Veterinary Microbiology 236 (2019) 108393

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Spatial and temporal variation of Marek's disease virus and infectious laryngotracheitis virus genome in dust samples following live vaccination of layer flocks

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ARTICLE INFO

Keywords:
Aggregated samples
Infectious laryngotracheitis virus
Marek's disease virus
Population monitoring
Poultry dust
PCR
Vaccine

ABSTRACT

Monitoring of Marek's disease virus (MDV) and infectious laryngotracheitis virus (ILTV) genome using poultry dust can be useful to monitor on-farm vaccination protocols but there are no set guidelines for collection of this sample type. This study assessed different dust collection methods for MDV and ILTV detection in a vaccinated layer flock (n = 1700) from day-old to 50 weeks of age. Birds were vaccinated against MDV at day-old, and ILTV by drinking water at week 6 and eye drop at week 12. Dust samples were collected weekly by settle plates (1–3 plates/15 m^2) or by scraping surfaces in the poultry shed and tested for ILTV and MDV genomic copies (GC) by PCR. ILTV GC were detected 4 weeks post water vaccination, peaked at weeks 12–14 and became mostly undetectable after week 18. MDV was detected in dust on week 1, peaked at weeks 3–6, declined 3 logs by week 26 and remained detectable at this level until week 50. There was no difference in the detection rates of ILTV and MDV collected from settle plates in different locations of the shed ($P > 0.10$). There was no difference between settle plate and scraped samples in ILTV GC load but higher MDV GC were found in scraped samples. The settle plate method appears to reflect the current level of vaccine virus in the flock while the scrape method likely represents a cumulative record of shedding. Assessment of viral GC in dust samples is a good candidate for a practical method of estimating successful vaccine administration.

1. Introduction

Marek's disease virus (MDV) and infectious laryngotracheitis virus (ILTV) of the *Alphaherpesvirinae* subfamily cause significant economic losses in the poultry industry worldwide (García et al., 2013; Morrow and Fehler, 2004). The disease caused by these viruses are currently controlled mostly by attenuated vaccines and biosecurity measures (García et al., 2013; Gimeno, 2004). MDV is an extremely contagious virus that can cause severe oncogenic changes in viscera and peripheral nerves (Witter and Schat, 2003) while ILTV causes upper respiratory disease in chickens (Bagust et al., 2000). MDV replicates in epithelial cells of feather follicles and the virus can spread in the environment through dust containing feather dander of infected chickens (Calnek et al., 1970; Islam et al., 2001). ILTV replicates in the upper respiratory tract and virus genome have also been detected in feather tips, faeces and dust samples of vaccinated birds (Bagust and Johnson, 1995; Davidson et al., 2016; Roy et al., 2015). Monitoring of vaccine administration has been previously performed using PCR testing for detection of MDV and ILTV DNA in samples collected from individual birds in commercial chicken flocks. Specifically, MDV has been detected in feather samples (Davidson et al., 2018; Ralapanawe et al., 2016), and ILTV has been detected in feathers (Davidson et al., 2018, 2016) and tracheal swabs (Groves et al., 2019). These methods have the potential to offer great information on the vaccine uptake but they require sampling of a great number of birds which is not always practical in commercial flocks.

Quantification of the MDV genome in poultry dust was first reported by Islam et al. (2006) and proved to be an effective tool to measure vaccinal and wild-type MDV shedding rates Islam et al., 2013; (Islam et al., 2008) under experimental conditions. In the field it has proved

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https://doi.org/10.1016/j.vetmic.2019.108393
Received 17 June 2019; Received in revised form 9 August 2019; Accepted 18 August 2019

useful to assess MDV incursions and vaccination success in layer, breeder and broiler chicken flocks (Kennedy et al., 2017; Ralapanawe et al., 2016; Walkden-Brown et al., 2013). A previous study has shown that MDV genome can be detected in dust 2–3 weeks after vaccination of commercial layer flocks and persists at high levels until 91 weeks of age (Ralapanawe et al., 2016). In the case of ILTV, detection at high levels in dust samples under experimental conditions was first reported by Roy et al. (2015) with subsequent detection in field samples (Roy et al., 2016) and promise as a marker of monitoring of vaccine water administration in broiler flocks (Ahaduzzaman et al., 2019). Dust collection is non-invasive and can be easily carried by farm staff, the sample material is dry so the genome is relatively stable and can be dispatched to the diagnostic laboratory in the normal mail without chilling. As a dust sample represents a population, a small number of samples is required to obtain population level information, making it cost effective (Walkden-Brown et al., 2013). For monitoring pathogen incursion, 1–6 sampling locations per poultry shed have been used, and it has been suggested that a single dust sample per shed would allow detection of a positive result in infected flocks (Kennedy et al., 2017; Walkden-Brown et al., 2013), although this recommendation has not been scientifically demonstrated. This is mainly due to the absence of guidelines for sampling of aggregate sample types, such as poultry dust, in contrary to well established strategies for collection of samples from individual animals (Cannon, 2001) and testing of pooled samples (Abel et al., 1999; Muñoz-Zanzi et al., 2006).

Although monitoring of MDV in dust has been used by the chicken industry in Australia (Walkden-Brown et al., 2013) and in the USA (Kennedy et al., 2017), there are no guidelines for collection of this sample type. Dust samples can be simply scraped from barn surfaces or be collected from dust deposited by settling in a collection apparatus. Kennedy et al. (2017) suggested that dust scraped from fan louvres provides a better representation of the current MDV status of a flock compared to dust scraped from wooden ledges. However, the usefulness of different dust collection methods for the detection of ILTV have not been explored. Unlike MDV, which is continuously shed in vaccinated birds (Davidson et al., 2018; Ralapanawe et al., 2016), ILTV shedding is expected to cease a week or so after vaccination, with episodic ILTV reactivation and shedding in a small proportion of birds afterwards (Davidson et al., 2018; Hughes et al., 1991).

The main goals of this study were to determine 1) the long term pattern of ILTV and MDV detection in dust following vaccination of layer chickens, 2) the influence of dust collection method (scraped from a surface or collected in a settle plate) on amount of dust collected and ILTV and MDV genome content of the dust, and 3) the effect of the location of dust collection in the shed on amount of dust collected and ILTV and MDV genome content of the dust. To achieve the study goals, two experiments were conducted. The profile of ILTV genome in tracheal swabs after vaccination in one of these experiments is also reported.

