°C for 3 hrs. The restriction endonuclease enzyme *Eae*I was used to digest the PCR product of gE gene, *Msp*I was used for those of gG and TK genes, *Hae*III for those of ICP4 and ICP18.5 genes and *Fok*I for that of ORFB-TK region. After digestion, the resultant DNA fragments were separated in an 11% polyacrylamide gel (for gE, gG and TK) or 15% (for ICP4, ICP18.5 and ORFB-TK) polyacrylamide gel. Restriction DNA fragments on polyacrylamide gels were visualised by silver staining as described previously (Herring *et al.*, 1982). Briefly, each gel was fixed by incubation in 10% ethanol and 0.5% glacial acetic acid for 10 min, stained in a 2% silver nitrate solution for 10 min, rinsed with distilled water for 1 min and then developed in 3% sodium hydroxide, 0.75% of 37% formaldehyde and 1% sodium thiosulphate. The developing process was stopped by rinsing the gel twice (each 5 min) with 0.75% sodium carbonate. The gel was placed in 5% glycerol and 10% ethanol for 10 min and subjected to digital imaging using a conventional scanner.

Analysis of DNA sequences

The DNA sequence was analyzed using computer programs provided by the Australian National Genomic Information Service.

Cluster analysis

Cluster analyses were performed using the computer software Free-Tree available at www.natur.cuni.cz/flegr/programs (Pavlicek *et al.*, 1999). Briefly, the absence or presence of a given restriction site in RFLP patterns were assigned 0 or 1, respectively, and resultant data were imported into Free-Tree for analysis. Similarity coefficients were calculated using the method described by Nei and Li (Nei & Li, 1979) and DICE. An unrooted dendrogram was constructed using the unweighted pair group method and statistical support for the dendrogram was obtained by bootstrapping using 500 resamplings.

Detailed Results

Selection of target genes and restriction endonuclease enzymes for PCR/RFLP

ILTV genes gE, gG, TK, ICP4, were selected as targets for PCR/RFLP analysis based on their potential for strain differentiation reported previously (Andreasen *et al.*, 1990; Chang *et al.*, 1997; Garcia & Riblet, 2001; Graham *et al.*, 2000; Han & Kim, 2001a; Han & Kim, 2001b; Han & Kim, 2003). The ILTV gene ICP18.5 was chosen principally because it contains one of the largest known ORFs in the ILTV genome (Johnson *et al.*, 1991). The ILTV genomic region spanning ORFB to TK genes was chosen because it contains a number of unique genes to ILTV (Ziemann *et al.*, 1998).

In order to select restriction endonuclease enzymes providing the highest number of fragments whilst delivering clear RFLP patterns, nucleotide sequences of the genes gE, gG, TK, ICP4, ICP18.5 and ORFB-TK from SA2 (Johnson *et al.*, 1991) were subjected to screening with several high frequency cutting enzymes. Consequently, restriction endonuclease enzymes *Eae*I, *Msp*I, *Hae*III, and *Fok*I were selected and used for restriction digestion of the PCR products of the genes gE, gG and TK, ICP4 and ICP18.5, and ORFB-TK, respectively.

PCR amplification of the gE, gG, TK, ICP4, ICP18.5 and ORFB-TK fragments from ILTV genome

The oligonucleotide primer set gE forward and gE reverse were used to amplify approximately 1.87 kbp fragments of gE from several ILTV isolates using the PCR procedure described in the materials and methods. Figure 1A shows examples of PCR products generated from isolates V3-02 and A20. All other strains/isolates under investigation produced gE PCR products of similar size (results not shown). Oligonucleotide primer sets TK forward and TK reverse, gG forward and gG reverse, ICP4 forward and ICP4 reverse, ICP18.5 forward and ICP18.5 reverse, and ORFB-TK forward and ORFB-TK reverse, were used to amplify ILTV genes TK, gG, ICP4, ICP18.5, and ORFB-TK, respectively. Figure 1 shows examples of gE, gG, TK PCR products generated from ILTV strains/isolates V3-02 and A20 (A), of ICP4 PCR products generated from ILTV strains/isolates N3-04, CSW and SA2 (B), and of ICP18.5 and ORFB-TK PCR products generated from ILTV strains/isolates N3-04, V3-02 and A20 (C). All ILTV strains/isolates generated PCR products of 1.87 kbp in gE PCR, 1.47 kbp in gG PCR, 2.22 kbp in TK PCR, 6.60 kbp in ICP18.5 PCR and 4.48 kbp in ORFB-TK PCR. All strains/isolates generated PCR products of 4.75 kbp in ICP4 PCR except for CSW that generated a PCR product of 5.13 kbp.

