Real-time PCR quantification of infectious laryngotracheitis virus in chicken tissues, faeces, isolator-dust and bedding material over 28 days following infection reveals high levels in faeces and dust

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Infectious laryngotracheitis (ILT) is an important disease of chickens caused by ILT virus (ILTV). We used the Australian SA2 and A20 vaccine strains of ILTV to determine tissue distribution and excretion characteristics of ILTV in specific-pathogen-free chickens and to determine whether ILTV is readily detectable in environmental samples such as faeces, bedding material and dust using real-time quantitative PCR. Three groups of 10 freshly hatched chicks were placed in isolators and infected orally with high doses of the two strains of vaccine virus or left unchallenged as controls. Over a 28-day post-infection (p.i.) period, faecal and serum samples were collected at frequent intervals from six individually identified chickens in each group. Dust and litter samples from the isolators were collected less frequently. Tissue samples were collected from three to four sacrificed or dead/euthanized birds at 6, 14 and 28 days p.i. Infection resulted in clinical ILT, a pronounced antibody response and sustained qPCR detection of the viral genome in the trachea, Harderian gland, lung and kidney up to 28 days p.i. A high level of the viral genome was also detected in faeces between 2 and 7 days p.i., declining by about approximately four orders of magnitude to low, but detectable, levels at 21 and 28 days p.i. The finding of high-level shedding of ILTV in faeces warrants further investigation into the epidemiological role of this, and the sustained high levels of ILTV observed in dust suggest that it may be a useful sample material for monitoring ILTV status in flocks.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a respiratory disease of chickens, caused by gallid herpesvirus 1 (GaHV-1), a member of the genus *Iltovirus*, subfamily *Alphaherpesvirinae* and family *Herpesviridae* (Davison *et al.*, 2005). The disease is prevalent worldwide and causes significant economic losses (Guy & Garcia, 2008). The disease affects chickens of all ages but most characteristic signs are found in adult birds. In acute cases, clinical signs include nasal discharge, moist rales followed by coughing and gasping (Kernohan, 1931). In severe cases, dyspnoea and expectoration of blood stained mucus is very characteristic. Morbidity varies from 90 to 100 % and mortality varies from 5 to 70 % (Hinshaw *et al.*, 1931; Jordan, 1958; Seddon & Hart, 1935). In mild enzootic form, chicks may show ill-thrift, conjunctivitis, swelling of infraorbital sinuses, mild tracheitis, persistent nasal discharge and decreased egg production. Morbidity is as low as 5 % and mortality varies from 0.1 to 2 % (Cover & Benton, 1958; Linares *et al.*, 1994; Pulsford & Stokes, 1953; Sellers *et al.*, 2004). Biosecurity measures and live attenuated vaccines have been used for the control of the disease outbreaks (Gelenczei & Marty, 1965; Samberg & Aronovici, 1969), but despite these measures ILT remains a threat to the poultry industry, with outbreaks recurring periodically (Blacker *et al.*, 2011; Dufour-Zavala, 2008). Since vaccination can result in latently infected carrier chickens, it is recommended only in endemic areas. It has also been observed that most outbreaks have been caused by vaccine-related ILTV strains (Blacker *et al.*, 2011; Creelan *et al.*, 2006; Neff *et al.*, 2008; Ojkic *et al.*, 2008).
2006; Oldoni & Garcia, 2007; Oldoni et al., 2008). In Australia, ILT is considered endemic in Victoria and New South Wales. Two different live vaccines are commonly being used in Australia, the SA2 ILT and A20 ILT (Fort Dodge Australia Pty Ltd, Baulkham Hills, NSW, Australia). The SA2 ILT vaccine strain originated from an Australian field isolate and was attenuated through sequential passages in chicken embryos (Purcell & Surman, 1974). The A20 vaccine strain was developed by further passages of the SA2 strain in chicken embryonic cell culture (Blacket et al., 2011).