2. Materials and methods

2.1. Birds, housing and sampling procedures

2.1.1. Profile of detection of ILTV DNA in dust and tracheal swab samples

The first experiment aimed to profile ILTV genomic copies (GC) content in dust and tracheal swabs following vaccination and to compare the sensitivity of detection of ILTV genome by the two methods as a proof of concept. MDV was not included in this experiment which used 238 Hy-Line Brown layer chickens from day-old to 30 weeks of age reared in a single shed in seven separate floor pens. New wood shavings were used as bedding material. The experiment was conducted at the Zootechny Research Facility (Austral, NSW, Australia). The facility comprises a single house containing 32 pens of 7 m^2 floor space with natural ventilation through side curtains. Each pen used for this study held 34 birds supplied with a single tube feeder, nipple drinkers and a nest box. The experiment was approved by the University of Sydney Animal Ethics Committee (approval no. 2017/1207).

Twelve birds (n = 2/pen) were randomly selected, individually identified, and sampled for tracheal swabs at days 4, 8, 11, 34 and 74 post ILTV vaccination which corresponds to weeks of age 8.5, 9, 9.5, 12 and 18. On day 4 post vaccination, tracheal swabs were collected from an additional five birds housed in the same pen. Five dust samples were collected from settle plates with a surface area of 520 cm^2 suspended at a height of approximately 1.4 m at weeks 8.5, 9, 9.5, 12, 14, 15, 16, 18 and 30. Swabs and individual dust samples were placed in plastic zip-lock bags and stored at −80 °C until further processing.

2.1.2. Longitudinal profile of ILTV and MDV DNA detection in dust samples

The second experiment aimed to profile ILTV and MDV GC content in dust following vaccination and to evaluate methodologies for dust sample collection for detection of these viruses. This experiment used 1700 Hy-Line Brown chickens from day old to 50 weeks of age and was divided into a pullet raising phase (phase 1) and a laying phase (phase 2) conducted at research facilities at the University of New England (Armidale, NSW). Rice hulls were used for bedding in both phases. The experiment was approved by the University of New England Animal Ethics Committee (AEC17-092).

Phase 1, from day-old to 16 weeks of age, was conducted at Kirby Poultry Facility (Fig. 1). This facility had three identical rooms, each with an area of 6.25 × 9.78 m, with two doors, two circulating fans, one roof fan and four double mini air entry vents. Each room was divided into three pens divided by wire mesh with a common shared air space. The air was pulled into the rooms through the double mini air entry vents and exhausted through the roof fans. Circulating fans pushed air downwards from the ceiling. Each room contained approximately 570 Hy-Line Brown layer chicks from day old.

At the start of phase 2 (17 to 50 weeks of age), pullets were moved to a free-range laying shed at Laureldale Poultry Facility (Fig. 1). Within a large fully-enclosed shed the chickens were maintained in nine wire mesh and shade cloth sided pens housing 154 chickens/pen with a common shared air space. Each pen had dimensions of 3.6 × 4.8 m and four of nine pens had an exhaust fan on the wall. Each pen had a single chicken mesh door and two pop-holes providing access to the free-range area. The pop-holes were opened daily between 9 h and 18 h from 25 weeks of age onwards.

Dust samples were collected from settle plates as described in the Section 2.1.1 and by scraping dust from walls and horizontal surfaces. During the pullet raising phase, individual dust samples from settle plates were collected weekly from four sites per room (near the mini air entry vents, under the circulating fan, under the roof exhaust fan and in the open area, Fig. 1). One pooled sample was created weekly by mixing individual samples. Two scraped dust samples were randomly collected from each room when birds were 7, 11, and 14 weeks old and one pooled sample was created weekly by mixing individual scraped samples.

During the laying phase, individual dust samples from settle plates were collected weekly from two settle plates placed at the door end and two settle plates placed at the pop-hole end of each pen (Fig. 1). A single scraped sample per pen was also collected weekly. One of the duplicate settle plate samples was used to create a pooled sample for the nine pens while the other was retained as an individual sample. A weekly pool of scraped dust was created by mixing 20 mg dust from each individual scraped sample every week. Individual and pooled samples were weighed and stored in plastic zip-lock bags at −20 °C until further processing.

2.2. Vaccination procedures

In the first experiment, birds were vaccinated with ILTV strain SA2 (Poulvac Laryngo SA2, Zoetis, Australia) by eye-drop at 8 weeks of age. In the second experiment, day-old chicks were vaccinated against MDV-