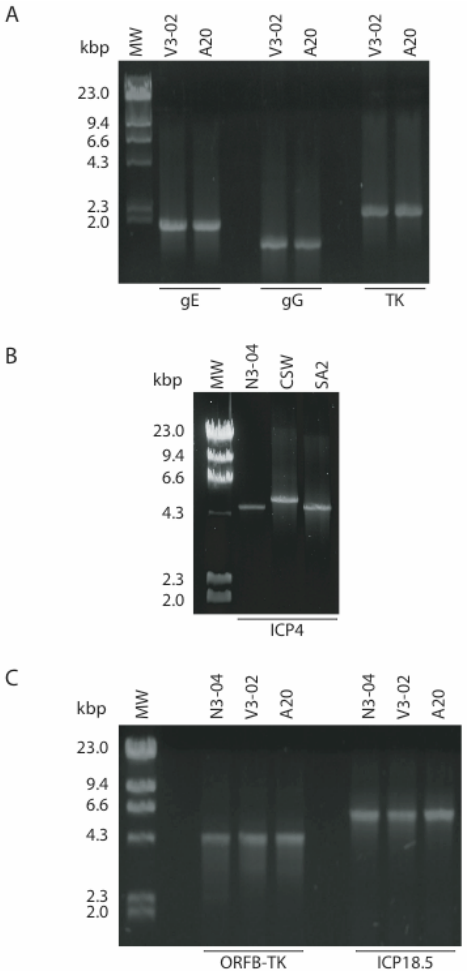

Fig. 1. (A) Agarose gel electrophoresis of PCR products of the genes gE, gG and TK from ILTV field isolate V3-02 and vaccine strain A20. (B) Agarose gel electrophoresis of PCR products of the ICP4 gene from ILTV field isolates N3-04 and CSW and the vaccine strain SA2. (C) Agarose gel electrophoresis of PCR products of the ILTV genes ICP18.5 and ORFB-TK from field isolates N3-04 and V3-02 and the vaccine strain A20. MW is λ DNA digested with *Hind*III.

RFLP patterns of gE, gG and TK genes

Restriction digestion of gE gene PCR product using endonuclease *Eae*I gave identical patterns in all ILTV isolates used. This pattern consisted of 3 bands of approximately 1.02, 0.45 and 0.25 kbp (Fig. 2.).

Restriction digestion of gE PCR product using endonuclease *Eae*I also produced two DNA bands of approximately 1.24 and 0.71 kbp which were presumably partially digested products (Garcia & Riblet, 2001).

Restriction digestion of gG PCR product using endonuclease *Msp*I gave 2 different patterns, A and B, in different ILTV isolates. Pattern A consisted of 9 bands of approximately 0.30, 0.28, 0.24, 0.19, 0.14, 0.10, 0.10, 0.08 and 0.05 kbp while pattern B consisted of 7 bands of 0.35, 0.30, 0.24, 0.22, 0.19, 0.10 and 0.08 kbp.

Restriction digestion of TK PCR product using endonuclease *Msp*I gave 2 different patterns, A and B in different ILTV isolates. Pattern A consisted of 7 bands of approximately 1.15, 0.45, 0.26, 0.22, 0.21, 0.14 and 0.13 kbp while pattern B consisted of 6 bands of 1.15, 0.67, 0.26, 0.21, 0.14 and 0.13 kbp.

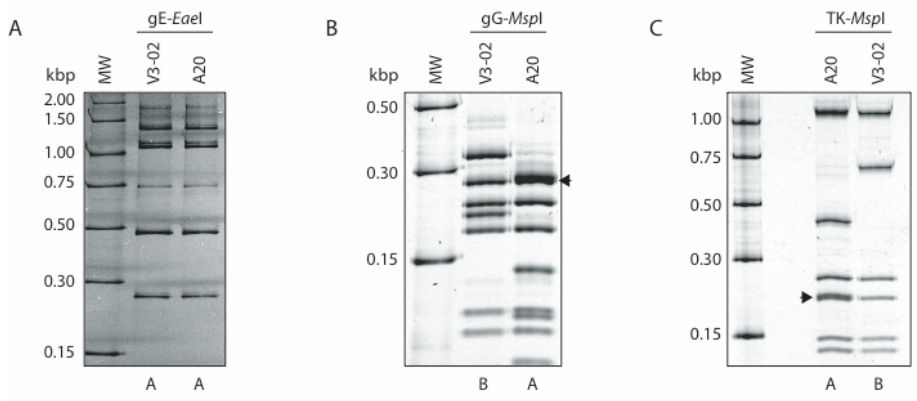

Fig. 2. Polyacrylamide gel electrophoresis of DNA fragments generated by restriction digestion of gE (A), gG (B) and TK (C) PCR products using enzymes *Eae*I, *Msp*I and *Msp*I, respectively. Genomic DNA from field isolate V3-02 and the vaccine strain A20 were used as template. RFLP fragments were visualized using silver staining. Arrow heads indicate the presence of two DNA bands of approximately similar sizes.
Letters "A" and "B" represent vaccine-type and non vaccine-like patterns respectively. MW is PCR marker (Sigma Pty Ltd).

RFLP patterns of ICP4, ICP18.5 and ORFB-TK genes

Restriction digestion of ICP4 using endonuclease *Hae*III gave 3 different patterns, A, B and C in different ILTV isolates. Pattern A consisted of 11 bands of approximately 0.65, 0.53, 0.50, 0.30, 0.27, 0.22, 0.20, 0.18, 0.15, 0.15 and 0.14 kbp, pattern B consisted of 14 bands of 0.65, 0.54, 0.53, 0.50, 0.31, 0.27, 0.22, 0.20, 0.18, 0.15, 0.15, 0.14, 0.12 and 0.11 kbp, while pattern C consisted of 15 bands of 0.79, 0.56, 0.53, 0.50, 0.31, 0.27, 0.22, 0.20, 0.18, 0.17, 0.15, 0.15, 0.14, 0.12 and 0.11 kbp. Note that the ICP4 PCR product generated from strain CSW was slightly larger than those produced from strain SA2 or isolate N3-04.