ILT is thought to enter the host via the nasal, oral or conjunctival routes with the source of infection suspected to be aerosolized exudates from the respiratory tract (Bagust et al., 2000). However, recent studies have indicated a wide range of tissues may be infected with the virus (Oldoni et al., 2009; Wang et al., 2013) and transmission of ILT has been demonstrated by intra-tracheal inoculation of suspensions of liver and spleen tissue from affected birds (Beach, 1931), raising the possibility of alternative modes of shedding and transmission. Another avian herpesvirus, gallid herpesvirus 2 (GaHV-2) or Marek's disease virus (MDV), is the causative agent of Marek's disease and is also transmitted by inhalation of infective material. For this virus, routine monitoring of large flocks based on quantitative PCR (qPCR) determination of MDV viral load in poultry house dust samples has been developed as a useful disease monitoring tool (Walkden-Brown et al., 2013). This method may be useful for other avian viral pathogens such as ILTV. We therefore designed the present study to investigate tissue tropism of ILTV, faecal excretion and presence of the virus in environmental samples such as dust and litter.

RESULTS

Mortality

Six chicks died in the SA2 group and one in the A20 group while there were no deaths in the control. Mortality occurred within 1 week of infection and was associated with clinical signs of ILT. Post-mortem lesions were consistent with ILT.

Tissue distribution of ILTV

ILT was detected in all of the four sampled tissues with viral load declining by 2–3 logs over the 28-day experimental period (Fig. 1). There was no difference in viral load between tissues collected from euthanized/dead birds that were showing clinical signs, and that from birds sacrificed at random (P=0.43), so data for the two types are combined. The effect of time post-infection (p.i.) was significant for all tissues for the A20 strain of ILTV but only trachea and lung for the SA2 strain (P<0.05). Viral load differed significantly between tissues, being highest in the Harderian gland and lung and lowest in the kidney (P<0.05, Fig. 2). Over all tissues, viral load was significantly higher for strain A20 than strain SA2 (P<0.05, Fig. 2).

Serological response to ILTV infection

Serum antibody titre directed against ILTV was significantly influenced by time after infection (P<0.0001) but not ILTV strain (P=0.31), with no interaction between these effects (P=0.23, Fig. 3). Titres rose sharply at 14 days p.i. then increased further significantly to 21 days p.i. with a smaller non-significant increase at day 28.

Faecal excretion of ILTV

ILT of both strains was detected in faeces at each sampling up to 28 days p.i. Viral load in faeces was significantly influenced by time after infection (P<0.0001) but not ILTV strain (P=0.53), with no interaction between these effects (P=0.76, Fig. 4). With minor variations between the two ILTV strains, excretion in faeces was high at 2 days p.i., peaked at 5 days p.i. and declined thereafter by about 4.5 logs to low levels at 21 and 28 days p.i. (Fig. 4).

ILT detection in dust and litter samples

ILT was detected at high levels in dust collected within the infected isolators (Fig. 5a) and in litter samples collected at the end of the experiment at 28 days p.i. (Fig. 5b). Analysis of dust data revealed a significant effect of time after infection (P=0.026), with a strong trend towards higher levels of viral genome load for SA2 than A20 (P=0.059, Fig. 5a). Only two litter samples were analysed for ILTV presence, so no formal analysis could be conducted. There appeared to be a trend towards higher ILTV viral genome load for SA2 than A20 (Fig. 5b).

Association between variables

There were generally strong positive linear associations between levels of ILTV in different tissues measured in the same bird. The strongest association was between those in the Harderian gland and trachea (R²=0.67, P<0.0001) and the weakest between lung and kidney (R²=0.24). The association between ILTV viral genome load in the trachea and the other tissues is shown in Fig. 6.

Anti-ILT antibodies in serum and ILTV viral genome load in faeces were measured repeatedly in the same chicken. There was a strong negative linear association between weekly measurements for the two variables in the same chicken as shown in Fig. 7.

