Vaccine 37 (2019) 5035–5043

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Uptake and spread of infectious laryngotracheitis vaccine virus within meat chicken flocks following drinking water vaccination

Peter J. Groves [a,*], Sarah L. Williamson [b], Sue M. Sharpe [b], Priscilla F. Gerber [c], Yuanshuo K. Gao [d], Tabitha J. Hirn [a], Stephen W. Walkden-Brown [c]

[a] Sydney School of Veterinary Science, Poultry Research Foundation, Faculty of Science, The University of Sydney, 425 Werombi Rd, Camden, 2570 NSW, Australia
[b] Birling Avian Laboratories, 975 The Northern Rd, Bringelly 2556, NSW, Australia
[c] School of Environmental and Rural Science, The University of New England, Armidale, NSW, Australia
[d] Zootechny Pty Ltd, Austral 2179, NSW, Australia

ARTICLE INFO

Article history:
Received 25 March 2019
Received in revised form 28 June 2019
Accepted 30 June 2019
Available online 9 July 2019

Keywords:
Infectious laryngotracheitis
Vaccination

ABSTRACT

Vaccination against infectious laryngotracheitis virus (ILTV) in commercial broiler flocks in the field, which is only undertaken in the face of a local outbreak, requires mass administration techniques, usually via drinking water. This is often fraught with difficulties such as variable vaccination "reactions" and sometimes, vaccination failure. Laboratory testing of the outbreak strains however invariably shows the vaccines in use to be protective. To investigate this paradox, the dynamics of an ILT vaccine virus was examined within broiler flocks during a natural outbreak. In an initial flock, 70 birds were individually identified and had tracheal swabs collected sequentially at intervals from 1 to 26 days after vaccination and submitted for ILTV detection using qPCR. This evaluation was extended by collection of tracheal swabs from 40 to 45 random birds at 4, 7–8, 12–13 and 25–26 days post vaccination (pv) across a further 7 flocks. The results showed a very variable early uptake of vaccine virus from the drinking water (between 3% and 52% of tested birds with detectable virus in trachea at 4 days pv) and revealed that actual vaccination of the flocks relied on bird to bird transmission of the vaccine virus. In flocks with very low (<10%) initial bird uptake, successful exposure of vaccine virus to the majority of the flock can be delayed, leaving a large proportion of birds as susceptible at the likely time of possible exposure to wild virus. This may explain the cases of apparent failure of vaccination in the field. The variable bird to bird spread can be associated with reversion to virulence, this may explain the rolling vaccine reactions often observed. The variation in initial vaccine uptake may be affected by some factors involved with the administration technique and this requires further study in a larger sample size.

1. Introduction

Infectious Laryngotracheitis (ILT) is a severe viral infection of the upper respiratory tract of chickens often producing high mortality and productive losses. Long lived commercial chickens (breeders and layers) are regularly vaccinated against ILT using live attenuated vaccine strains predominantly by the eye-drop route [1]. However, vaccination is not regularly employed in commercial meat chickens unless they are deemed to be in a high risk situation. ILT outbreaks in localities containing many commercial meat chicken farms become problematic due to a large number of unvaccinated young birds in close proximity. When this occurs, administration of ILT vaccines to young meat chickens is done via mass administration using the drinking water route due to the large numbers of birds in commercial flocks [2]. Issues such as poor uniformity of protection, reversion to virulence and possible transmission of vaccine virus to other farms are recognized with drinking water administration of ILT vaccines, particularly the chick embryo origin (CEO) types [3]. Problems related to effective take of live vaccines delivered via mass vaccination procedures have been described [4]. Reports in outbreak situations frequently

Abbreviations: ILT, infectious laryngotracheitis; ILTV, infectious laryngotracheitis virus; qPCR, quantitative polymerase chain reaction; ELISA, enzyme linked immunosorbent assay; pv, days postvaccination; BAL, Birling Avian Laboratories; UNE, The University of New England; USYD, The University of Sydney.

* Corresponding author.
E-mail addresses: peter.groves@sydney.edu.au (P.J. Groves), sarah.williamson@baiada.com.au (S.L. Williamson), sue.sharpe@baiada.com.au (S.M. Sharpe), pgerber2@une.edu.au (P.F. Gerber), karen_gao@bigpond.com (Y.K. Gao), thir5445@uni.sydney.edu.au (T.J. Hirn), swalkden@une.edu.au (S.W. Walkden-Brown).

https://doi.org/10.1016/j.vaccine.2019.06.087
0264-410X/

suggest poor or incomplete protection in the field [5] but when these outbreak strains (Classes 8 and 9) are tested in the laboratory, protection against them with the vaccines in use has been confirmed [6,7].

Robertson and Egerton (1981) [8] demonstrated the impediments for ILT vaccine virus to access significant target tissues (conjunctivae, nasal cavity or trachea) using drinking water as the vehicle and concluded that "successful" vaccination of chickens using this route was essentially accidental.

Laboratory studies that have compared the recovery of ILTV from trachea following either eye drop or drinking water administration depict a slower establishment of vaccinal infection with the drinking water route [9,10]. This results in peak viral copies at 8 days instead of 4 days post vaccination as would be seen with eye drop application.

Several studies have investigated the effectiveness and subsequent viral replication of ILT vaccines given to chickens by the drinking water route [8,9,11,12] but these have used older layer strain chickens and open drinker types. No laboratory studies of which we are aware have looked at water administration to younger meat type chickens using nipple drinker systems.

The objective of this study was to investigate the dynamics of ILT vaccine virus uptake by and subsequent spread throughout the target birds, and to evaluate the presence and distribution of wild ILT virus within these flocks if ingress was to occur. Specifically: to determine the optimum sample size and timing necessary to estimate ILTV infection prevalence in a vaccinated flock and, to detect the incursion of wild ILTV in a vaccinated flock if naturally challenged.

2. Materials and methods

2.1. Meat chicken flocks and study design

The study was instigated during a naturally occurring field outbreak of ILT in commercial meat chicken (broiler) flocks in the greater Sydney basin (New South Wales, Australia). Within this wider region, two outbreak virus strains were detected from clinical cases and identified from each outbreak by Birling Avian Laboratories (BAL, Bringelly, NSW). Based on restriction fragment length polymorphism (RFLP) analysis, Class 9 was identified in western Sydney [13] and a newly emerged novel class with the RFLP pattern BGC (designated Class 14) (S. Williamson, unpublished). A table showing the history of notified ILT outbreaks in NSW is provided in supplementary information (Table S1). The local poultry industry adopted drinking water ILT vaccination in this area. Companies with farms in this region agreed on the use of the Serva strain of ILTV vaccine (Nobilis ILT, MSD) in this outbreak. This vaccine strain is typed as Class 7 [13] and is of chick embryo origin. The study was carried out on 8 flocks of chickens on four farms in two phases as described below. The University of Sydney (USYD) research team observed and recorded vaccination procedures without intervention and subsequently collected samples for detection of ILTV at various times post vaccination. A meaningful proportion of ILTVs detected were typed to identify the presence of vaccine or wild strains. All birds in the studies were Cobb 500 broiler strain, supplied from two commercial hatcheries.