Restriction digestion of ICP18.5 using endonuclease *Hae*III gave 3 different patterns, A, B and C in different ILTV isolates. Pattern A consisted of 11 bands of approximately 1.89, 0.97, 0.83, 0.75, 0.50, 0.45, 0.41, 0.33, 0.25, 0.24 and 0.22 kbp, pattern B consisted of 9 bands of 2.19, 1.12, 0.97, 0.83, 0.50, 0.42, 0.33, 0.24 and 0.22 kbp, while pattern C consisted of 10 bands of 2.19, 0.97, 0.83, 0.75, 0.50, 0.45, 0.41, 0.33, 0.24 and 0.22 kbp. RFLP of ORFB-TK using restriction endonuclease enzyme *Fok*I gave 2 different patterns, A and B in different ILTV isolates. Pattern A consisted of 6 bands of approximately 2.52, 0.58, 0.44, 0.36, 0.24 and 0.14 kbp, while pattern B consisted of 5 bands of 2.52, 0.97, 0.36, 0.24 and 0.14 kbp.

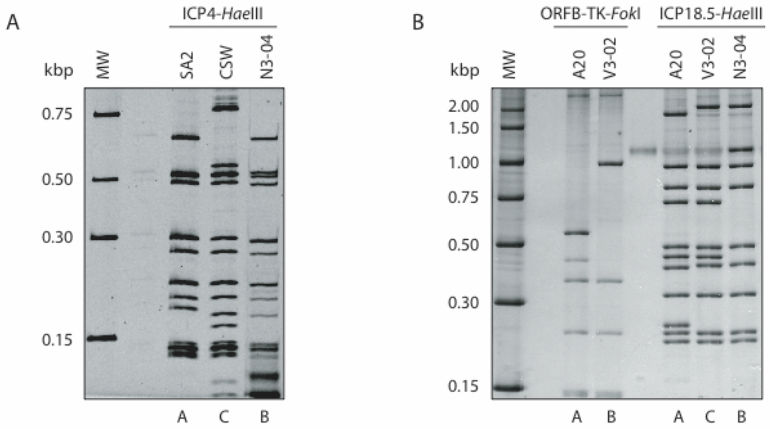

Fig. 3. (A) Polyacrylamide gel electrophoresis of DNA fragments generated by restriction digestion of ICP4 PCR products using enzyme *Hae*III. Genomic DNA from field isolates N3-04 and CSW and the vaccine strain SA2 were used as template. (B) Polyacrylamide gel electrophoresis of DNA fragments generated by restriction digestion of ICP18.5 and ORFB-TK PCR products using enzymes *Hae*III and *Fok*I respectively. Genomic DNA from field isolates N3-04 and V3-02 and the vaccine strain A20 were used as template. RFLP fragments were visualized using silver staining.
Letter "A" represents vaccine-like patterns while letters "B" and "C" represent non vaccine-like patterns. MW is PCR marker (Sigma Pty Ltd).

Classification of ILTV isolates

Classification of the ILTV isolates using RFLP patterns generated from genes gG, TK, ICP4, ICP18.5 and ORFB-TK revealed that ILTV isolates fell into 5 different classes: 1, 2, 3, 4 and 5 (Table 3.). All NSW isolates (N1-03, N1-04, N2-04 and N3-04) exhibited class 2 RFLP pattern while all SA isolates (S1-04, S2-04, S3-04 and S4-04) exhibited class 3 RFLP pattern. Note class 2 and 3 differ in ICP4 and ICP18.5 genes. Victorian isolates exhibited class 2, 3 or 5. Most QLD isolates exhibited class 1, similar to the vaccine strains SA2 and A20. Cluster analysis of the data generated from all RFLP patterns for each class of ILTV strain/isolate (Fig 4) revealed that class 3 and class 4 are closely related but both are distinct from class 5 and class 1.

Discussion of Results

In this study, PCR-RFLP of a number of ILTV genes/genomic regions including gE, gG, TK, ICP4, ICP18.5 and ORFB-TK were utilized to examine a number of historical and contemporary ILTV isolates. While PCR-RFLP of gE has previously been used to distinguish between different viral subpopulations (Garcia & Riblet, 2001) or study the relationship of ILTV isolates and vaccine strains in Australia (Bagust, T. J. 2005, pers. comm.), it failed to distinguish between any of the isolates used in this study. PCR-RFLP of gG, TK and ORFB-TK divided all the isolates into two groups.

Results from PCR-RFLP of gG and TK correlated well, with all the isolates that were determined to be non-vaccine type in gG PCR-RFLP were also non-vaccine type in TK PCR-RFLP. Results from either gG or TK PCR-RFLP were also highly correlated with those of ORFB-TK except that isolate V1-03 was classified as non-vaccine type by gG and TK PCR-RFLP but indistinguishable from vaccine strains by ORFB-TK PCR-RFLP. PCR-RFLP of ICP1 8.5 and ICP4 divided the isolates into three different groups with ICP18.5 and ICP4 PCR-RFLP distinguishing 14 and 8 isolates from vaccine strains respectively. By combining the groupings generated from PCR-RFLP of the genes gG, TK, ICP4, ICP18.5 and ORFB-TK, the ILTV isolates could be divided into 5 different groups.

Table 3. Comparisons of RFLP patterns generated by restriction digestion of PCR products of selected genes from different ILTV isolates.