DISCUSSION

In the present experiment, high-dose, oral challenge of specific-pathogen-free (SPF) chicks with two vaccine strains of ILTV resulted in clinical ILT, a pronounced antibody response to infection and persistent detection of the
viral genome in the trachea, Harderian gland, lung and kidney up to 28 days p.i. Furthermore, the viral genome was also detected in faeces up to 28 days p.i., indicating sustained replication and shedding of the virus. The ILTV viral genome was also readily detected in dust and litter samples with sustained high levels found in dust. There is considerable age-related resistance to infection with ILTV (Clarke et al., 1980; Fahey et al., 1983) and high-dose ILTV vaccination at 1-day-old in the present experiment undoubtedly resulted in the induction of more severe ILT than would have been the case had the birds been challenged at 3–5 weeks of age, which is more typical of ILTV challenge studies (Coppo et al., 2012b; Oldoni et al., 2009; Wang et al., 2013). This probably resulted in higher levels of viral genome detection, but, as discussed below, where similar measurements permit comparison, the results are broadly consistent with other studies.

**Fig. 1.** Viral load of ILTV (log10 viral copy number mg⁻¹ tissue, LSM ±SEM) detected in Harderian gland (a), trachea (b), lung (c) and kidney tissues (d) over a 28-day period after infection of SPF chickens at hatch (day 0). Columns not sharing a common letter in the superscript within challenge virus are significantly different, indicating a significant effect of days p.i. within virus (P<0.05).
**Fig. 2.** Viral load of ILTV (log_{10} viral copy number mg\(^{-1}\) tissue, LSM ± SEM) showing overall differences between tissues (a) and ILTV strain used for infection (b). Data are overall least-squares means for samples collected at 6, 14 and 28 days p.i. Columns not sharing a common letter in the superscript are significantly different (P<0.05).

**Fig. 3.** Serum anti-ILTV antibody titres (LSM ± SEM) in SPF chicks over the 28 days following infection of SPF chicks at hatch (day 0) with ILTV strains A20 or SA2. Letters indicate the overall significance of time p.i. with days post-infection not sharing a common letter having significantly different mean titres (P<0.05). Differences between ILTV strains were not significant.

**Fig. 4.** Viral load of ILTV in faeces (log_{10} viral copy number g\(^{-1}\), LSM ± SEM) over a 28-day period following infection of SPF chicks at hatch (day 0) with ILTV strains A20 or SA2. Letters indicate the overall significance of time p.i. with days post-infection not sharing a common letter having significantly different mean titres (P<0.05). Differences between ILTV strains were not significant.
ILTV, like other herpesviruses, induces persistent infection of the host with latency and reactivation from latency as key features (Bagust et al., 2000; Guy & Garcia, 2008; Williams et al., 1992). The present study could not distinguish clearly between latent and active infection in tissues but ongoing detection in faeces is suggestive of active infection in some tissues with release of virus into the gastrointestinal tract. It is known that ILTV is usually present in tracheal tissues and secretions for 6–8 days post-inoculation (Bagust et al., 1986; Hitchner et al., 1977; Purcell & McFerran, 1969) and this coincides with the period of clinical ILT. This was true in the present experiment where high levels of the viral genome were found in tissues at 6 days p.i. following or during clinical ILT and there was sustained high-level detection of the viral genome in faeces between 2 and 7 days p.i. Other studies have reported reliable isolation of virus from tracheal swabs up to 10 days p.i. with intermittent isolation up to 59 days p.i., presumably due to reactivation of the virus within individual chickens (Williams et al., 1992), or sustained detection of the ILTV genome for up to 60 days following vaccination with ILTV strain SA2 (Coppo

**Fig. 5.** (a) Viral load of ILTV in dust collected in isolator settle plates (log_{10} viral copy number mg^{-1}) over a 28-day period following infection of SPF chicks at hatch (day 0) with ILTV strains A20 or SA2. (b) Viral load of ILTV in litter (log_{10} viral copy number g^{-1}) collected at 28 days p.i. from the same groups.