Initial sampling numbers were estimated based on published laboratory study data [10] assuming ability to detect a prevalence between 0.3 and 0.5 with 90% confidence and a precision bound of no more than 0.1 [14]. This required a sample size between 55 and 67 birds. Hence a sample size of 70 was selected to allow for some inability to identify and retrieve individually marked birds at each sampling time.

All flocks in this study were vaccinated against Infectious Bronchitis (MSD S strain) and Newcastle Disease (Zoetis V4 strain) by coarse spray at the hatchery. Presence or ingress of wild strains of either of these respiratory pathogens into the studied flocks was not determined in this study although clinical issues with either of these diseases had not been reported in the study period or within the preceding 12 months.

All work involving live birds was conducted under the supervision of the Animal Ethics Committee of the University of Sydney (approval number 2017/1207) in compliance with the NSW Animal Research Act 1985. The first phase concentrated on one house on a five house farm undergoing vaccination in western Sydney (coded as Case-01). The house for observation was selected at random prior to commencement of vaccination. The selected house held 15,800 as hatched Cobb 500 chickens when placed on 29 August 2017. The vaccination procedure was conducted when the birds were 9 days of age. At one day post vaccination, 70 birds were selected at random from five different regions within the brooding area. A laryngeal swab was collected using a flocked swab (mini-tipped FLOQSwab 501CS01) for birds up to 28 days of age and regular tipped FLOQSwab 502CS01 for older birds (Copan, supplied by Interpath Services, Melbourne Australia). Birds were individually identified by a mark made with a permanent marker on the shank of both legs (using a system comprised of three symbols, "I", "V" or "T", marked at various 90° rotations to indicate a number from 0 to 9). The tips of both wings of the birds were sprayed with blue or green stock mark to allow them to be found within the house on the next visit

On post vaccination (pv) days 4, 8, 12, 18, 21 and 26, as many of these birds as could be identified were located and caught and they again had a laryngeal swab collected. Each bird was observed for any signs of conjunctivitis, nasal discharge, dyspnoea, respiratory râles or presence of visible blood staining on the laryngeal swab on each occasion.

If clinical ILT was observed in the flock after the sampling period, samples from affected birds were also collected to identify the ILTV class associated with the clinical signs.

After collection, swabs were placed on ice immediately. After the full collection on each day the samples were immediately transported to BAL for storage (frozen) and then processed for DNA extraction. The extracted DNA was transferred to the University of New England (UNE) for ILTV qPCR. All positive laryngeal swab samples at pv day + 26 were submitted for ILTV typing at BAL.

Based on the findings from phase 1, a further 7 houses were selected for wider study comprising phase 2 of the study. As it appeared that the field outbreak was concluding and the local industry was discontinuing ILT vaccination, the houses selected for phase 2 were limited to only three farms in the NSW Central Coast region. Details of the houses used (locations, bird numbers, age of vaccination, house floor area, ventilation style) are shown in Table 1. Sample size for a random selection in each house was adjusted assuming similar prevalence of ILTV detection as in phase 1. Each vaccination procedure was observed and 45 randomly selected birds were sampled in three houses (Case-02, 03 and 04) while 40 randomly selected birds were sampled in four houses (Case-05, 06, 07 and 08) on four occasions: days 4, 8, 12 or 13 and 25 or 26 pv. Each bird was examined and laryngeal swab samples were collected from each bird as in phase 1.

2.2. Vaccination procedures

All cases used MSD Nobilis ILT vaccine (Serva strain,). Vaccine was supplied in either 5000 (batch 1707904) or 10,000 (batch 1610904) dose vials. Dosage was calculated to the nearest appropriate vaccine vial size for the number of birds in the house at

Table 1
Details of meat chicken houses used in the study.

Case No.	Farm	Location	House ventilation style	Total floor space m^2	Floor space when vaccinated[c] m^2	Water source	No. birds placed	Age ILT vaccinated (days)	No. birds at vaccination	Total number of houses on farm
01	A	Western Sydney	Conventional[a]	1026	447	Town	15,800	9	15,500	5
02	B	Peat's Ridge	Conventional	893	447	Bore	14,300	7	14,106	4
03	B	Peat's Ridge	Conventional	893	447	Bore	14,300	7	14,092	4
04	C	Peat's Ridge	Conventional	1116	625	Bore	17,460	9	17,366	4
05	D	Kulnura	Tunnel[b]	2646	1323	Bore	51,110	14	50,351	4
06	D	Kulnura	Tunnel	2646	1323	Bore	51,160	13	50,522	4
07	D	Kulnura	Tunnel	2646	1323	Bore	51,110	13	50,286	4
08	D	Kulnura	Tunnel	2646	1323	Bore	51,110	14	49,954	4

[a] Curtain or shutter-sided house, natural ventilation.
[b] Tunnel ventilation system.
[c] Brooding area.

the time of vaccination. This gave a slight variation in actual label doses of vaccine applied per bird (Table 2).

Vaccination against ILT via drinking water in meat chicken flocks was supervised or conducted by trained supervisory personnel or the company veterinarian following documented standard operating procedures. These are similar between companies and are based on recommendations from the vaccine manufacturers. All involve common procedures including protected preparation of the vaccine which generally entail:

- avoidance of use of other water medications or sanitizers within 24 h of vaccination,
- estimation of the amount of drinking water required for 2–3 h consumption by the target flock,
- deprivation of water to the birds for a time prior to administration, addition of a stabilizer to the water prior to addition of the vaccine,
- pre-mixing of the vaccine in a smaller volume of stabilized water and thorough mixing of the prepared vaccine into the header tank,
- allowing the birds rapid access to the vaccine by flushing the drinker lines until vaccinated water is distributed through all the lines, and
- walking the house soon after administration to encourage chicks to move away from the walls and access the drinkers.