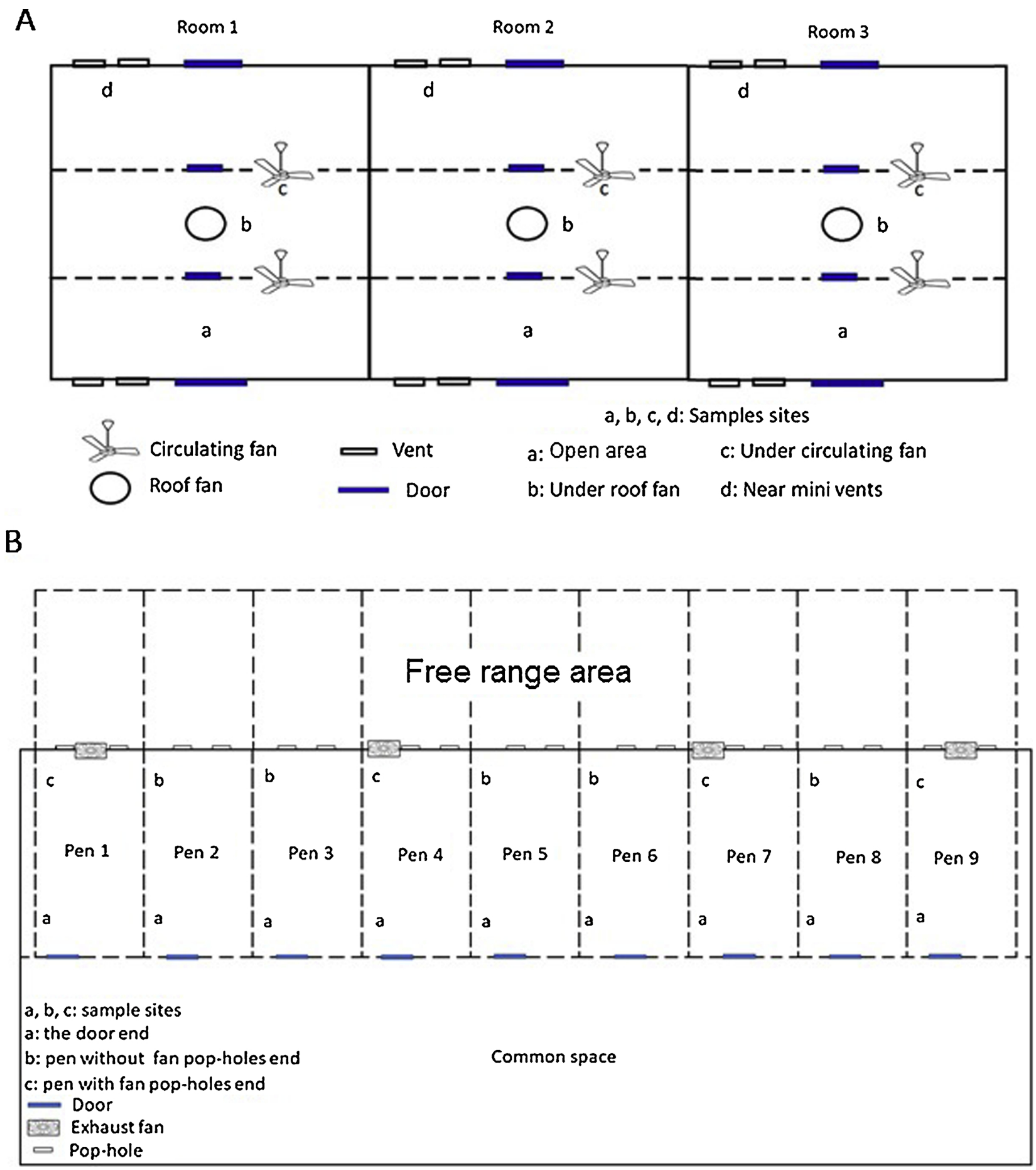

Fig. 1. Facilities and sample collection location in the shed during (A) phase 1 (0–16 weeks), and (B) phase 2 (17–50 weeks) for the MDV and ILTV longitudinal profiling in dust samples. In phase 1, birds were allocated to three rooms, each room was divided by mesh wire into three pens but sharing the same air space. In phase 2, birds were allocated in a single shed divided by wire mesh wire and shade cloth into nine pens but sharing the same air space.

1 strain Rispens CVI988 (Vaxsafe RIS, Bioproperties Pty Ltd, Australia) by subcutaneous injection at the hatchery. The birds were vaccinated against ILTV strain A20 (Poulvac Laryngo A20, Zoetis) by drinking water in nipple drinkers at 6 weeks of age and re-vaccinated with ILTV strain SA2 by eye drop at 12 weeks of age. Vaccine administration was performed according to industry practices in Australia.

2.3. DNA extraction and real-time PCR assay for ILTV and MDV detection

Tracheal swabs were placed in 0.8 ml of sterile phosphate buffered solution and vortexed for 10 s before DNA extraction. DNA was extracted from 200 µl of tracheal swab wash or approximately 5 mg of dust samples using the Bioline ISOLATE II Genomic DNA kit (Roy et al., 2015). All DNA extracts were stored at −20 °C until further analysis by qPCR. Dust and tracheal extracts were tested by qPCR for the ILTV glycoprotein C gene (Callison et al., 2007) and dust extracts from Experiment 2 were tested for Rispens vaccine virus presence based on polymorphism of the pp38 gene (Baigent et al., 2016). Results were reported in $\log_{10}$ genomic copies (GC) per milligram of dust or per tracheal swab reaction.

2.4. Statistical analysis

Statistical analyses were conducted using JMP 14 (SAS Institute Inc., Cary NC, USA). The genome copy number of viruses were transformed into $\log_{10}$ before analyses ($\log_{10}$ GC + 1).

For Experiment 1, $\log_{10}$ GC of ILTV on dust and swabs was analysed by using a restricted maximum likelihood (REML) model fitting bird age (weeks) and location of sample collection in the shed (pen number) as fixed effects. Within main effects, significance of differences between individual means was determined by using Tukey's honestly significant difference test. For continuous variables, least squares means (LSM) and standard error means (SE) are presented unless otherwise specified.

For Experiment 2, analysis was conducted separately for the pullet raising phase and laying phase. For individual samples collected from settle plates, the $\log_{10}$ GC of ILTV or MDV were analysed by using a REML model fitting sample as a random effect and bird age (weeks), dust deposition rate (mg/100 cm^2/chicken/day), room or pen, location of the dust collector in room or pen (phase 1, open area, circulating fan, roof exhaust fan, air entry vents; phase 2, at the door end, at the pop-holes end), type of pen (phase 2, with or without exhaust fan) and their interactions as fixed effects. For individual scraped samples, the $\log_{10}$ GC of ILTV or MDV was analysed by using an REML model fitting sample as a random effect and bird's age and room or pen as fixed effects. For the pooled samples, the effects of bird age on virus GC were tested using a linear model fitting bird age as a fixed effect.

Discrete data (positive or negative for virus DNA) for different groups (pooled and individual dust samples; settle and scraped dust samples) were subject to contingency table analysis with significance between means determined by the Chi-Square test for independence. The proportion of overall agreement was calculated between sample types (settle and scraped samples; pooled and individual samples). For individual samples, a sample collection day was considered positive for a given collection method when at least one of the samples collected on a given day was positive for virus DNA. A P-value of less than 0.05 was set as the statistically significant level throughout.

Table 1
Detection rates and ILTV $\log_{10}$ GC levels in tracheal swabs and settle plate dust samples. Birds were vaccinated against ILTV via eye-drop at 8 weeks of age.

Age (weeks)	Tracheal swab		Settle plate dust	
	N. positive/ total (%)	ILTV $\log_{10}$ GC/ reaction (LSM ± SE)	N. positive/ total (%)	ILTV $\log_{10}$ GC/ mg dust (LSM ± SE)
8.5	17/17 (100)	6.53 ± 0.43^{A}	5/5 (100)	6.43 ± 0.70^{A}
9	9/12 (75)	2.79 ± 0.51^{B}	5/5 (100)	6.64 ± 0.70^{A}
9.5	7/12 (58)	2.08 ± 0.51^{B}	2/2 (100)	7.14 ± 1.15^{A}
12	8/12 (66)	2.75 ± 0.51^{B}	5/5 (100)	5.27 ± 0.70^{A}
14	–	–	4/5 (80)	3.79 ± 0.70^{AB}
15	–	–	2/5 (40)	1.86 ± 0.70^{BC}
16	–	–	2/5 (40)	1.32 ± 0.70^{BC}
18	6/12 (50)	1.85 ± 0.51^{B}	0/5 (0)	0^{C}
30	–	–	2/5 (40)	1.25 ± 0.70^{C}

Different superscripts ($^{A, B, C, D}$) within column for each level of factors indicate significant difference for $\log_{10}$ ILTV GC ($P < 0.05$); – not tested.