**Fig. 6.** Linear regression relationship between ILTV viral load in tracheal tissue and load in the Harderian gland, lung and kidney. Each data point represents one chicken (n=17). P values and coefficients of determination are shown on each figure.
In the present study, repeated samples from the same chicken were only obtained for faeces. These revealed a marked decline overall by 4–5 logs in faecal shedding between the peak at 5 days p.i. and low levels at 21–28 days p.i. Examination of individual bird profiles revealed a marked decline overall by 4–5 logs in faecal shedding between the peak at 5 days p.i. and low levels at 21–28 days p.i. Examination of individual bird profiles from the 12 birds was indicative of sustained faecal shedding of virus, and thus active infection in some tissues over the period, rather than the intermittent pattern reported by Williams et al. (1992). This finding is consistent with the sustained detection of the SA2 strain ILTV in trachea up to 60 days after oral (drinking water) vaccination (Coppo et al., 2012b). These differences may reflect ILTV strain differences, the greater sensitivity of the PCR method, the high challenge doses used or the route of application (oral or intra-tracheal). The latter is possible as Coppo et al. (2012a) reported qPCR detection of the Serva ILTV vaccine genome in trachea for up to 28 days following oral (drinking water) vaccination but only 16 days following eye drop application.

ILTV may be isolated from trachea, larynx, lung, conjunctiva or exudate from these sites (Guy & Garcia, 2008; Jordan, 1966), with tracheal scrapings, tracheal exudates and lung being the preferred tissues for isolation (Tripathy & Garcia, 1998). Oldoni et al. (2009) detected the ILTV genome in the conjunctiva, sinuses, trachea, trigeminal ganglia, caecal tonsils, thymus and cloacal swabs from chickens challenged with a range of field and vaccine strains of ILTV in the USA. Virus was isolated on adult chick kidney cell culture from the conjunctiva and sinus of all infected chickens and from the trachea and thymus some infected chickens. No virus was isolated from the lung, spleen, bursa, caecal tonsils, cloacal swabs and the trigeminal ganglia. The finding of the viral genome in the trigeminal nerve supported an earlier report that it is a likely site of latency for ILTV (Williams et al., 1992). Wang et al. (2013) challenged 30-day-old SPF chicks with a Chinese field strain of ILTV and compared viral genome presence by standard and real-time PCR with virus isolation in chick embryos at 5 days p.i. There was successful viral genome detection and virus isolation from trachea, lung and thymus confirming previous findings, but also from the throat, oesophagus, caecum, kidney and pancreas. There was no viral genome detected or virus isolated from the bursa of Fabricius, proventriculus, spleen, liver, heart or brain.

In the present study, qPCR detection of the ILTV genome was extended to the Harderian gland, which contained the highest levels of the viral genome. The Harderian gland is an exocrine gland found in the orbit of the eye. It is comparatively large in the chicken and the main function is thought to be lubrication of eyeball and nictitating membrane (Mobini, 2012; Payne, 1994). Given the involvement of conjunctivitis in ILT and physical connections of Harderian gland (via the lachrymal duct) with the pharynx and trachea, it is perhaps not surprising that the ILTV viral genome was detected in this organ, although the very high levels observed suggest that it may be a site of viral replication. This requires confirmation using other methods. Clarke et al. (1980) reported infection of both the conjunctiva and the naso-lachrymal duct epithelium of spray vaccinated chickens with ILTV vaccine strain SA2. Detection in kidneys supports the finding of Wang et al. (2013) and, as in that study, the genome was detected at a comparatively low level. This may simply reflect presence of viraemic virus in a highly vascular tissue. Although no clear evidence is thought to exist for a viraemic phase of infection (Guy & Garcia, 2008), infection of macrophages in vitro has been demonstrated, particularly for vaccine strains (Calnek et al., 1986) and these could be the source of virus in tissues outside the respiratory tract. In addition to isolation of ILTV from thymus (Oldoni et al., 2009), caecum, kidney and pancreas (Wang et al., 2013), transmission of ILT has been demonstrated by intra-tracheal inoculation of suspensions of liver and spleen tissue from affected birds (Beach, 1931).