Stabilizer is used to neutralize any residual chlorine or salts in the water and stabilize pH so that no deleterious effects on the vaccine virus may occur. Choice of stabilizer may vary between companies or farms depending on their drinker systems. Choices of stabilizer include skim milk powder, liquid skim milk or a proprietary product containing a blue dye (Vac-Pac Plus®, Animal Science Products Inc., Nacogdoches, Texas, USA, supplied by BEC Feed Solutions Pty Ltd QLD). Drinking water samples were collected from the

Table 2
Vaccination procedures, vaccination factors and point prevalence of ILTV detection in laryngeal/tracheal swabs for each case flock.

Case	ILT vaccine doses[a]/ vial used	Calculated vaccine label doses/ bird	Total drinking water volume used (L)	Water mL/b	Stabilizer used (amount)	Water stabilization time[c] (m)	Vaccine preparation time[d] (m)	Time[e] till house till walked (m)	Time to consume (m)	Point prevalence of ILTV in trachea on day post vaccination			
										4	8	12–13	25–26
01	10,000	1.29	500	32.26	Skim milk[b] (1.5 kg)	26	20	52	122	0.029	0.071	0.304	0.986
02	5000	1.06	400	28.36	Liquid skim milk (10 L)	26	28	2	108	0.522	0.578	0.889	0.422
03	5000	1.06	400	28.38	Liquid skim milk (10 L)	42	41	3	110	0.244	0.511	1.000	0.511
04	5000	0.86	500	28.79	Skim milk[a] (1.5 Kg)	33	20	1	82	0.200	0.289	0.667	0.667
05	5000	0.99	1400	27.80	Skim milk[a] (1.5 Kg)	46	9	0	90	0.175	0.250	0.800	0.700
06	5000	0.99	1400	27.71	Dye preparation (200 g)	5	25	0	90	0.050	0.050	0.150	0.475
07	5000	0.99	1400	27.84	Skim milk[a] (3 Kg)	36	14	1	85	0.325	0.700	0.925	0.750
08	5000	1.00	1400	28.00	Dye preparation (200 g)	9	16	1	151	0.100	0.025	0.300	0.100

[a] One label dose is specified as ≥ 2.8 $\log_{10}$ EID_{50} per bird.
[b] Powdered skim milk.
[c] Time drinking water in the header tank was allowed to sit with stabilizer added prior to addition of vaccine.
[d] Time from vaccine preparation until the vaccine-containing water was available to the birds.
[e] Time from vaccinated water being available to the birds until the farmer walked through the house.

header tank at time of vaccine preparation, from the end of a drinker line following flushing and again from the end of the drinker line 1 h later and assayed by qPCR for ILTV in cases 05 through 08.

Vaccination procedures recorded for each house are shown in Table 2.

2.3. Laboratory procedures

2.3.1. ILTV DNA extraction and detection

The DNA was extracted from the swabs using a fully integrated QIAsymphony SP™ DNA extraction platform (Qiagen) and Qiagen DSP Pathogen / Virus Midi kits (Complex 400_V4_ DSP) according to the manufacturer's instructions.

Each DNA sample was split into two, with half retained at BAL and the other half transported to UNE for testing for the presence of ILTV DNA. All extracts were tested by a real-time qPCR targeting the gC gene of ILTV by using primers FWD (5′-CCTTGCGTTTGAAT TTTTCTGT-3′), REV (5′-TTCGTGGGTTAGAGGTCTGT-3′) and probe (5′-FAM-CAGCTCGGTGACCCCATTCTA-BHQ1-3′) previously described [16]. The PCR was carried out in 72-well discs (Qiagen, Doncaster, Victoria, Australia), each reaction containing 12.5 μl of KAPA Probe Fast qPCR universal master mix (KAPA Biosystems, Woburn, MS, US), 0.5 μl of each primer (10 μM), 0.5 μl of the probe (10 μM), and 5 μl of DNA template in a total reaction volume of 25 μl. The cycling parameters consisted of 95 °C for 3 min, followed by 40 cycles consisting of 95 °C for 5 sec and 60 °C for 30 sec. Amplification and data acquisition were carried out by using a RotorGeneQ instrument (Qiagen). Absolute quantification of viral copy number was determined against a standard curve based on a plasmid preparation of the target sequence.

2.3.2. ILTV typing

All samples positive on day 26 of the initial study (Case-01) by qPCR were typed by BAL, based on the RFLP method of Hpa I digest of the TK gene, Hae III digest of the ICP4 and IPC18.5 genes as described in Kirkpatrick et al 2006 [16]. Similarly, 77 positive tracheal samples with a virus copy number greater than 10^3 were submitted from those collected from 12 to 26 days pv in Cases 02–08 (between 10 and 16 samples per house) for typing. Fifty-eight of these had sufficient DNA to allow successful typing.

2.4. Statistical analysis

For phase 1, the individual sequential bird study, data is presented with 95% confidence intervals as the incidence of birds having ILTV detected in their laryngeal swab for the first time, point prevalence of birds with detectable ILTV in laryngeal swab at each time point and cumulative prevalence at each sampling time graphically. Individual quantitative detection of ILTV in laryngeal swabs over time for each individual bird and their patterns were examined graphically.

ILTV detection rates from phase 2 were used to calculate point prevalence at each sample time and the appropriate point prevalence results for the phase 1 study were included. Prevalence patterns were examined graphically and designated as reflecting poor or better vaccination outcomes based on early prevalence of detection of vaccine ILTV in laryngeal swabs and the subsequent time to peak prevalence. Contingency table analyses (χ^2 analysis) were used to search for statistically significant associations between factors identified during vaccine application and vaccination outcome category. Spearman rank correlation coefficients were also used to assess association of the factors with vaccination outcome.

3. Results and discussion

3.1. The outbreak and location

ILT is a notifiable disease in all states and territories of Australia. Supplementary Table S1 details the outbreaks of ILT in the region studied that were notified by NSW Department of Primary Industries during the outbreak in question. The two areas affected were western Sydney and the Central Coast region (Greater Sydney). Meat chicken farms in western Sydney have progressively succumbed to the urban sprawl of Australia's largest city and the remaining farms tend to be of the older curtain-sided or shutter style sheds relying on natural ventilation and fogging systems for temperature control. Many of these farms have disappeared or have turned over to alternate uses, such as commercial duck production. The area however is still well populated with egg layer farms which may serve as a reservoir of ILTV. The Central Coast region occupies the plateau of the NSW central tablelands and consists of several high and relatively narrow ridges (Mangrove Mountain, Peats Ridge, Central Mangrove, Kulnura and Somersby localities) separated by deep valleys. Each ridge has one central main road, with some side roads along ridge branches. The majority of poultry farms in this region are in close proximity to each other and generally close to the roads. This configuration makes the region prone to higher risk of disease spread. Proximity to a major road is recognized as a major risk factor for the introduction of ILTV [17,18].