PCR product	gE	gG	TK	ICP4	ICP18.5	ORFB-TK	Pattern Combination [A]	Class [B]
Restriction endonuclease	*Eae*I	*Msp*I	*Msp*I	*Hae*III	*Hae*III	*Fok*I		
Isolate/strain	RFLP PATTERNS							
SA2	A	A	A	A	A	A	AAAAA	1
A20	A	A	A	A	A	A	AAAAA	1
CSW	A	B	B	C	C	B	BBCCB	4
V1-99	A	B	B	B	B	B	BBBBB	2
V2-02	A	B	B	B	B	B	BBBBB	2
V3-02	A	B	B	A	C	B	BBACB	3
V1-03	A	A	A	A	A	B	AAAAB	5
V1-04	ND[C]	B	B	A	C	B	BBACB	3
N1-03	A	B	B	B	B	B	BBBBB	2
N1-04	A	B	B	B	B	B	BBBBB	2
N2-04	ND	B	B	B	B	B	BBBBB	2
N3-04	ND	B	B	B	B	B	BBBBB	2
S1-04	A	B	B	A	C	B	BBACB	3
S2-04	ND	B	B	A	C	B	BBACB	3
S3-04	ND	B	B	A	C	B	BBACB	3
S4-04	ND	B	B	A	C	B	BBACB	3
Q1-95	A	A	A	A	A	A	AAAAA	1
Q1-96	ND	A	A	A	A	A	AAAAA	1
Q1-00	ND	A	A	A	A	A	AAAAA	1
Q1-01	A	B	B	B	B	B	BBBBB	2

[A] Combination of RFLP patterns for genes gG, TK, ICP4, ICP18.5 and ORFB-TK.
[B] Classes of ILTV isolates according to their pattern combination.
[C] Not Defined.

It was also observed from the current study that classes 5 and 1 are identical in PCR-RFLP of all genes except for ORFB-TK PCR-RFLP. Class 5 contained only a single ILTV isolate, V1-03. Interestingly, this isolate was obtained from a flock of broiler breeders which had a history of

respiratory symptoms along with having been vaccinated with the vaccine strain A20 approximately 10 days prior to this isolation. Histopathological examination of trachea of the affected birds had revealed hyperplastic epithelial changes and mononuclear inflammatory cell infiltration into lamina propria suggestive of chronic tracheitis. It is therefore tempting to speculate that alteration of the ORFB-TK region in A20, from vaccine type (A) to wild type (B), may have been linked to an increase in virulence for an otherwise avirulent vaccine strain. The ORFB-TK region consists of several unique genes including ORFB, ORFC, ORFD, ORFD and ORFE that are known to be unique to ILTV and of gH (UL22) and TK (UL23).

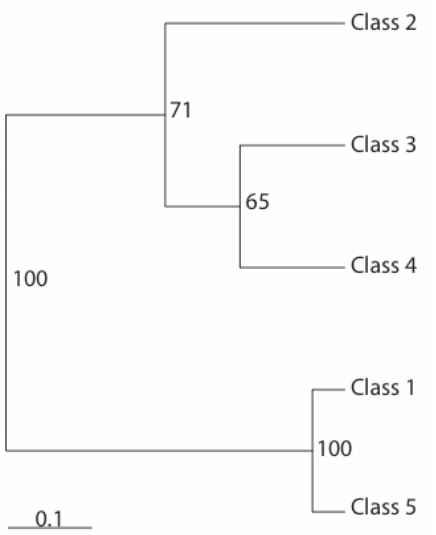

Fig. 4. Dendrogram based on cluster analysis of restriction endonuclease cleavage data for the five classes of ILTV isolates. Similarity coefficients were calculated according to the method of Nei-Li and DICE. The branch lengths represent the genetic distances between the classes, and the number on the branches are bootstrap values (using 500 resampling).

Implications

Using the techniques described in this study, the majority of the recent ILTV isolates isolated from outbreaks in commercial flocks were found to be distinct from one of the vaccine strains SA2 and A20. The exceptions were the isolates Q1-95, Q1-96 and Q1-00, each of which had identical RFLP patterns to the vaccine strains. This finding suggests that these isolates may have originally derived from vaccine strains, which then underwent an increase in virulence (Guy *et al.*, 1991). The alternative explanation is that these isolates are perhaps unrelated to the vaccine strains but the PCR-RFLP analysis described here was unable to distinguish between them. No clear vaccination history was associated with these cases suggesting that the virus may have probably been introduced into the respective flocks from an infected flock.

The results from this study reveal the potential of analyzing the ILTV gene region ICP18.5 by RFLP for epidemiological investigations and also emphasize that examination of multiple gene regions should be examined if ILTV strain identifications are to be reliably undertaken. A possible role in virulence for the ILTV unique genes ORF-E was suggested. Further research is necessary to investigate the association of classifications described here with the virulence of the ILTV isolates.

Recommendations

The results presented in this study suggests that, where the primary objective of an investigation in a face of an ILT outbreak is to determine the relationship between the ILTV strain associated with the disease and the vaccine strain, PCR-RFLP of either gG or TK can be used as a primary screening technique. However, where more extended strain identification is required, as to determine the possible relationship of the causative ILTV strain with historical ILTV isolates, PCR-RFLP of ICP18.5 or a combination of TK, ICP4, ICP18.5 and ORFB-TK PCR-RFLP should be used.

Virulence of the Australian Infectious Laryngotracheitis Viruses

Introduction

Infectious laryngotracheitis (ILT) is a significant respiratory disease of chickens in many countries and may also involve in reduced egg production and predisposition to other respiratory pathogens (Guy & Bagust, 2003). Outbreaks of mild to moderate forms of ILT are not uncommon in commercial layer flocks worldwide, while sporadic outbreaks of ILT in broiler flocks have also been recognised in recent years as an emerging problem in several countries including Australia (Critchley, 2004; Wells, 2004). Two live attenuated vaccines, SA2 and A20 (Fort Dodge, Australia, Pty. Ltd.), are used to immunize chickens against ILT in Australia. While the A20 vaccine is considered relatively safe and can be used in very young chickens, the SA2 vaccine is less attenuated and is only recommended for use in adult birds or as a booster vaccine after A20 administration.