3. Results

3.1. *The profile of detection of ILTV GC in dust and tracheal swab samples over a period of 10 weeks post-vaccination is similar*

The individual results of ILTV GC detection in dust and tracheal swabs are shown in Table 1 and Fig. 2. All tracheal swabs (n = 17) were positive at 4 days post-vaccination (week 8.5) with a decrease in the number of positive samples (P = 0.02) and ILTV GC levels (P < 0.0001) from 7 days post-vaccination onwards (Table 1 and Fig. 2). From the birds longitudinally sampled, 4/12 had tracheal swabs ILTV GC positive only in the first week post-vaccination, 3/12 were intermittently positive and 5/12 had ILTV GC positive samples at all sampling points. Pen had a significant effect in the number of positive tracheal swabs (P = 0.01) and ILTV GC load (P = 0.003), as birds sampled in one of the pens were ILTV GC positive only on the first week post-vaccination.

All dust samples were ILTV GC positive from weeks 8.5 to 12, afterwards the number of positive samples (P = 0.003) and the levels of ILTV GC (P < 0.001) in dust samples decreased (Table 1). At week 18, all five dust samples were negative for ILTV GC (Table 1). In contrast to the pen effect observed in the ILTV detection in the tracheal swab

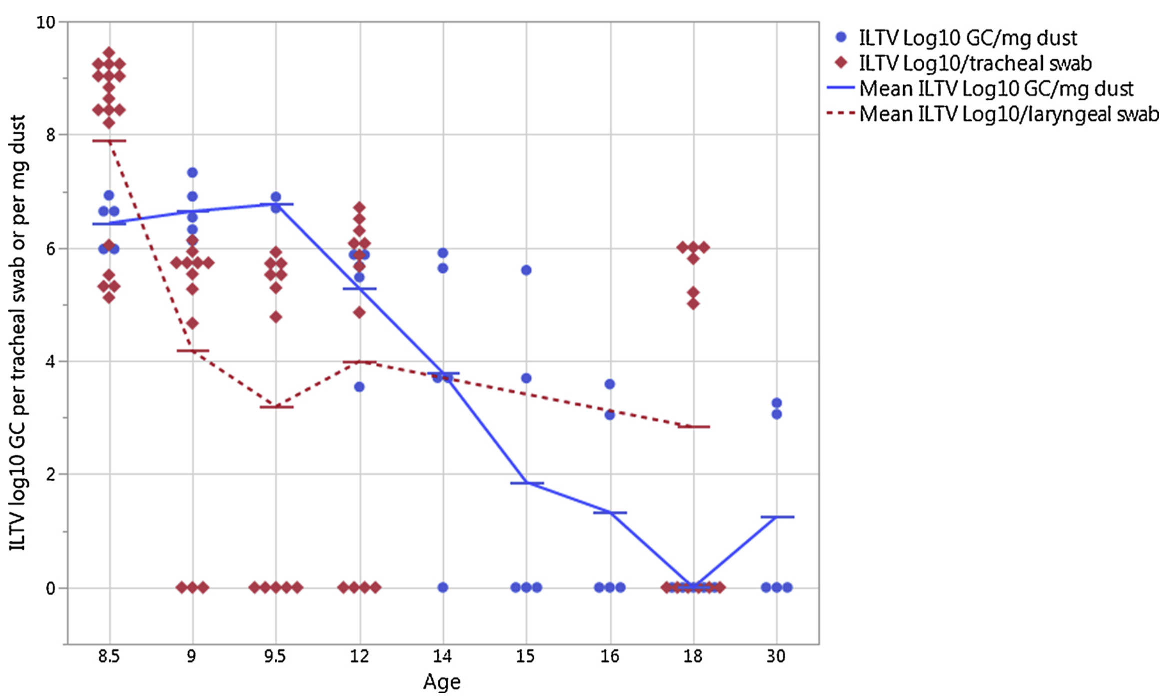

Fig. 2. ILTV log10 GC per milligram of settle plate dust (blue circles) or per tracheal swab (red rhombus) in individual samples for the proof of concept study. The blue solid line indicates the mean ILTV log10 GC/mg of dust and the red interrupted line indicates the mean ILTV log10 GC/tracheal swab. Birds were vaccinated against ILTV by eye-drop at 8 weeks of age. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

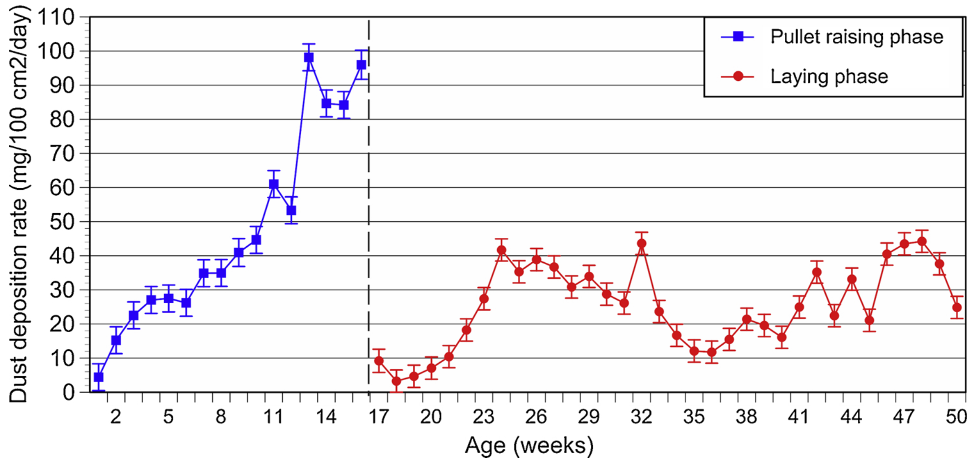

Fig. 3. Least squares mean dust (± SE) deposition rate in settle plates for the MDV and ILTV longitudinal profiling in dust samples. The interrupted line indicates that birds were relocated from the pullet raising facility (weeks 1 to 16), to the laying facility (weeks 17 to 50).

samples described above, the location in which dust samples were collected had no effect in ILTV GC detection (P = 0.46).

Overall, ILTV GC detection rates in tracheal swabs (48/66, 72%) and settle plate dust samples (27/42, 64%) were similar (P = 0.35) and there was a strong agreement (80%, 4/5) between sample types in the classification of a collection point as positive except at week 18 when ILTV GC was detected in 6/12 tracheal swabs but 0/5 dust samples (Table 1).

3.2. Dust deposition rate varies within the poultry shed and the variation is related to its relative position to exhaust and circulation fans

Dust deposition rate profiles measured in the longitudinal profile study varied significantly between management phases (Fig. 3).

In the pullet rearing phase, the rate of dust deposition rapidly increased from week 1 (4.8 ± 0.3 mg/ 100 m^2day) to week 16 (100.3 ± 3.5) (P < 0.0001) (Fig. 3). Dust deposition rates were higher under the circulating fan (64.7 ± 0.2) compared to the other sampled areas (40.0 ± 1.5 to 46.0 ± 1.5) (P < 0.0001). Dust deposition rate was lower in room 1 (42.7 ± 1.3) than rooms 2 (50.0 ± 1.3) and 3 (51.15 ± 1.34) (P < 0.0001).