The first notified outbreak in the western Sydney region occurred in June 2017 and was followed by further notifications through July and August. These were identified as being due to Class 9 ILTV, which is the predominant outbreak strain seen in eastern Australia since 2009 [15]. In comparison, the first outbreak seen in the Central Coast region was notified in August 2017 and the virus type identified was designated to be Class 14 by BAL. Class 14 continued to be detected in outbreaks in that region throughout September and October 2017 [19]. Vaccination began in August 2017 in western Sydney and in September on the Central Coast using Serva strain vaccine (Class 7). The majority of notified ILT cases showed the presence of Class 7 isolates from late October onwards, perhaps indicating displacement of the outbreak strains and possible spread of the vaccine virus itself, producing some degree of clinical signs in unvaccinated flocks. Notification of outbreaks ceased in February 2018 (Table S1) and poultry companies considered cessation of meat chicken vaccination during March 2018, hence the research team expedited the project to complete within this time frame (vaccination ceased end of March 2018; Y. Gao, pers. comm). No further outbreaks were notified in this region until June 2018.

3.2. Phase 1

Case-01 was vaccinated at 9 days of age by a company service person with assistance from the farm owner (the "grower"). All houses on the farm were vaccinated sequentially, beginning around 0900 h, with Case-01 being the first house done. This farm differed from the usual for this company in that the water deprivation, which is usually accomplished by lifting the drinker lines, in this house required draining of the drinker lines as the brooding surrounds hindered the ability to raise them. There were two unintended departures from the standard operating procedure for ILT drinking water vaccination used by this company. A bypass water valve was inadvertently left open and the lines did not therefore drain completely. This delayed vaccination about 30 min and may not have achieved the desired water deprivation. As the second house on the farm was vaccinated, a blockage of the water

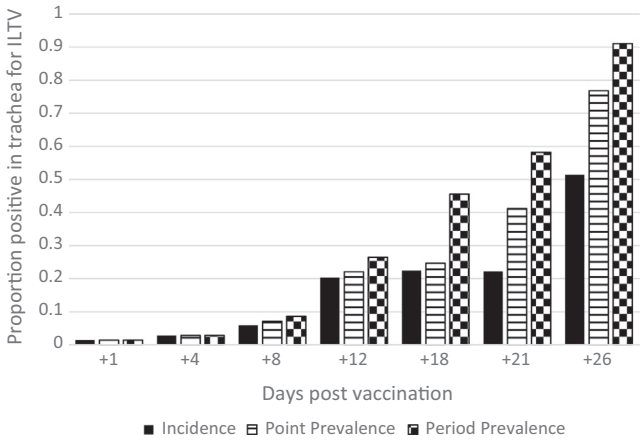

Fig. 1. Case-01 individual bird study: Incidence, point prevalence and period prevalence for positive ILTV detection at each sequential sampling point for individually identified birds from laryngeal/ tracheal swabs.

lines occurred. This delayed the grower from being able to walk through the first house (Case-01) while attending to the blockage.

One day after vaccination, 70 chicks were randomly selected from different regions of the brooding area and were identified individually as described above. Satisfyingly, every one of the identified birds was retrieved at every sampling time. The identity marks on the shanks had faded but were generally decipherable and allowed confident chick identification. Wing tags were not allowed by the company as it was feared that foreign material may progress through to the final meat product if one or more birds could not be located during the procedure.

Incidence, point prevalence and period prevalence of ILTV detection by qPCR in laryngeal/tracheal swabs at each sampling point are shown in Fig. 1. Less than 9% of birds had positive detections before 12 days pv. Incidence of detection rose to around 20% on days 12 through 21 and then rose to over 50% on day 26 pv. The point prevalence at 26 days pv thus reached 91%.

Viral copy numbers in swabs at each time point for individual birds are shown in Fig. 2. Only two birds (2.86% of those sampled)