Although antigenically homogenous, ILTv strains may vary in their virulence in chickens, chicken embryo and cell culture. Differentiation of ILTv strains of varying virulence, particularly wild-type and modified live vaccine viruses is an important practical problem. In a recent study, PCR-RFLP of a number of ILTv genes/genomic regions was utilized to examine a number of historical and contemporary Australian ILTv field and vaccine strains. The ILTv strains were classified into 5 different classes, with most strains distinguishable from the vaccine strains.

Objectives

The main purpose of the present study was to determine and compare the tissue tropism and virulence of the ILTv strains belonging to different PCR-RFLP classes by assessment of clinical symptoms, mortality, pathological lesions and effects on weight gain.

Methodology

Viruses

The origin of the ILTv strains used in this study has been previously described (Kirkpatrick *et al.*, 2006). The commercial ILTv vaccine strains SA2 and A20 (Fort Dodge) and several recent ILTv strains were used in this study (Table 4). The vaccine strain SA2 was originated from an Australian field isolate by attenuation through sequential passages in chicken embryo (Purcell & Surman, 1974a) while strain A20 originated from SA2 through further passages in chicken embryonic cell culture to lessen residual virulence (Bagust, T. J. 2005, personal communication). All field strains were isolated from the upper respiratory tract of infected birds during outbreaks of ILT in Australia. Field strains V1-99 and Q1-96 were isolated by the Australian government diagnostic laboratories (Department of Primary Industries, Victoria/Queensland). The field strain CSW was isolated from the upper respiratory tract of infected birds (Fahey *et al.*, 1983). The field strains N3-04 and S2-04 were isolated within our laboratory during the course of a previous study. Strains and isolates were chosen based on their different PCR-RFLP patterns of several ILTv genes including gE, gG, TK, ICP4 and ORFB-TK as described previously (Kirkpatrick *et al.*, 2006).

Titration of virus strains

All virus strains were propagated on the chorioallantoic membrane (CAM) of 10 day old chicken embryos using the standard techniques (Tripathy & Hanson, 1980) and purified by picking single plaques from infected CAMs. The virus strains were titrated also on the CAM of embryonated

chicken eggs. The stock for each virus strain was diluted to obtain a median embryo infected dose (EID_{50}) of 10^4/ml and stored at –70° C.

Table 4. Details of the ILTv strains used in this study and comparisons of RFLP patterns generated by restriction digestion of PCR products of selected genes.

Strain	Year of isolation	Origin	Pattern Combinationa	Class[b]
N3-04	2004	Broiler	BBBBB	2
S2-04	2004	Layer	BBACB	3
Q1-96	1996	Unknown	AAAAA	1
V1-99	1999	Layer	BBBBB	2
CSW	1970	Layer	BBCCB	4
SA2	C1966	Vaccine	AAAAA	1
A20	1983	Vaccine	AAAAA	1

[a] Combination of RFLP patterns for genes gG, TK, ICP4, ICP18.5 and ORFB-TK.
[b] Classes of ILTv strains according to their pattern combination.

Experimental design

One hundred and twenty eight 3-week-old specific pathogen free hybrid White Leghorn chickens (Charles River Laboratories Pty, Ltd., Woodend, Victoria, Australia) were randomly separated into 8 groups of 16, housed in separate isolators and provided irradiated feed and water *ad libitum*. Birds within each group were weighted and then inoculated within isolators with 10^3 EID_{50} of the relevant ILTv strains in a 300µl volume per bird by the intra-tracheal route. Dilutions were made in sterile phosphate buffered saline (PBS). The control birds received 300µl of sterile PBS only. Four days post inoculation, 8 birds from each group were examined for clinical symptoms, weighed, euthanised by halothane and then subjected to pathological, histopathological and virological examinations. The remaining 8 birds within each group were kept within isolators, monitored for clinical signs and mortality pattern, and euthanised at 21 days post inoculation.

Scoring of clinical symptoms

Four days post inoculation, clinical signs were recorded digitally (recording 30s for each bird) and scored subsequently. Clinical signs were scored on the basis of the bird's breathing, the condition of their conjunctiva and their general demeanour. Breathing was scored on a scale of 0 (normal breathing), 1 (mild dyspnoea), 2 (mild dyspnoea and open mouth breathing), 3 (gasping) and 4 (gasping with an extended neck). The condition of the conjunctiva was scored on a scale of 0 (normal), 1 (swollen and/or partial closure of the eyes) and 2 (complete closure of the eyes). The demeanour was scored on a scale of 0 (normal), 1 (depressed) and 2 (severely depressed). A sum of scores for each clinical sign was then calculated for each bird.

Histopathology

Transverse segments of upper and lower trachea were fixed in Bouin's solution overnight, embedded in paraffin and stained with Haematoxylin and Eosin for histopathological examinations. Microscopic lesions were scored according the method described previously (Guy *et al.*, 1990).

Real-Time PCR

A transverse section of proximal trachea was collected during the post mortem examination and transferred to a microcentrifuge tube containing 500 µl of 4M guanidinium isothiocyanate, 15 mM PIPES (pH 7.6) and 5 µl of ß-mercaptoethanol. Viral DNA from these sections was extracted using

Qiaex II suspension (Qiagen) for detection and quantification of ILTv genome by a quantitative PCR. The quantitative PCR was performed using the Mx4000 thermocycler (Stratagene Pty Ltd) and Platinum SYBR Green qPCR SuperMix (Invitrogen). The forward (TTGCTGTCGTATTTCGCGTG) and reverse (GTAAATCGTTAGTGCGGCAT) primers used amplified a 115-bp region from the ILTv UL15 gene. The template was 2 µl of extracted DNA (or sterile water as control). In each set of reaction, or 5 µl volumes of 10 fold dilutions of 4.0×10^8 copy number of the ILTv UL15 sequence in pGEM-T were included to form a standard curve. This assay provided a threshold of detection of 2.98 ILTv genome equivalents (GE) per section of trachea.