In the laying phase, the dust deposition rate also varied through the observational period (P < 0.0001), with highest deposition rates at week 44 (37.5 ± 2.0) and 48 (37.3 ± 2.0) (Fig. 3). The pen door end (18.1 ± 0.5) had lower dust deposition rate than the pop-hole and exhaust fan end of the pen towards which air was drawn (25.9 ± 0.5) (P < 0.0001). However, pens without an exhaust fan had a higher dust deposition rate (24.3 ± 0.4) than pens with an exhaust fan (19.8 ± 0.5) (P < 0.0001).

3.3. There is a strong agreement in the longitudinal profiles of ILTV and MDV GC between pooled scraped and pooled settle plate dust samples

The level of ILTV and MDV GC in pooled samples from settle plates or scraped samples in the longitudinal profile study is shown in Fig. 4.

The ILTV GC levels and percentage of positive samples varied with bird age in both phases (P < 0.001). ILTV GC was first detected at week 10, four weeks after the first vaccination by drinking water. This detection was delayed compared to Experiment 1 in which ILTV GC was detected in dust samples 4 days after bird vaccination via eye-drop. ILTV GC peaked between weeks 12 and 14 with a detection period of 9 weeks (Fig. 4). After moving to the laying shed at 16 weeks of age, ILTV GC was detected for the first three weeks in scraped samples and the first two weeks in settle plate samples. Both sample types were ILTV GC positive at low levels at week 43 (Fig. 4). An additional positive scrape sample was detected at week 22. There was a strong overall agreement (33/36, 92%) between scraped and settle samples on the classification of a sample on a given week as positive or negative.

The MDV GC levels varied with bird age in both phases (P < 0.0001). MDV was first detected at week 1 and peaked at approximately 6 logs/mg at weeks 3–6 followed by a slow decrease until week 26 for both sample types (Fig. 4B). Thereafter to week 50, the MDV GC varied between 3 and 4 logs/mg with some undetectable values (Fig. 4B). Overall, the detection rates for weekly pooled scraped samples (35/37) were similar to weekly pooled settle plates (45/50) (P = 0.42). Likewise, the MDV GC levels in pooled scraped samples (3.7 ± 0.2) were similar to those for pooled settle plate samples (3.6 ± 0.2) (P = 0.85). There was an overall strong agreement (33/37, 89%) between scrape and settle samples on the classification of a collection day as positive or negative.

3.4. Detection rates of ILTV GC in individual dust samples has moderate agreement with pooled dust samples while strong agreement was observed for MDV GC in both sampling strategies

Detection of viral genome in individual settle plates and scraped samples was assessed at specific ages during the longitudinal profile study (summarised in Table 2 for ILTV and Table 3 for MDV) based on the virus detection in pooled samples.

During the pullet raising and laying phases, there was a significant effect of age of birds (weeks 11, 13, 14, 15, 17, 18, 20, 26, 32) on the detection and load of ILTV GC (P < 0.0001). In the pullet raising phase, all settle plate and scraped dust samples were positive for ILTV GC apart from week 11 (Table 2). In the laying phase, the detection rate in individual samples varied from 0% on week 43 to 100% in week 20 for both scraped and settle plate samples (Table 2). Surprisingly, both scraped and settle plate pooled samples were positive on week 43 but negative at week 20. There was an overall moderate agreement between pooled and individual samples on the classification of a sampling day as ILTV DNA positive (5/8 sampling days classified as positive by both pooled and individual settle plate samples and 4/7 for scraped samples).

All of the individual dust samples tested were MDV GC positive in the pullet raising phase (weeks 2, 4, 7, 11, 14) (Table 3). Bird age had no effect on MDV GC levels in dust collected by settle plate or scrape (P > 0.10). The highest log_{10} MDV GC load (5.04 ± 0.11) was at 4 weeks of age with a gradual decline in viral load (Table 3). During the laying phase (weeks 17, 18, 20, 25, 32) all samples were positive until week 20 and 50–88% samples were positive thereafter. Both scraped (8/9) and settle plate (9/18) samples were positive on week 32, in which scraped pooled samples were also positive but the pooled settle plate sample was negative. For MDV, there was a strong agreement