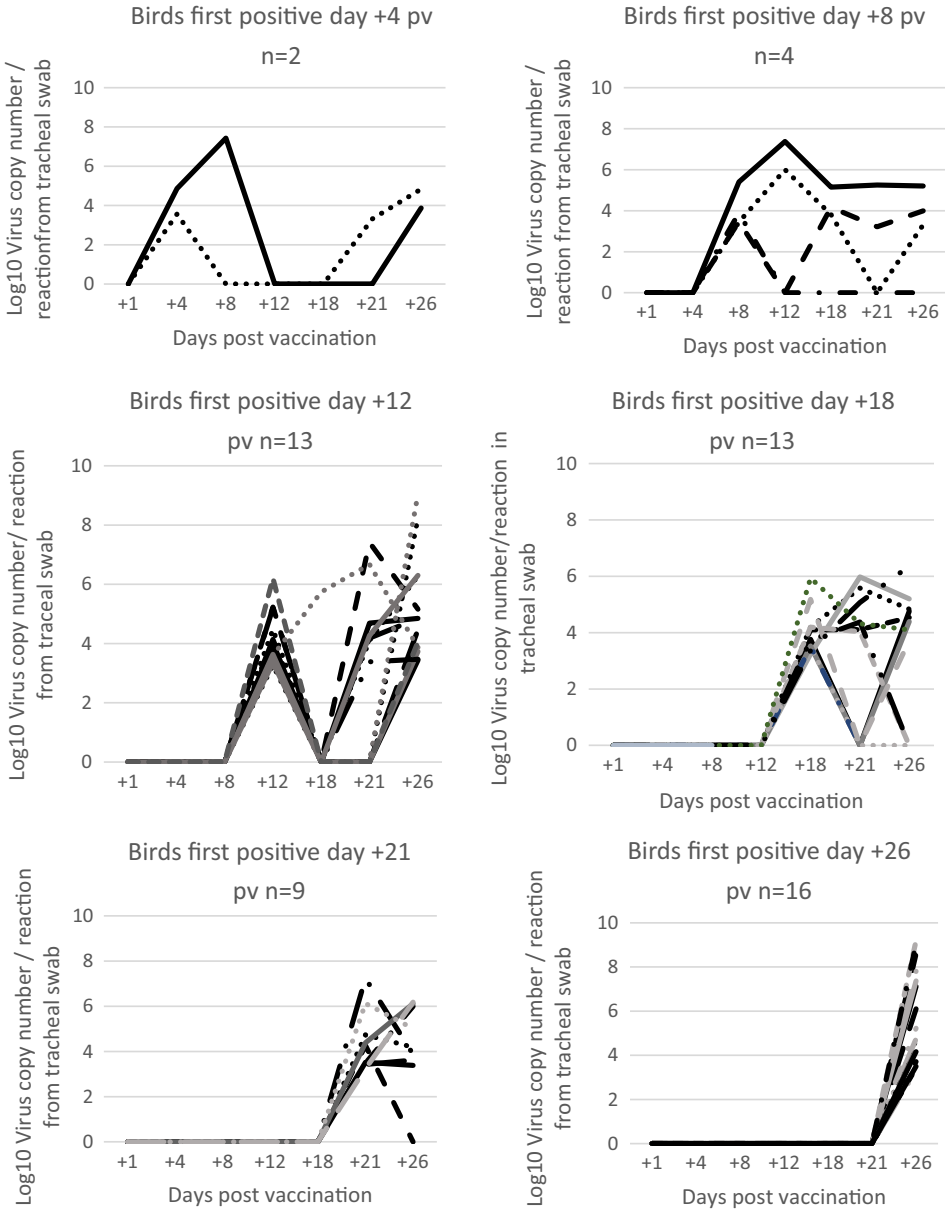

Fig. 2. Case-01: Individual plots of virus copy number detected in trachea over the course of the study (n = 70). Each plot shows birds which showed first detection on days 4, 8, 12, 18, 21 and 26 post vaccination respectively.

gave a positive detection of ILTV 4 days pv. One of these did not show detection on days 8 or 18 pv but was again positive on days 21 and 26 pv and in the other bird virus could not be detected on days 12 and 21 pv but was again positive on day 26 pv. A similar intermittent pattern of viral detection from laryngeal swabs was seen for each bird following its first positive result (Fig. 2). Many birds (18%) did not show a positive ILTV detection in trachea until 26 days pv and almost 13% never showed detection. Virus number in individual birds over time showed an intermittent presence which resembled the pattern described by Rodriguez-Avila et al (2007) [10] for birds becoming infected by contact with vaccinated birds at various times after vaccination via drinking water. The phasic presence of ILTV has been attributed [13] to the intermittent production of mucosal antibody in the trachea [20,21].

Recent laboratory studies using the same vaccine strain [9–11] indicated that a peak virus number would occur in trachea at 8 days pv following drinking water administration, which was seen to be similar to in-contact birds, rather than at 4 days pv with eye drop instillation. Thus water administration would appear to inherently lead to some bird to bird spread of the virus. This was not observed in the current study where peak viral copy numbers did not occur until days 12–21 pv (Fig. 2). It appeared that only very few birds actually obtained a vaccine virus infection from the drinking water application and the majority of the flock became exposed by subsequent contact with positive birds. Most of the flock became infected as a result of gradually increasing passage from bird to bird.

Supporting this conclusion was the presence of clinical conjunctivitis in a small number of birds in the general flock from around 8 days pv. None of the sample birds exhibited this but it was noticeable in a small membership of the general flock. Replication of ILTV in the conjunctiva, and hence the appearance of conjunctivitis signs has been linked to infection occurring by natural exposure [22] and hence to vaccine virus spread between naïve birds. By the end of the flock's life (after the sampling time frame), the grower reported culling a small number of birds (about 8) which were exhibiting marked dyspnoea typical of clinical ILT.

All positive viral DNA samples from day 26 pv were subjected to typing at BAL and all proved to be Class 7, consistent with the vaccine strain used. No wild ILTV was detected within Case-01. A small number of birds in another house on this farm showed clinical signs consistent with ILT and tracheal swabs from some of these birds yielded ILTV determined to be Class 9 (the local outbreak strain). Hence a wild challenge virus was present on this farm but this was not detected within the study house.

3.3. Phase 2

From virus detection results from Case-01 a re-evaluation of the number of birds necessary to sample to detect a similar prevalence of infected birds was made. Assuming that 3% of the subjects in the population have detectable ILTV in trachea at 4 days pv, the study would require a sample size of 45 for estimating the expected proportion with 5% absolute precision and 95% confidence [23].

Details of houses designated as Cases 02 to 08 are shown in Table 1 and their recorded vaccination procedure factors and ensuing point prevalence of detection of tracheal ILTV are shown in Table 2. All houses involved in phase 2 were vaccinated between 7 and 14 days of age by the grower and the company veterinarian following the standard operating procedure, except that for Cases 06 and 08 where the company allowed the use of a dye-type commercial water stabilizer rather than their standard use of a skim milk stabilizer. This provided a direct comparison of stabilizer types in two out of the four houses on farm D under otherwise similar administration practices. All houses used medication tanks (either gravity fed or through a pump) for drinking water supply. Prevalence of ILTV DNA detection from laryngo-tracheal swabs revealed a wide variation in time of detectable presence and the pattern of subsequent prevalence over time between houses (Table 2). Presence of the vaccine virus in a susceptible respiratory tissue is assumed necessary for the birds to produce a protective immunity [8].

Point prevalence at day 4 pv in each of the houses revealed very wide variation (between 0.05 and 0.52, Table 2). An effective "herd immunity" level is regarded as 95% of the population effectively vaccinated [24]. Some of the houses in this study only approached this level of vaccine virus detectability in tracheas by 12–13 days pv. Houses which reached a prevalence of at least 0.20 at 4 days pv reached a point prevalence above 0.80 by 12–13 days pv (Table 2). Given the age of vaccination, reaching high vaccine virus prevalence in tracheas by 12–13 days pv places the birds at between 21 and 27 days of age. Most of the field outbreaks were