The sustained detection of the ILTV genome in faeces observed within this experiment is a novel finding but its significance in the epidemiology of ILT is unknown. It is consistent with previous reports of ILTV genome detection in cloacal swabs (Oldoni et al., 2009) and the caeca (Wang et al., 2013). The virus presumably originates from the trachea and pharynx and at least some of the viral genome survives transit through the gastrointestinal tract. Whether this is indicative of the presence of infective virus in faeces was not examined in this study, but while Oldoni et al. (2009) failed to isolate ILTV from cloacal swabs, Wang et al. (2013) were able to isolate it from caeca. It is unclear from the latter report whether the caecal samples included caecal contents. In an early study using ILTV vaccine strain SA2, Robertson and Egerton (1981) reported isolation of the virus from the cloaca for 6 days following cloacal vaccination, but not following vaccination via drinking water or the conjunctival route, suggestive of viral replication at

![Fig. 7. Linear regression relationship between anti-ILTV titre in serum and ILTV viral load in faeces from the same chicken in the same week. Each data point represents values for one chicken in one week of the experiment (n=48).](image-url)
this site. The literature is silent on the precise details of transmission of ILTV. Natural entry to the host is via the nasal, oral or conjunctiva with the source of infection being infected, in-contact birds or contaminated litter or other fomites (Bagust et al., 2000). The source of the infective material is suspected to be aerosolized exudates from the respiratory tract (Bagust et al., 2000). Vaccinal ILTV has been demonstrated to both survive in, and transmit to other birds from, litter (Giambrone et al., 2008) and infected water lines (Ou et al., 2011), but in the case of contaminated litter, whether the virus originates from the respiratory tract or the faeces is unknown, as is the precise route of infection. The faeces appear not to have been investigated as a significant source of infective virus and faecal-oral cycling of the virus is not postulated. Our results suggest that this is worthy of investigation.

Dust samples collected within the isolators showed extremely high levels of ILTV nucleic acid with only modest decline of approximately 1.5 logs between 7 and 28 days p.i. Poultry dust is a composite of feather dander and fine particulate matter from bedding materials, feed and faeces. It is not clear in the current experiment whether the dust contamination came principally from faecal excretion of virus, or excretion via the respiratory tract or other route. The sustained high levels of ILTV observed in dust indicate that it may be a useful sample material for monitoring ILTV status in flocks, as has been shown to be the case for another avian herpesvirus, MDV (Walkden-Brown et al., 2013). Dust as a sample material for monitoring of poultry viruses has a number of advantages including non-invasive collection, low sample number required, no cold chain required during initial storage and transportation to the laboratory and integration of information from many individual chickens to provide a flock sample (Walkden-Brown et al., 2013). These results show that viral monitoring in dust is not only feasible for viruses shed in feather dander such as Marek’s disease, but for viruses shed via alternative routes.

There was a strong antibody response to infection, with no difference in response between the two strains used. The response was associated with protection as demonstrated by the strong negative association between antibody titre and detection of ILTV in faeces within individual birds. Whether this association is causative is not known. It has been reported that ELISA titres for ILTV over 400 are protective against challenge (Sander & Thayer, 1997) but also that bursectomy and inoculation with anti-ILTV antibody have no or limited effect on pathogenesis (Fahey et al., 1983). Levels above this were developed by 14 days p.i. and maintained until the end of the experiment at 28 days p.i. Interestingly, the high antibody levels were not associated with a cessation of viral excretion, although it was reduced by roughly four orders of magnitude by days 21 and 28. It is important to note that the present experiment was carried out in SPF chickens, which are free of maternal antibodies directed against ILTV. Hence, they are highly susceptible to infection and the pathogenesis of infection is likely to be somewhat different in chicks derived from ILT-vaccinated parents.

There were few consistent differences between ILTV vaccine strains SA2 and A20 in the present experiment and both were able to induce ILT when administered at high doses to chicks not protected by maternal immunity. The higher level of disease severity induced by SA2 may reflect the higher dose of virus used, the lower level of attenuation or both. While the results of infection with these strains of ILTV are broadly consistent with earlier reports of viral detection and localization following challenge with them (Clarke et al., 1980; Coppo et al., 2012b; Purcell & Surman, 1974), detection of viral genomes in a wider range of tissues and other materials was observed generally over a longer time period post-infection. An exception to the latter is the detection of the SA2 vaccine viral genome in trachea for up to 60 days post-vaccination, reported by Coppo et al. (2012b). While not formally tested, active excretion of virus in both groups suggests that the challenge strains would transmit readily, as has been previously demonstrated for the SA2 vaccine (Coppo et al., 2012b). Transmission of vaccine strains is important epidemiologically as several outbreaks of ILT have been attributed to revertant vaccine strains (Andreasen et al., 1990; Guy et al., 1990) and recombinant strains derived from vaccine strains (Lee et al., 2012). While most ILTV vaccines are efficacious, many of them possess considerable residual virulence, which can further increase during back passages in chickens (Guy et al., 1991).