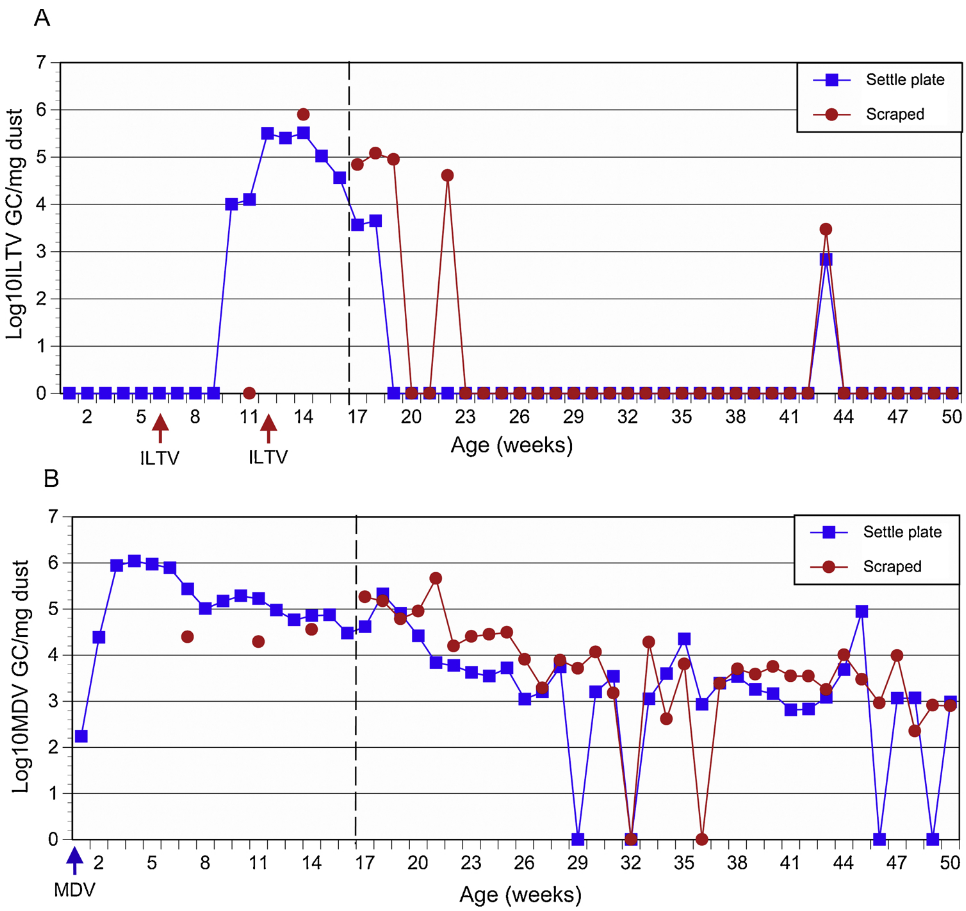

Fig. 4. Least square mean log_{10} GC/mg of dust over time in pooled settle plate samples or pooled scraped dust samples for the longitudinal profiling of ILTV (A) or MDV (B). The interrupted line indicates that birds were relocated from a pullet raising facility to a laying facility after week 16 sampling. Birds were vaccinated against MDV at day old (blue arrow), and against ILTV at weeks 6 (strain A20 in drinking water) and 12 (strain SA2 by eye drop) (red arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Proportion of ILTV positive samples and log_{10} GC/mg (LSM ± SE) of individual dust samples collected by settle plate or scraped from surfaces during phase 1 (pullet raising) and phase 2 (laying period).

Phase	Factors	Settle plate			Scraped		
		N	Positive samples	Log_{10} GC/mg	N	Positive samples	Log_{10} GC/mg
1	Age (weeks)	48	**P < 0.0001**	**P < 0.0001**	12	**P = 0.005**	**P = 0.0005**
	11	12	2/12 (17%)	0.00 ± 0.66^{B}	6	2/6 (33%)	0.72 ± 0.47
	13	12	12/12 (100%)	6.10 ± 0.53^{A}	–	–	–
	14	12	12/12 (100%)	5.64 ± 0.30^{A}	6	6/6 (100%)	5.18 ± 0.47
	15	12	12/12 (100%)	5.09 ± 0.26^{A}	–	–	–
	Room	48	P = 0.88	P=0.30	12	P = 0.69	P=0.71
	1	16	13/16 (81%)	3.59 ± 0.34	4	3/4 (75%)	3.34 ± 0.57
	2	16	12/16 (75%)	4.54 ± 0.43	4	2/4 (50%)	2.82 ± 0.57
	3	16	13/16 (81%)	4.34 ± 0.34	4	3/4 (75%)	2.68 ± 0.57
	Location	48	P = 0.91	P = 0.54		–	–
	Air entry vents	12	10/12 (83%)	3.80 ± 0.28		–	–
	Open area	12	10/12 (83%)	4.57 ± 0.41		–	–
	Circulating fan	12	9/12 (75%)	4.53 ± 0.74		–	–
	Roof exhaust fan	12	9/12 (75%)	3.73 ± 0.81		–	–
2	Age (weeks)	70	**P < 0.0001**	**P < 0.0001**	45	**P < 0.0001**	**P < 0.0001**
	17	–	–	–	9	5/9 (55%)	2.54 ± 0.51^{B}
	18	17	10/18 (55%)	2.04 ± 0.27^{B}	9	5/9 (55%)	1.73 ± 0.51^{BC}
	20	17	17/17 (100%)	4.58 ± 0.28^{A}	9	9/9 (100%)	4.82 ± 0.51^{A}
	26	18	2/18 (11%)	0.36 ± 0.27^{C}	9	1/9 (11%)	0.61 ± 0.51^{BC}
	43	17	0/17	0^{C}	9	0/9	0^{C}
	Exhaust fan	70	P = 0.93	P = 0.80	45	P = 0.94	P = 0.56
	In pen	31	13/31 (41%)	1.71 ± 0.21	20	9/20 (45%)	2.07 ± 0.34
	In next pen	39	16/39 (41%)	1.78 ± 0.18	25	11/25 (44%)	1.80 ± 0.30
	Location	70	P = 0.46	P = 0.28	–	–	–
	Door end	35	16/35 (45%)	1.90 ± 0.19	–	–	–
	Pop-holes/ exhaust fan end	35	13/35 (37%)	1.58 ± 0.20	–	–	–

Different superscripts ($^{A, B, C, D}$) within column for each level of factors indicate significant difference for log_{10} ILTV GC ($P < 0.05$).
- not tested.

Table 3
Proportion of MDV positive samples and log10 GC/mg (LSM ± SE) of individual dust samples collected by settle plate or scraped from surfaces during phase 1 (pullet raising) and phase 2 (laying period).

Phase	Factors	Settle plate			Scraped		
		N	Positive samples	Log_{10} GC/mg	N	Positive samples	Log_{10} GC/mg
1	Ages (weeks)	60	60/60	**P < 0.0001**	18	18/18	P = 0.29
	2	12	12/12 (100%)	4.58 ± 0.10^{D}	–	–	–
	4	12	12/12 (100%)	6.16 ± 0.10^{A}	–	–	–
	7	12	12/12 (100%)	5.51 ± 0.10^{B}	6	6/6 (100%)	5.78 ± 0.10
	11	12	12/12 (100%)	5.05 ± 0.10^{C}	6	6/6 (100%)	5.60 ± 0.10
	14	12	12/12 (100%)	4.46 ± 0.10^{D}	6	6/6 (100%)	5.83 ± 0.10
	Room	60	60/60	P = 0.07	18	18/18	P = 0.71
	1	20	20/20 (100%)	5.29 ± 0.08	6	6/6 (100%)	5.68 ± 0.10
	2	20	20/20 (100%)	5.01 ± 0.08	6	6/6 (100%)	5.80 ± 0.10
	3	20	20/20 (100%)	5.15 ± 0.08	6	6/6 (100%)	5.74 ± 0.10
	Location	60	60/60	P = 0.13	–	–	–
	Air entry vents	15	15/15 (100%)	5.23 ± 0.09	–	–	–
	Open area	15	15/15 (100%)	5.31 ± 0.09	–	–	–
	Circulating fan	15	15/15 (100%)	5.03 ± 0.09	–	–	–
	Roof exhaust fan	15	15/15 (100%)	5.05 ± 0.09	–	–	–
2	Ages (weeks)	71	**P < 0.0001**	**P < 0.0001**	45	P = 0.39	**P < 0.0001**
	17	–	–	–	9	9/9 (100%)	4.85 ± 0.21^{AB}
	18	18	18/18 (100%)	5.22 ± 0.18^{A}	9	9/9 (100%)	5.12 ± 0.21^{A}
	20	17	17/17 (100%)	4.39 ± 0.19^{B}	9	9/9 (100%)	4.98 ± 0.21^{AB}
	25	18	16/18 (88%)	3.10 ± 0.18^{C}	9	9/9 (100%)	3.99 ± 0.21^{B}
	32	18	9/18 (50%)	1.09 ± 0.18^{D}	9	8/9 (88%)	1.44 ± 0.25^{C}
	Exhaust fan	71	P = 0.49	P = 0.64	45	P = 0.36	P = 0.55
	In pen	32	26/32 (81%)	3.49 ± 0.13	20	18/20 (90%)	4.15 ± 0.17
	In next pen	39	34/39 (87%)	3.41 ± 0.12	25	24/25 (96%)	4.01 ± 0.15
	Location	71	P = 0.70	P = 0.80	–	–	–
	Door end	36	31/36 (86%)	3.47 ± 0.13	–	–	–
	Pop-hole / exhaust fan end	25	29/35 (82%)	3.43 ± 0.13	–	–	–