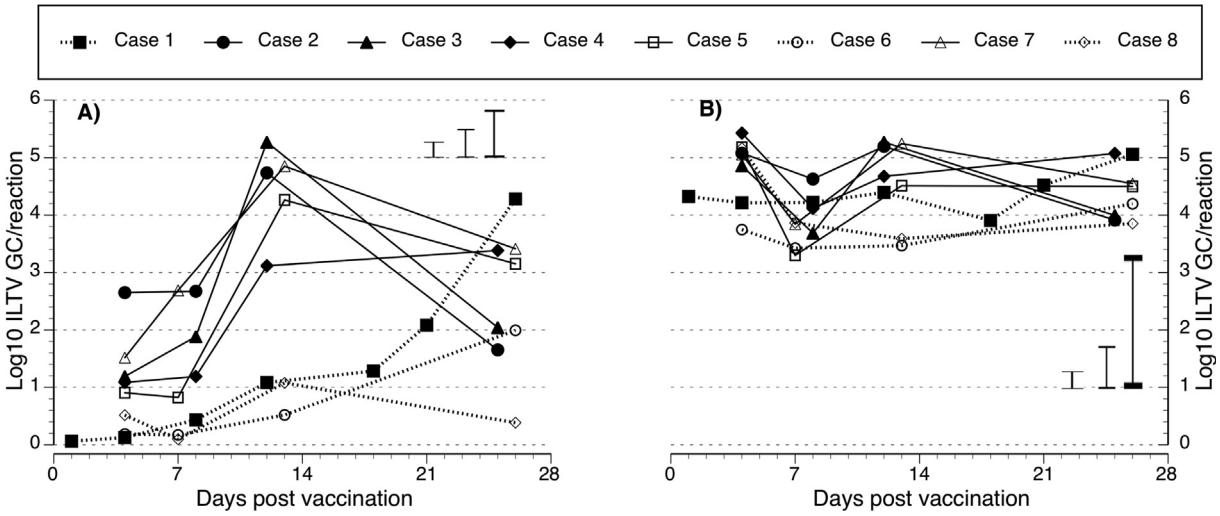

Fig. 3. Mean ILT viral genome copies from laryngeal swabs taken at various times post vaccination in the eight case studies. Panel A shows means of all samples including negatives (ascribed 0 value) while panel B shows means of samples that were positive for ILTV. The three cases with poor ILTV prevalence post vaccination have dotted lines. The error bars represent 2 × standard error representing the minimum, mean and maximum SE values for the various mean values (SE values 0.11, 0.28, 0.41 and 0.13, 0.36 and 1.17 for A and B respectively).

observed clinically to occur after 35 days of age (Table S1) and assuming an incubation period of 6–12 days [25] we could assume that infection begins commonly sometime after 23 days of age. Hence we could hope that most of the houses in this study would have achieved sufficient herd immunity by that age to be protected. However we observed several houses which never reached this degree of detectable vaccine presence in tracheas and must be considered to be susceptible at the likely time of infection.

All viral DNA samples typed from samples collected at days 12–13 and 25–26 pv from cases 02–08 were subjected to typing at BAL and all those with sufficient DNA to allow typing proved to be Class 7, consistent with the vaccine strain used.

3.4. Combined analysis

From Table 2 it can be observed that the prevalence patterns of detection of ILTV in trachea in each house fell into one of two broad categories. The mean viral copy numbers per qPCR reaction from tracheal swabs at each time point for each case (the mean here includes negative results) are shown in Fig. 3A. Fig. 3B shows mean viral copy numbers from only the tracheal swabs with positive detection of ILTV. There are no significant differences between viral copy numbers from only positive swabs indicating that the differences shown in Fig. 3A reflect prevalence, rather than a difference in viral infection once birds are exposed. None of the houses achieved a prevalence of ILTV in tracheas at 4 or 8 days pv which could be considered adequate herd immunity [24]. As can be resolved from Table 2 and Fig. 3A, three of the houses (Cases 01, 06 and 08) showed a very poor establishment of the vaccine early while the other five could be considered to have produced a "better" result. The mean prevalence by age for these two groupings are expressed graphically in Fig. 4. To achieve a higher point prevalence of detectable tracheal vaccine virus by 12–13 days pv, it appears necessary for the birds to have a point prevalence of at least 0.175 on day 4 pv. Below this day 4 pv prevalence, a good protection level either failed or was either much delayed, and the flock could have been assumed unprotected during the period of higher risk of infection. On this basis, the houses were classified as either having a "poor" or "better" vaccine take. This classification was then used as the dependent variable in contingency table analysis or as Spearman rank correlations across the other factors recorded (Table 2) during the vaccination process. Analysis results from this are shown in Table 3. As this is only a small dataset (8 houses only), the analytical results must be considered only as indications, but provide insight into further required studies. Factors showing statistically significant associations with the relative success of vaccination were the choice of water stabilizer (skim milk products superior to dye preparation – Fig. 5) and the amount of time that water stabilizer was present in the header tank prior to addition of the vaccine (Fig. 6). These factors were somewhat confounded as the use of the dye product had very low water stabilization times (a promoted feature of the product) but water samples drawn from various points of the drinker lines during vaccination revealed a significantly lower vaccine virus copy number for those houses utilizing the dye product, even after the pH was adjusted to neutral using soda ash. Table 4 shows the differences in detection of virus from drinking water at various times during vaccination,

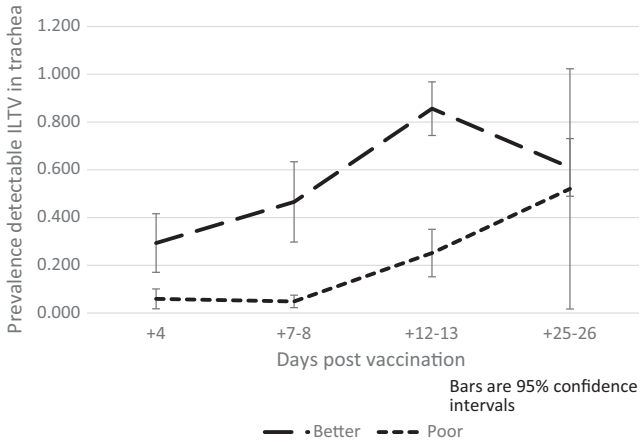

Fig. 4. Mean prevalence of detection of ILTV from laryngo-tracheal swabs over time categorised into "poor" and "better" groupings by prevalence of ILTV detection over time.

Table 3
Cross tabulation of vaccination procedures and defined "success" level of vaccination as assessed by prevalence of ILT vaccine virus detection in trachea at 12–13 days post vaccination.

Vaccination procedure measurement	Category[a]	No. flocks with Poor[b] vaccination success (n = 3)	No. flocks with Better[c] vaccination success (n = 5)	Fisher's exact test, 2-tailed P=	Spearman Rank R=	Spearman Rank P=
Hatchery	A	3	1	0.07	−0.77	0.02
	B	0	4			
Flock size	<20,000 birds	1	3	1.00	−0.25	0.57
	>20,000 birds	2	2			
Water deprivation	≤55 m	1	2	1.00	−0.25	0.54
	>55 m	2	3			
Water stabilized with	Dye product	2	0	0.107	−0.75	0.034
	Skim milk	1	5			
Water stabilization time[d]	>30 m	0	4	0.14	−0.77	0.024
	<30 m	3	1			
Time after vaccination house walked	≤5 m	2	5	0.38	−0.49	0.22
	>5 m	1	0			
Time to consume vaccine	≤100 m	1	3	1.00	−0.26	0.54
	>100 m	2	2			
Label doses of vaccine administered	<1/bird	1	3	1.00	−0.26	0.54
	≥1/bird	2	2			

[a] Times are in minutes.
[b] Flocks with <60% detection of vaccine ILTV at 12–13 days post vaccination.
[c] Flocks with >60% detection of vaccine ILTV at 12–13 days post vaccination.
[d] Time between addition of water stabilizer product and addition of vaccine.