In summary, we have found that high-dose, oral challenge of SPF chicks with two vaccine strains of ILTV resulted in clinical ILT, a pronounced antibody response to infection and persistent qPCR detection of the viral genome in the trachea, Harderian gland, lung and kidney up to 28 days p.i. A high level of virus was also detected in faeces between 2 and 7 days p.i., declining by about approximately four orders of magnitude to low, but detectable, levels at 21 and 28 days p.i., indicating sustained replication and shedding of the virus. ILTV was also readily detected in dust and litter samples. The sustained high levels of ILTV observed in dust suggest that it may be a useful sample material for monitoring ILTV status in flocks, as it has several practical advantages over alternative methods.

METHODS

Ethics statement. The experiment was approved by the University of New England Animal Ethics Committee (AEC No. UNE AEC13/045).

Vaccine strains. Two different live commercial ILT vaccine strains SA2 (Poulvac Laryngo SA2) and A20 (Poulvac Laryngo A20) were used in this study (Fort Dodge Australia Pty).

Experimental inoculation of SPF chickens. Thirty individually identified 1-day-old SPF White Leghorn chickens (Australian SPF Services) were divided into three treatment groups of 10 chickens each in separate isolators. One group (SA2) received $2.5 \times 10^{9}$ p.f.u.
of SA2 ILT vaccine individually at 1 day old (day 0) while a second group (A20) received 1.23 × 10^5 p.f.u. of the A20 ILT vaccine. Vaccines were administered in 200 μl water into the oral cavity using a disposable 1 ml syringe. These doses are approximately 20 and 40 times the minimum recommended doses of 12.6 × 10^3 and 3.2 × 10^3 p.f.u. for SA2 and A20 vaccines, respectively, and were chosen to ensure a high level of infection, as litter from this experiment was used subsequently to test litter-based transmission of ILTV. Giambrone et al. (2008) used a vaccine challenge dose 100 × the recommended dose to achieve a similar purpose. The third group (CON) was kept as an unvaccinated negative control. The experiment was terminated at 28 days of age. The experiment was conducted in three identical positive pressure isolators supplied with temperature-controlled HEPA filtered air. All the birds were provided commercial feed and water ad libitum for the course of experiment. The isolator floors were covered with fresh pine shavings as bedding material.

Measurements and sample collection. Mortality was recorded and dead/euthanized or sacrificed chickens were sampled for the trachea, lung, Harderian gland and kidney at 6, 14 (except for SA2) and 28 days p.i. for the quantification of viral load by qPCR. Due to differences in the level of induced clinical ILT, sample numbers were not identical for the two challenge virus groups. In the SA2 group, samples were collected from five dead/euthanized and one sacrificed chickens at 6 days p.i. and from three sacrificed chickens at 28 days p.i. In the A20 group samples were collected from two sacrificed chickens at 6 days p.i. and three sacrificed chickens at 14 days p.i. and 28 days p.i. Six chickens were individually marked in each group and individual faecal samples collected from marked chickens at 2, 3, 5, 7, 10, 14, 18, 21 and 28 days p.i. to assess the viral load by qPCR. Dust samples from the inside of each isolator were collected using settle plates at weekly intervals to assess the viral load by qPCR. Litter samples were collected at the end of experiment (28 days p.i.) for assessment of the viral load by qPCR. Sera samples were collected from all the individually marked chickens (six from each group) at weekly intervals for assessment of ILTV specific antibody levels. All the samples were stored at −20 °C until further processing.

DNA extraction from tissues and dust samples. DNA was extracted from 25 mg of each tissue sample using the Bioline ISOLATE II Genomic DNA kit according to the manufacturer's instruction (Bioline) and stored at −20 °C. DNA was eluted in a final volume of 100 μl. For dust samples, DNA was extracted from 5 mg samples using the same kit according to the manufacturer's instruction but after lysis with buffer G3, undigested particles were removed by centrifugation and DNA was finally eluted in a final volume of 50 μl.