Statistical analysis

The Mann-Whitney U-test was used to compare the median figures for clinical sings, histological tracheal scores and viral copy numbers. The Student t-test was used for comparison of the mean percent weight changes. The Fisher's exact test was used to compare the incidence of mortalities. *P*-values of <0.05 were considered to be significant.

Detailed Results

Percentage weight gain

Mean percentage weight gain 4 days post inoculation of the bird groups with the ILTv strains are shown in Table 5. All groups except for the Q1-96 had positive percentage weight gains and all, except for the group N3-04, had a significantly ($P \leq 0.002$) different average weight gain to that of the non-challenged group. The mean percentage weight gain of the birds inoculated with the strain Q1-96 was negative and significantly ($P \leq 0.001$) different to those of all other groups.

Table 5. Mean percent weight gain ± SD of 4-week-old chickens at 4 days post inoculation with different strains of ILTv.

Group	Mean % weight gain[a] ± SD
NC	$38.3^{A} \pm 10.3$
N3-04	$22.5^{AB} \pm 35.8$
S2-04	$14.3^{B} \pm 5.8$
Q1-96	$-14.4^{C} \pm 19.9$
V1-99	$14.9^{B} \pm 17.0$
CSW	$19.5^{B} \pm 18.4$
SA2	$11.9^{B} \pm 16.6$
A20	$13.3^{B} \pm 10.4$

[a] Significantly different figures are shown by different superscript letters.

Clinical signs

Median and range of scores for clinical sign for each group are shown in Table 6. The group inoculated with strain Q1-96 showed only very minimal clinical symptoms related to breathing with a median of score that was significantly ($P \leq 0.005$) different to those of groups inoculated with other ILTv strains, but not to that of the negative control group. Most birds of the group Q1-96 showed clinical symptoms of conjunctivitis and/or closure of the eyes. The median of conjunctiva related clinical symptoms of the group Q1-96 was 1.0, that except for that of the group N3-04, was significantly ($P \leq 0.046$) higher than those of all other groups. The group inoculated with Q1-96 also showed a moderate to severe signs of depression and had a significantly ($P = 0.022$) different median score for demeanour to that of the negative control group. The median scores for demeanour of the groups SA2, A20, CSW, V1-99, N3-04 and S2-04 were not significantly different to that of the

negative control group. The sum of scores for breathing, condition of conjunctiva and demeanour of all the groups inoculated with an ILTv strain were not significantly different from each other but were all significantly ($P \leq 0.001$) different to that of the negative control group.

Table 6. Scores for the median (range) clinical signs of 4-week-old chickens at 4 days post inoculation with different strains of ILTv.

Group	Median[a] (range)			
	Breathing	Conjunctiva	Demeanour	Sum
NC	0^{A} (0-0)	0^{A} (0-0)	0^{B} (0-0)	0^{A} (0-0)
N3-04	1^{B} (0-2)	0^{AB} (0-2)	0^{AB} (0-1)	2^{B} (1-3)
S2-04	1^{B} (0-4)	0.5^{A} (0-1)	0^{B} (0-0)	2^{B} (0-5)
Q1-96	0^{A} (0-1)	1^{B} (0-2)	1^{A} (0-2)	2^{B} (0-4)
V1-99	1^{B} (0-3)	0^{A} (0-0)	0^{B} (0-1)	1^{B} (0-3)
CSW	1^{B} (0-2)	0^{A} (0-2)	0^{AB} (0-2)	1^{B} (1-4)
SA2	1^{B} (0-2)	0^{A} (0-0)	0^{B} (0-1)	1^{B} (0-3)
A20	1^{B} (0-4)	0^{A} (0-2)	0^{AB} (0-1)	2^{B} (1-4)

[a] Significantly different figures are shown by different superscript letters.

Microscopic tracheal lesions

Median histological lesion scores and range of upper and lower trachea for each group are shown in Table 7. All ILTv strains induced moderate to severe microscopic lesions in upper trachea with medians of scores that were significantly ($P \leq 0.005$) different to that of the negative control group. Except for A20, all ILTv strains induced no or only minor microscopic lesions in lower trachea and had medians of scores that were not significantly different to that of the negative control group. The medians of microscopic lower tracheal lesion scores for the A20 was significantly ($P = 0.005$) different from that of the negative control group. The sum of medians for upper and lower tracheal microscopic lesions of the groups SA2, A20, CSW, V1-99 and N3-04, but not SA-04 or Q1-96, were significantly ($P \leq 0.032$) different to that of the negative control group.

Table 7. Scores for the median (range) microscopic tracheal lesions for upper trachea (UT), lower trachea (LT) and total trachea (T) of 4-week-old chickens at 4 days post inoculation with different strains of ILTv.