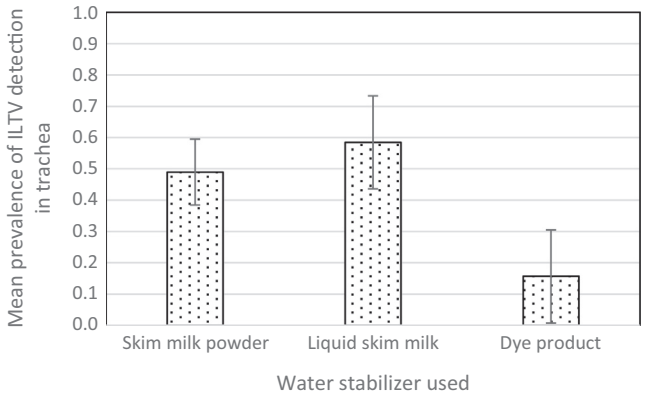

Fig. 5. Repeated measure ANOVA least square mean prevalence of ILTV detected following vaccination as affected by product used for water stabilization. Vertical bars denote 0.95 confidence intervals; P = 0.02.

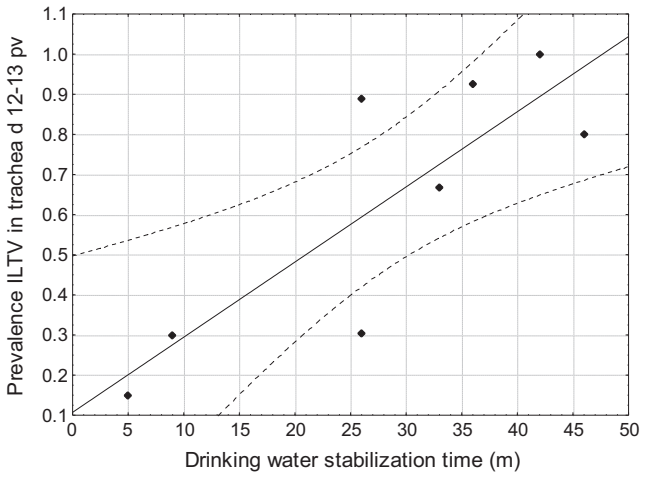

Fig. 6. Correlation of water stabilization time and prevalence of ILTV detection in trachea at days 12–13 post vaccination (0.95 confidence limits shown). Spearman Rank Order Correlation R = 0.78, P < 0.05.

Table 4
Log_{10} ILT vaccine virus copy number in water samples collected at various points of the drinking water supply during vaccination (2 houses per treatment on the same farm: Cases 05, 06, 07 and 08).

Sample site and time after vaccine preparation	Vaccine mean Log_{10} virus copy number/ mL for each type of water stabilizer used (Std Err) (Expected value[a] approx. 4.85 Log_{10} VCN/mL)	
	Skim milk powder	Dye product
Drinker tank - fresh vaccine	4.575 (0.002)	2.990 (0.805)
End of drinker line after flush	4.565 (0.005)	1.550 (0.075)
End of drinker line after 1 hr	4.500 (0.004)	1.465 (0.367)

[a] Based on the label dose of ≥2.25 EID_{50}/bird being equivalent to 8 Log_{10} VCN.

indicating that where the dye product was used significantly lower viral numbers were recovered than if skim milk was used as stabilizer.

There was also an observation of the effect of source of chicks (hatchery) (Table 3) which approached significance (P = 0.07). The putative effect here could be related to the hatchery, breeder flock, age of breeder flock, chick quality, transport or other factors which are not discernible from the available information but this requires further evaluation and confirmation.

4. Conclusions

Vaccination against ILTV via the nipple drinking water route appears to be a gamble, with few birds acquiring a colonizing viral infection in respiratory tissues from contact with the vaccine virus in the water. Effective vaccination of these flocks relied upon extensive bird to bird transmission. Repeated bird passage is known to allow reversion to virulence of vaccinal strains [3,26]. Hence this may explain the field observation of so called "vaccine reactions", where low level clinical signs (conjunctivitis, mild tracheitis) may be seen with only vaccine virus detectable in trachea. A poor initial uptake of the vaccine (perhaps < 17% of birds with detectable ILTV in trachea at day 4 pv) results in delayed spread or even failure of spread of vaccine virus through the flock. In the latter case, a majority of the flock may remain susceptible to ILTV infection at the likely time of wild viral exposure. Hence when a wild ILTV enters one of these poorly protected flocks, a clinical outbreak may easily be explained in the face of vaccination. This would indicate that such vaccination "failures" may be more associated with inadequate vaccine administration rather than the vaccine itself being non-protective [5–7].

Successful vaccination with ILTV vaccines requires the vaccine virus to access susceptible oculo-respiratory tissues (conjunctiva, nasal cavity, larynx or trachea), while contact with the mouth, tongue, oesophagus and the gastrointestinal tract is ineffective [8]. Drinking water vaccination achieves such contact only accidentally [8]. The field practice of examining the proportion of birds with stained tongues when a dye preparation is used as stabilizer to judge successful uptake of vaccine therefore appears to be not useful with ILT vaccination. To overcome this, other research [4] has indicated that it is necessary to increase the vaccine dose rate, perhaps by as much as tenfold, to achieve success via drinking water with ILT vaccines. This however would pose serious problems of affordability and adequate vaccine supply in large outbreaks.

This present study identified several putative associations between the type of water stabilizer used, the time that the stabilizer was present in the vaccination water prior to the addition of the vaccine and potential success of vaccination. A possible influence of chick source was also incriminated in vaccine success. However these analytical outcomes are only based on a small sample size and these associations require a further, larger study for their validation and increased understanding. Such further studies will be essential to identify factors in administration technique which may improve the success and consistency of mass vaccination of meat chickens against ILT.

No wild type ILT virus was detected in any of the houses within this study and hence the objective of examining the ingress of this was not able to be evaluated in this project.

Funding

This work was supported by AgriFutures Australia (project number 010639).