Different superscripts ($^{A, B, C, D}$) within column for each level of factors indicate significant difference for $\log_{10}$ MDV GC ($P < 0.05$).
- not tested.

between pooled and individual samples with 8/9 sampling days classified as positive by both pooled and individual settle plate samples and all 8/8 sampling days classified as positive for both pooled and individual scraped samples.

3.5. Sampling location has no effect in ILTV and MDV GC detection

Sampling location had no significant effect on the ILTV and MDV GC detection in scraped and settle plate dust samples (Tables 2 and 3). For both sample types, there was no difference in the number of positive samples or viral load in different rooms during the pullet rearing phase or between pens with or without an exhaust fan during the laying phase (Tables 2 and 3).

Similarly, location within rooms or pens had no effect in the detection of ILTV and MDV GC (Tables 2 and 3), although there was a significant effect of location on dust deposition rates in settle plates (Fig. 5A, B). During the pullet rearing phase, the dust deposition rate was higher under the circulating fan compared to other sampled areas ($P < 0.001$, Fig. 5). During the laying phase, the dust deposition rates were significantly higher ($P < 0.0001$) in pens with the exhaust fan in a neighbouring pen (24.34 ± 0.47) compared to pens with an exhaust fan in the pen itself (19.84 ± 0.53). It was also higher at the exhaust fan end of pens (25.99 ± 0.50) to which air was drawn, compared to the door end (18.19 ± 0.50).

3.6. There is strong agreement between the detection of ILTV GC and MDV GC in individual scraped and individual settle plate dust samples

The detection of viral genome in individual settle plates and scraped samples collected in the longitudinal profile study is summarised in Fig. 6.

There was no difference in the ILTV GC load in scraped and settle plate sample types during the pullet rearing phase and laying phase (Fig. 6). There was no difference in the number of ILTV GC positive samples in dust samples (P = 0.35) collected during the pullet rearing phase by settle plate (38/48, 79%) or scraped (8/12, 66%). Likewise, there was no difference in the number (P = 0.98) of ILTV GC positive samples between dust samples collected during the laying phase by settle plate (29/70, 41%) or scraped (15/36, 41%). There was 100% agreement in the classification of a collection day (n = 6) as positive or negative by both sampling methods.

There was no difference in the MDV GC load in scraped and settle plate sample types during the pullet raising phase but there was a higher MDV GC load in scraped samples during the laying phase (Fig. 6). All scraped (n = 18) and settle plate (n = 36) individual samples tested for MDV GC during the pullet rearing phase were positive (Table 3). During the laying phase, the proportion of samples positive for MDV GC was higher in scraped samples (35/36, 97%) compared to settle plate samples (60/71) (P = 0.04) although both sample types classified all tested collection days (n = 8) as positive.

4. Discussion

Dust samples were collected from multiple locations and times post vaccination in two layer flocks and tested for ILTV and MDV GC to determine temporal profiles of detection in dust and provide guidelines for dust sample collection. There was a good agreement in the detection of ILTV GC in dust samples collected by settle plates and tracheal swabs of individual birds after eye drop vaccination, although dust samples were ILTV GC negative when detection in individual tracheal swabs were close to the PCR detection limit. In this experiment, ILTV GC was readily detected in dust samples and tracheal swabs as soon as four days post eye-drop vaccination, while ILTV GC was detected in dust samples only four weeks after water vaccination in the longitudinal dust profiling experiment suggesting a poor vaccination take after drinking water vaccination (Coppo et al., 2012; Groves et al., 2019; Robertson and Egerton, 1981; Roy et al., 2015).

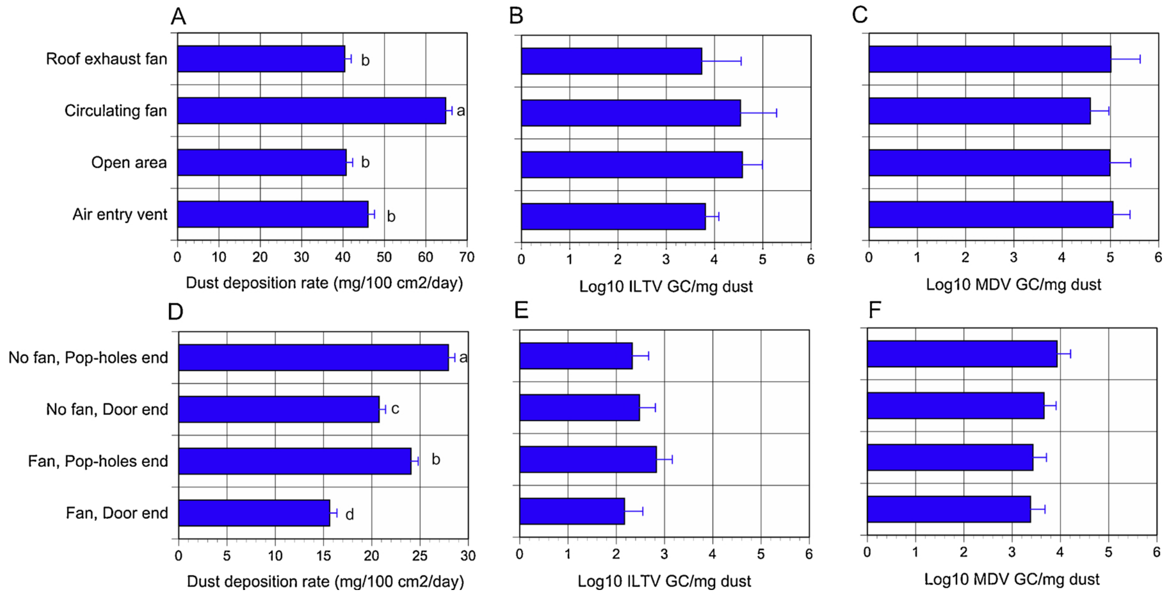

Fig. 5. Dust deposition in settle plates and viral detection in samples collected in different locations of the shed for the MDV and ILTV longitudinal profiling in dust samples. A) Dust deposition, B) ILTV GC levels and C) MDV GC levels in different locations of the rooms during the pullet rearing phase. D) Dust deposition, E) ILTV GC levels and F) MDV GC levels in different locations of the rooms during the laying phase. Different superscripts (a, b, c, d) indicate significantly ($P < 0.05$) different group means.