Group	Median[a] (range)		
	UT	LT	T
NC	0.0^{A} (0.0-0.3)	0.0^{A} (0.0-0.0)	0.0^{A} (0.0-0.3)
N3-04	2.8^{BC} (0.0-5.0)	0.0^{AB} (0.0-4.5)	2.8^{BC} (0.0-9.5)
S2-04	0.5^{BC} (0.0-5.0)	0.0^{AB} (0.0-5.0)	0.5^{AC} (0.0-9.5)
Q1-96	2.3^{B} (0.0-3.0)	0.0^{A} (0.0-2.0)	2.3^{AC} (0.0-4.5)
V1-99	3.0^{BC} (0.0-4.0)	0.0^{A} (0.0-2.5)	3.0^{BC} (0.0-6.0)
CSW	4.0^{C} (2.5-5.0)	0.0^{A} (0.0-2.0)	4.3^{BC} (2.5-6.0)
SA2	3.0^{C} (2.0-4.0)	0.8^{AB} (0.0-3.0)	4.3^{BC} (2.0-7.0)
A20	3.5^{C} (1.0-4.0)	2.5^{B} (0.0-2.5)	6.0^{B} (1-6.5)

[a] Significantly different figures are shown by different superscript letters.

Viral copy numbers

The results of quantitative PCR for extracted DNA from the tracheas of birds sacrificed at the 4 days post inoculation are summarised in Table 8. ILTv DNA was detected in all the birds in groups CSW, SA2 and A20, in few of the birds in groups N3-04 and S2-04, in most of the birds in groups Q1-96

and V1-99, but in none of the birds in the negative control group. Among ILTv strains, A20 produced the highest median viral copy number, while N3-04 produced the lowest median viral copy number in trachea.

Table 8. Median and range of viral copy numbers (determined by quantitative PCR) in trachea of 4-week-old chickens at 4 days post inoculation with different strains of ILTv.

Group	Median[a]	Range
NC	1^{A}	1-1
N3-04	1^{AB}	1-8863
S2-04	10^{BD}	1-16411
Q1-96	238^{BE}	1-5916
V1-99	1028^{B}	1-5394
CSW	1498^{CDEF}	321-38361
SA2	2615^{BFG}	221-7760
A20	7596^{EG}	616-22481

[a] Significantly different figures are shown by different superscript letters.

Mortality

Pattern and the rate of mortalities (including the birds culled because of severe clinical symptoms) after inoculation of chickens with different ILTv strains or sterile PBS are shown in Figure 5. Mortality occurred in all groups inoculated with an ILTv strain. Generally, the highest mortality occurred between days 6-9 post-inoculation. Three groups, SA2, V1-99 and N3-04, produced greater than 80% cumulative mortality per group that were significantly ($P \leq 0.033$) different to those of the control group. Groups S2-04, Q1-96 and CSW, produced 25%, 50% and 62.5% cumulative mortality per group, respectively. The group inoculated with A20 was the only group to have a single mortality before the first time point of 4 days post inoculation, after which no mortality was produced.

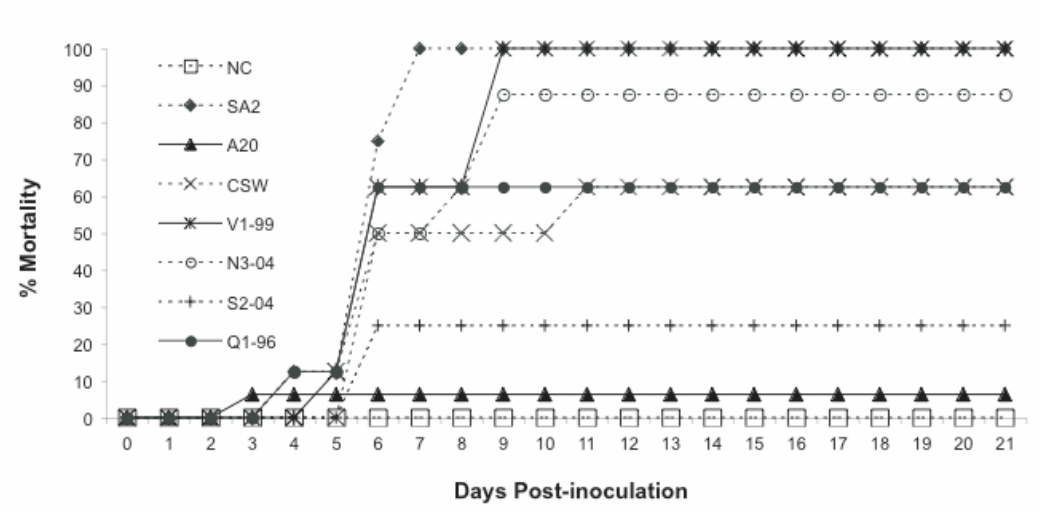

Figure 5. Mortalities of chickens after inoculation with different ILTv strains or sterile PBS. The figures represent birds found dead as well as birds culled before termination of experiment (21 days post inoculation) due to severe clinical symptoms.

Discussion of Results

The ILTv field strain CSW and the vaccine strains SA2 and A20 were found to have a high affinity for trachea but little affinity for conjunctival tissues. In contrast, the ILTv strain Q1-96 was found to have a particularly high affinity for conjunctival tissues but less affinity than most of the other ILTv strains for tracheal tissues. Interestingly, the affinity of the ILTv strain Q1-96 for conjunctivitis was found to be associated with a significant reduction in body weight. This is likely to be caused by severe conjunctivitis and inability to locate the feed provided to the birds.