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

Acknowledgements

We gratefully acknowledge the assistance provided by Joy Gill, Wade Chen, Kylie Warr and Adrian Dam (Poultry Research Founda-

tion, The University of Sydney), Maria King (Zootechny Pty Ltd), Nguyen Van Trong, Mohammed Ahaduzzaman and Yugal Bindari (The University of New England), David Parrot and Felicity Reddy (Cordina Chickens) and the contract growers of Cordina Chickens for assistance with vaccination administration and sample collection during this study and Sithara Ralapanawe (UNE) and Meghan Jones (BAL) for assistance with molecular testing of the samples. Funding provided by AgriFutures Australia, Australia is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.087.

References

[1] Coppo MJC, Noormohammadi AH, Browning GF, Devlin JM. Challenges and recent advancements in infectious laryngotracheitis virus vaccines. Avian Pathol 2013;42:195–205. https://doi.org/10.1080/03079457.2013.800634.
[2] Keck L. ILT vaccination decisions can be difficult balancing act in broilers. Poultry Health Today. <https://poultryhealthtoday.com/ilt-vaccination-decisions-can-be-difficult-balancing-act-in-broilers/>; [accessed 13 May 2018].
[3] Menendez KR, García M, Spatz S, Tablante NL. Molecular epidemiology of infectious laryngotracheitis: a review. Avian Pathol 2014;43:108–17. https://doi.org/10.1080/03079457.2014.886004.
[4] De Wit JJ. Underestimation of the difficulties of vaccination against viral respiratory diseases by mass application methods. In: Proceedings XVIIIth congress of the world veterinary poultry association, session J: viral respiratory diseases 2: IBV, metapneumovirus, others. 19–23 August, Nantes, France 2013; 18:63–7
[5] Groves PJ. Class 8 infectious laryngotracheitis virus in Sydney. In: Proceedings scientific meeting of australasian veterinary poultry association, 11–12 February, 2009. Sydney, Australia.
[6] Arzey GG, Arzey KE. ILT-protection against Class 8 NSW ILTV – challenge trial 2008. In: Proceedings scientific meeting of australasian veterinary poultry association, 11–12 February, 2009. Sydney, Australia.
[7] Korsa MG, Browning GF, Coppo MJC, Legione AR, Gilkerson JR, Noormohammadi AH, et al. Protection induced in broiler chickens following drinking-water delivery of live infectious laryngotracheitis vaccines against subsequent challenge with recombinant field virus. PLoSONE 2015. https://doi.org/10.1371/journal.pone.0137719.
[8] Robertson GM, Egerton JR. Replication of infectious laryngotracheitis virus in chickens following vaccination. Aust Vet J 1981;57:119–23.
[9] Coppo MJC, Devlin JM, Noormohammadi AH. Comparison of the replication and transmissibility of an infectious laryngotracheitis virus vaccine delivered via eye-drop or drinking water. Avian Pathol 2012;41:99–106. https://doi.org/10.1080/03079457.2011.643222.
[10] Rodríguez-Avila A, Oldoni I, Riblet S, García M. Replication and transmission of live attenuated infectious laryngotracheitis virus (ILTV) vaccines. Avian Dis 2007;51:905–11.
[11] Coppo MJC, Devlin JM, Noormohammadi AH. Comparison of the replication and transmissibility of two infectious laryngotracheitis virus chicken embryo origin vaccines delivered via drinking water. Avian Pathol 2012;41:195–202. https://doi.org/10.1080/03079457.2011.660132.
[12] Davidson I, Raibshtein I, Altori A, Elkin N. Infectious laryngotracheitis virus (ILTV) vaccine intake evaluation by detection of virus amplification in feather pulps of vaccine chickens. Vaccine 2016;34:1630–3.
[13] Agnew-Crumpton R, Vaz PK, Devlin JM, O'Rourke D, Blacker-Smith HP, Konsak-Ilievski B, et al. Spread of newly emerging infectious laryngotracheitis viruses in Australia. Inf Gen Evol 2016;43:67–73. https://doi.org/10.1016/j.meegid.2016.05.023.
[14] Hancock DD, Blodgett D, Gay CC. The collection and submission of samples for laboratory testing. Vet Clinics N Am: Food An Practice 1988;4:33–59.
[15] Kirkpatrick C, Mahmoudian A, O'Rourke D, Noormohammadi AH. Differentiation of infectious larynotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. Avian Dis 2006;50:28–34.
[16] Callison SA, Riblet SM, Oldoni I, Sun S, Zavala G, Williams S, et al. Development and validation of a real-time Taqman PCR assay for the detection and quantitation of infectious laryngotracheitis virus in poultry. J Virol Methods 2007;139:31–8.
[17] DAF, Queensland. Infectious laryngotracheitis; 2017. <https://www.daf.qld.gov.au/business-priorities/animal-industries/animal-health-and-diseases/list/infectious-laryngotracheitis> [accessed 13 May 2018].
[18] Johnson YJ, Gedamu N, Colby MM, Myint MS, Steele SE, Salem M, et al. Wind-borne transmission of infectious laryngotracheitis between commercial poultry operations. Int J Poult Sci 2005;4:263–7.
[19] Williamson SL, Sharpe SM, Jones M, Weaving L, Pavic A. ILT, classes detected in NSW between 2014 – present. In: Proceedings scientific meeting of australasian veterinary poultry association, 20–21 February, 2019. Sydney, Australia.
[20] York JJ, Young JG, Fahey KJ. The appearance of viral antigen and antibody in the trachea of naïve and vaccinated chickens infected with infectious laryngotracheitis virus. Avian Pathol 1989;18:643–58.
[21] Da Silva Martins NR, Mockett APA, Barrett ADT, Cook JKA. Local and systemic antibody class responses to an infectious laryngotracheitis virus vaccine strain. Avian Pathol 1992;21:97–106.
[22] Rodríguez-Avila A, Oldoni I, Riblet S, García M. Evaluation of the protection elicited by direct and indirect exposure to live attenuated infectious laryngotracheitis virus vaccines against a recent challenge strain from the United States. Avian Pathol 2008;37:287–92.
[23] Dhand NK, Khatkar MS. Statulator: An online statistical calculator. Sample Size Calculator for Estimating a Single Proportion. 2014. <http://statulator.com/SampleSize/ss1P.html> [accessed 28 January 2018]
[24] Finn A. Vaccine Knowledge Project. Oxford Vaccine Group; 2018. <http://vk.ovg.ox.ac.uk/herd-immunity> [accessed 10 January 2019]
[25] Guy JS, Bagust TJ. Laryngotracheitis. In: Saif YM, editor. diseases of poultry. Ames, Iowa: Iowa State Press; 2003. p. 121–34.
[26] Guy JS, Barnes HJ, Smith L. Increased virulence of modified-live infectious laryngotracheitis vaccine virus following bird-to-bird passage. Avian Dis 1991:348–55.