In the present study, the levels of ILTV GC detected in dust significantly decreased around 8 weeks post-vaccination but ILTV GC was sporadically detected in dust samples at low levels after 10 weeks of vaccination in both experiments, suggesting release of latency during these periods. ILTV is known for causing life-long persistent infection with intermittent release of latency (Bagust et al., 2000).

Detection of the MDV GC at the first week following vaccination is consistent with Baigent et al. (2005) who first detected the Rispens virus in feathers at 7 days post vaccination and with previous experimental (Islam et al., 2013) and field (Ralapanawe et al., 2016) studies in which MDV GC were readily detected in the first week following vaccination. In both of the latter studies high levels of MDV GC were present in the first 8–10 weeks following vaccination, consistent with the present findings, but the decline observed following this period appeared to be greater in the present study than that of Ralapanawe et al. (2016). The higher MDV GC in scraped samples compared to settle plate samples in the later stages of the laying phase, probably reflects the accumulation of old dust in the scraped samples compared to settle plate samples, which capture dust only for the immediate period between plate collections. It may also explain the higher levels of MDV GC observed in dust samples from older birds in the study of Ralapanawe et al. (2016) for which scraped, rather than settle plate samples were used. MDV is highly stable in the environment and can survive for at least 200 days at room temperature (Carrozza et al., 1973). Difference of ILTV GC between sample types was not observed in this study, perhaps due to its shorter shedding period in dust.

In the present study, the location of settle plates had no significant effect on the ILTV and MDV GC load and detection rates despite differences in dust deposition rates between locations, indicating that any location of the shed would be appropriate to collect dust samples. This somewhat contradicts a previous study that suggested that the spatial variation of MDV in dust collected from ledge-like surfaces is higher than the dust collected on fan covers or louvers (Kennedy et al., 2017). In that study, this difference may reflect differences in the degree of cleaning of these different surfaces between batches of chickens as settle plates were not use.

The agreement of MDV and ILTV GC detection rates in individual and pooled samples were high for samples with high GC during the pullet rearing phase but low for ILTV GC during the laying phase in which low shedding of virus was detected in dust samples. In this case, there was a higher chance of positive detection when individual samples were tested. These suggest that pooling samples collected in various locations of the shed may decrease the sensitivity of the test when virus levels are low. Further studies are needed to elucidate the required number of samples to be collected for detection of a positive sample for different virus prevalence.

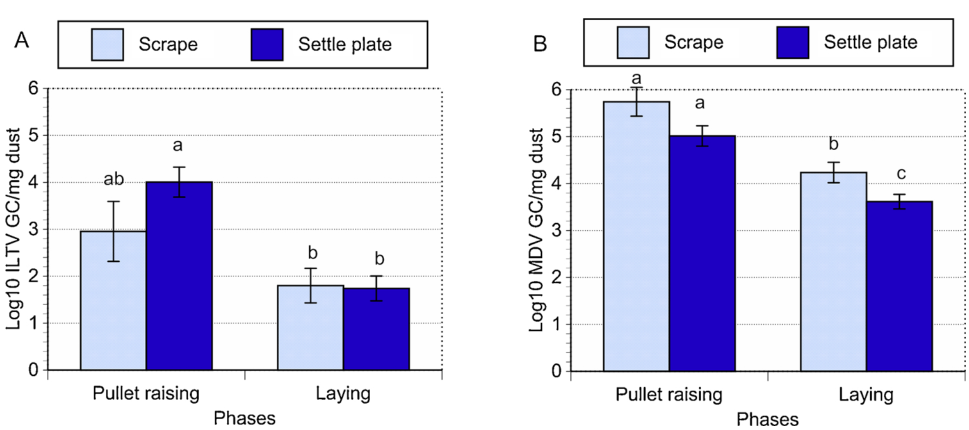

Fig. 6. Log_{10} GC/mg of dust (LSM ± SE) in individual settle plate samples or individual scraped dust samples for ILTV (A) or MDV (B) during the pullet raising (weeks 1–16) and laying (weeks 17–50) phases for the MDV and ILTV longitudinal profiling in dust samples. Individual settle plate samples ($n = 12–18$) and scraped samples ($n = 6–9$) were tested on weeks 11, 14, 18, 20 and 26 for ILTV GC and on weeks 7, 11, 14, 18, 20, 25 and 32 for MDV GC.

Dust deposition rates were sufficient for samples to be collected weekly from the placement of the birds as only 5 mg of dust is required for testing. The dust deposition rate increased rapidly with chicken age as expected as poultry dust is a combination of feathers, litter, feed, and excreta of birds (Fakhrul Islam et al., 2008), which increases as birds become older. The deposition rate is likely to be affected by the airflow in the flock, which is consistent with the findings of this study. Higher dust collection was achieved at the end of pens closest to the exhaust fans as air (and dust) were drawn through the pen to this end.

During the laying phase, the dust deposition rate varied across the weeks of the study possibly because of the structure of the free-range shed, including the opening of pop-holes daily and managing activities such as addition and raking of the litter, and movement of manure. Higher dust deposition rates at the pop hole and exhaust fan end of the pens in this phase is consistent with the direction of airflow towards the exhaust fan end across the birds whereas at the mesh door end the air and dust is being drawn from an empty common area in the shed. As expected, pens without exhaust fans had the higher dust deposition rates, presumably because reduced airflow allowed a higher proportion of the airborne dust to settle out.

In summary, the results show that MDV and ILTV are readily detected from dust samples after vaccination and this may provide a tool to monitor population level responses to vaccine application, although the proportion of adequately vaccinated birds cannot be directly inferred from the detected viral load. Dust samples can be collected in settle plates from the first week age of chicken placement. Dust collected in settle plates are likely to reflect the current level of viral genome load within a population in the dust while scraped samples may reflect the historical accumulation of viral genome in dust. There was no systematic bias in the place of sample collection in the shed and detection of MDV and ILTV. Testing of pooled samples can be used for detecting MDV and ILTV shortly after vaccination when virus load is expected to be high but testing of a single pooled sample may increase the chance of false negatives when virus shedding is expected to be low. Future studies in commercial flocks are necessary to further confirm and expand the present work.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was partially supported by PoultryHub Australia (project numbers 2017-20 and 18-424). We thank Tim Dyall (CSIRO), Andrew Cohen-Barnhouse, and Danielle Smith (UNE) for technical support.

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