An interesting finding of this study was that the vaccine strains, in particular A20, had a high tendency to cause microscopic lesions in trachea. The effect of A20 was noticeable even in lower trachea. It is known that the use of strain SA2, particularly in young chickens, may be associated with respiratory symptoms (Purcell & Surman, 1974b) but such an observation has not been reported with the vaccine strain A20. Despite a high affinity for tracheal tissues, infection with the ILTv strain A20, but not with SA2, was associated with only a minimal mortality in chickens suggesting that mortality is not necessarily correlated with tracheal lesions in ILT. This finding is also supported by a previous study by *Guy et al.* (Guy *et al.*, 1990) where some field isolates such as 87-30900 scored relatively moderate (2.8) in terms of tracheal pathology while in contrast the vaccine strain 4 scored relatively high (4.5). Analysis of the results obtained in our study found a high correlation (Pearson's correlation of 0.812) between the scores for microscopic tracheal lesions and viral copy numbers in trachea of the birds. This result suggests that enhanced tracheal pathology is perhaps due to a high rate of viral replication in this tissue.

Unlike vaccine strains A20 and SA2, the field ILTv strain N3-04 did not show a high affinity for tracheal tissue as evidenced by a relatively low genomic copy number as well as a moderate microscopic tracheal lesion score. In addition, only minimal conjunctiva-related clinical symptoms were seen in the birds infected with this strain. Nevertheless, this strain proved to be highly virulent causing severe mortality in infected birds. These results challenge the paradigm that ILTv is a local disease limited to the respiratory system (Bagust *et al.*, 1986; Hitchner *et al.*, 1977) and suggest that ILTv may also induce some systemic effects.
It was found that the ILTv strains V1-99 and N3-04 that belong to the same RFLP class (Kirkpatrick *et al.*, 2006) scored very closely for virulence determined by criteria used in this study (clinical signs, mortality, tracheal lesions and effects on weight gain). However this finding was less expandable to some other ILTv strains such as A20, SA2 and Q1-96 that belonged to the same class (1) and clearly exhibited different tissue tropisms, mortality rates and clinical symptoms.

Implications

Results from this study revealed that ILTv strains vary considerably in their capacity to induce mortality, clinical symptoms and lesions in different tissues and suggest that perhaps factors other than the variation in RFLP patterns of genes reported in our previous study (Kirkpatrick *et al.*, 2006) are involved in virulence of the ILTv strains. Further studies examining differential expression of ILTv genes may be necessary to reveal the genetic mechanism of virulence/attenuation of an ILTv strain.

Recommendations

Results presented in this study emphasize the use of appropriate vaccine for vaccination of young chickens. The ILTv strain SA2 was found to cause clinical symptoms, mortality and reduced weight gain comparable to some of the field strains. This vaccine strain obviously still retains residual virulence and needs to be used only as a second vaccination (i.e. after vaccination with a less virulent

strain such as A20) and its application should be only restricted to older birds. It is also recommended that clinical signs and mortality, as opposed to tracheal pathology, should be used for determination of ILTv virulence.

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Compendium Summary

Project Title:	Molecular Epidemiology and Pathogenesis of Infectious Laryngotracheitis Viruses in Australia
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Objectives	The main objectives were to determine the virulence and spread of the Australian ILTV isolates, to investigate possible genetic/virulence differences between the vaccine re-isolates and the original vaccine and to establish a genetic marker for ILTV virulence which can be developed as an epidemiological tool for investigation of ILTV infection.
Background	Outbreaks of mild to moderate forms of ILT have been reported in commercial layer flocks in some regional areas. In addition, sporadic outbreaks of ILT in broiler flocks has been recognised as an emerging problem as evidenced by the increasing number of ILT outbreaks in recent years. Some of these outbreaks are suspected to be caused by Australian vaccine strains.
Research	PCR-RFLP of a number of ILTV genes/genomic regions including gE, gG, TK, ICP4, ICP18.5 and open reading frame (ORF) B-TK was utilized to examine a number of historical and contemporary Australian ILTV isolates and vaccine strains. Seven groups of SPF chickens were inoculated with 5 different Australian ILTv strains and were examined for clinical signs, weigh loss, pathological and histopathological lesions and mortality.
Outcomes	A combination of groupings including gG, TK, ICP4, ICP18.5 and ORFB-TK PCR-RFLP classified the ILTV isolates under investigation into 5 different groups with most isolates distinguishable from vaccine strains. There was considerable variation among ILTv classes in their tropism for trachea or conjunctiva with the ILTv strains with high affinity for conjunctiva can severely affect weigh gain.
Implications	Where the primary objective of an investigation in a face of an ILT outbreak is to determine if the ILTV strain associated with the disease is derived from the vaccine strain, PCR-RFLP of either gG or TK can be used. However, where more extended strain identification is required, PCR-RFLP of ICP18.5 or a combination of TK, ICP4, ICP18.5 and ORFB-TK PCR-RFLP should be used. Clinical signs and mortality, as opposed to tracheal pathology, should be used for determination of ILTv virulence. The ILTv strain SA2 should only be used as a second vaccination and its application should be restricted to older birds.
Publications	Kirkpatrick, N.C., Mahmoudian, A., O'Rourke, D. & Noormohammadi, A.H. (2006). Differentiation of infectious laryngotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. *Avian Diseases, In Press* Kirkpatrick, N.C., Mahmoudian, A., Colson, C. A., Devlin, J. M. & Noormohammadi, A.H. (2006). Relationship Between Mortality, Clinical Symptoms and Tracheal Pathology in Infectious Laryngotracheitis, *Submitted for publication* Naomi C. Kirkpartick, Denise O'Rourke and Amir H. Noormohammadi Molecular Techniques for Differentiation of the Australian Infectious Laryngotracheitis Virus Strains. In: *Proceedings of the Australian Veterinary Poultry Association Scientific Meeting*, (P. 52). Melbourne, Australia.