DNA extraction from faecal samples. A 150 mg subsample of faeces was mixed with 1000 μl digestion buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 25 mM EDTA), 2 % SDS and 20 μl proteinase K, vortexed vigorously and incubated overnight at 65 °C. After further vortexing, the mixture was centrifuged, 400 μl of the supernatant collected and step 4 onwards of the Bioline ISOLATE Faecal DNA kit (Bioline) was followed. DNA was eluted in a final volume of 100 μl.

DNA extraction from litter samples. Poultry litter (10 g) was mixed with 100 ml digestion buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 25 Mm EDTA), 0.33 % SDS, mixed well and incubated overnight at 60 °C in a shaker incubator. After vigorous mixing, 1 ml of the suspended liquid was taken and mixed with 20 μl proteinase K, vortexed and further incubated at 60 °C for 30 min. After centrifugation, 400 μl supernatant was collected and step 4 onwards of the Bioline ISOLATE Faecal DNA kit (Bioline) was followed. DNA was eluted in a final volume of 100 μl.

DNA quantification. Prior to use in the qPCR assay, extracted DNA was quantified using spectrophotometer (NanoDropND-1000 UV-Vis) for DNA yield, purity and further dilution in qPCR assays. The purity of DNA samples was determined by the A260/A280 ratio and also A260/A230 ratio. An A260/A230 ratio of ~1.8 is generally accepted as pure for DNA but lower values are suggestive of protein contamination. Expected A260/A230 values for DNA ranges between 2.0 to 2.2 and lower ratios are suggestive for the presence of non-protein contaminants.

Real-time qPCR. Real-time qPCR was performed using primers that amplify a 103 bp product from the gc gene of ILTV as described previously (Callison et al., 2007). The published primer sequences were used: forward (5’-CTTGGGTGATTTTTCTGT-3’), reverse (5’-TTCGTGTTAGGCTGTCG-3’) and probe (5’-FAM-CAGCTCGGTGACCCCATTCTA-BHQ-3’). A 25 μl reaction mixture was used containing 12.5 μl of 2 × master mix, 0.5 μl of each primer (0.5 μM), 0.5 μl of probe (10 μM), 0.5 μl of template DNA and 6 μl of MilliQ water. qPCR was performed using a Rotor Gene 3000 real-time PCR instrument (Corbett Research) with thermocycle conditions of 2 min hold at 95 °C followed by 45 cycles of 94 °C for 20 s, 60 °C for 40 s and 25 °C hold for 2 min. Absolute quantification of viral copy number was determined against a standard curve based on a plasmid preparation of the target sequence.

Serological testing for anti-ILTV antibody. Antibody levels against ILTV in sera samples were assessed by using a commercial ELISA kit (ProFLOK Fowl Laryngotracheitis Virus Antibody Test kit, Synbiotics Corporation) following the manufacturer’s recommendations.

Statistical analysis. Analyses were performed with JMP11 software (SAS Institute, NC USA). Continuous variables measured once on a chicken (e.g. viral load in tissues) were subject to ANOVA, testing the effects of challenge strain, whether birds were sacrificed or dead/euthanized, days p.i., and the interaction between challenge strain and days p.i. Continuous variables measured repeatedly on the same chicken (e.g. faeces, antibody levels) were subjected to repeated-measures ANOVA, testing the effects of challenge strain, days p.i. and the interaction between these. The model used was a mixed REML model with individual bird fitted as a random variable and the other effects fitted as fixed effects. Viral load was transformed log_{10}+1 prior to analysis to normalize the variances. Following a significant main effect or interaction, the significance of differences between means was determined using Student’s t-test. Association between variables was assessed using standard correlation and linear regression analysis. Data are presented as least-squares means (LSM) ± SEM. A significance level of P<0.05 is used throughout.

ACKNOWLEDGEMENTS

We are grateful for the support this work has received. It was partly funded by the Poultry CRC, established and supported under the Australian Government’s Cooperative Research Centres Program and was undertaken while P. R. was the recipient of an Australian Endeavour Executive Award.

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