Final Report

Project code: 18-424

Prepared by: Priscilla Gerber

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Monitoring of key infectious pathogens using poultry dust for controlling diseases

Milestone 3

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Monitoring of key infectious pathogens using poultry dust for controlling diseases

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Project Summary

Project Title	Monitoring of key infectious pathogens using poultry dust for controlling diseases
Project No.	18-424
Date	Start: 31/07/2018 End: 30/10/2019
Project Leader(s)	Priscilla Gerber
Organisation	University of New England
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Project Aim	The main objective of this project was to provide guidelines for on-farm collection of poultry dust samples for monitoring of live vaccine administration and diseases. It also mapped the live vaccines and microorganisms that can be detected in poultry dust.
Background	Traditional health flock monitoring require invasive sampling of a large number of individual birds which makes it cost-prohibitive and generally underused on a large scale. Population level based monitoring of Marek's disease virus has been used by the poultry industry but there were no guidelines for collection of this sample type and the applicability of this approach to monitoring of other diseases needs investigation.
Research	The profile of live vaccines that can be detected in dust were determined by PCR-testing of dust samples collected weekly on layer and broiler breeder flocks. Proof-of-concept on detection of coccidiosis and necrotic enteritis in dust was obtained from experimentally infected broiler flocks.
Impacts and Outcomes	Availability of new sampling and testing methods will enable a more systematic approach for disease and live vaccine administration monitoring. This project has mapped live vaccines and some economically important pathogens that can be detected in poultry dust. Further research is needed to relate microbial levels in dust and prevalence of positive results in individual birds using reference sampling methods. Dust samples can be collected in settle plates from the first week age of chicken placement. Dust collected in settle plates are preferred and 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. Samples can be collected at any location of the poultry shed. Testing of pooled samples can be used for detecting microorganisms shortly after vaccination when microbial load is high but testing of a single pooled sample may increase the chance of false negatives when microbial load is expected to be low. Overall, this approach provides for an inexpensive, practical, and welfare friendly method to monitor chicken flocks.
Publications	1. Nguyen T, Ahaduzzaman M, Campbell D, Groves P, Walkden-Brown S, Gerber P. Spatial and temporal variation of Marek's disease virus and infectious laryngotracheitis virus genome in dust samples following live vaccination of layer flocks. Vet Microbiol. 2019:108393. 2. Ahaduzzaman M, Gerber P, Keerqin C, Musigwa S, Morgan N, Kheravii S, Wu S, Walkden-Brown S. Detection and quantification of Clostridium perfringens and netB toxin gene from poultry dust using real-time PCR. Proceedings of the 21st World Veterinary Poultry Association Congress, p. 161, Bangkok, Sept 16-20.

Executive Summary

A test based on molecular detection of Marek's disease virus (MDV) in poultry dust samples has been proved an effective tool to monitor the need for MDV vaccination and to assess the efficacy of vaccination where it is used. Dust collection is non-invasive and so can be easily carried by farm staff, the sample material is dry so the genome is relatively stable and do not require chilling during transportation to the diagnostic laboratory. As a dust sample represents a population, a small number of samples is required to obtain population level information, making it cost effective. The approach has applicability beyond MDV and data using dust samples to monitor infectious laryngotracheitis virus vaccine administration has been promising.

The overall aim of this project was to extend this principle of population based molecular for monitoring vaccine administration and to improve control of economically important infectious diseases. It investigated the profile of detection of several live vaccines and important gut disruptors such as coccidia and necrotic enteritis in poultry dust and provided guidelines for collection of this sample type on commercial farms.

To achieve the aims above, dust samples were collected from several locations of experimental and commercial layer, broiler breeder, and broiler poultry houses and tested by PCR targeting live vaccines and specific pathogens. Selected samples from each operation type were selected or high-throughput sequencing analysis for a comprehensive overview on the microbial populations that can be detected in dust.

This project mapped live vaccines and some economically important pathogens that can be detected in dust, which is the first step in the validation of dust molecular based tests for specific pathogens and live vaccines. This approach is inexpensive, practical, and a welfare friendly method to monitor chicken flock health and live vaccine administration.

The key findings of this project were:

- All eight live vaccines tested in this study could be detected by PCR in dust samples after vaccination indicating that this method offers promise to monitor administration of a wide range of live vaccines
- Monitoring of success of vaccine administration for live vaccines will require further testing of individual birds to correlate with findings in dust.
- Nucleic acid of *C. perfringens*, *netB* toxin (necrotic enteritis causative agents), and coccidia species from experimentally challenged flocks were readily detected in dust by PCR. These findings will be further explored in commercial flocks.
- The differences in microbial communities in dust samples collected from different production systems and age groups may provide a biomarker to evaluate gut health in commercial flocks. These findings will be further explored in commercial flocks.
- Settled dust collected weekly reflects the current level of genome load while samples scraped from surfaces may reflect historical accumulation and lead to false-positives for detection of microorganisms that persist in the environment such as coccidia.
- Settle plate dust collected at any place in the shed is representative of the population for detection of nucleic acids which means that there is no "best" place in a shed to collect dust samples.
- Testing of pooled samples can be used for detecting live vaccines shortly after vaccination when microbial load is high but testing of a single pooled sample may increase the chance of false negatives when microbial shedding is expected to be low.

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1 Introduction

Currently, farm-level monitoring of diseases and vaccine administration success are challenged by the larger population size common to contemporary poultry productions sites. Environmental and composite samples offer non-invasive monitoring options at the farm level to detect the presence and load of pathogens using sensitive and specific molecular assays such as PCR. This strategy reduces considerably the number of samples necessary to be tested to achieve representativeness of a population and are stress free to staff and animals (Mosher et al., 2017; Murai et al., 2014; Rotolo et al., 2017). Traditional disease monitoring and diagnosis based on routine collection and testing of statistically meaningful numbers of individual animals becomes cost-prohibitive in large populations. The general need for a new surveillance approach to assure animal health, welfare and producer profitability reflects the requirement to adapt to the current production practices and to the availability of new sampling and testing methods.

A test based on molecular detection of Marek's disease virus (MDV) in poultry dust samples has been successfully developed at University of New England (UNE) and implemented by industry in Australia (Walkden-Brown et al., 2013) and in the USA (Kennedy et al., 2017). This approach consists in collecting dust from representative sheds and assaying for detection of MDV by PCR. Dust samples can be collected by a single person without stress to birds or people. Another advantage of this method is that this sample type is dry and stable at room temperature and does not require a cold chain for transport. Under commercial conditions, it has been proved an effective tool to monitor the need for MDV vaccination and to assess the efficacy of vaccination where it is used. The approach has applicability beyond MDV and data using dust samples to monitor infectious laryngotracheitis virus (ILTV) vaccine administration has been promising (Ahaduzzaman et al., 2019).

The overall aim of this project is to extend this principle of population based molecular monitoring of vaccination success and to improve control of economically important infectious diseases. It investigated the profile of detection of several live vaccines and important gut disruptors such as coccidia and necrotic enteritis in poultry dust. It has also provided guidelines for collection of this sample type on-farm.

2 Objectives

The main goals of this project were to optimise dust sampling collection on-farm and sample processing in the laboratory, and to assess which live vaccines and microorganisms can be detected from dust.

The original specific aims of this project were to:

1. Determine which pathogens and live vaccines can be detected in poultry dust.
2. Provide optimised dust collection guidelines.
3. Optimise high-throughput automated extraction methods for dust.
4. Determine the dust profile in different operation types.

3 General Methods

3.1 Sample collection

Settle plate dust. Dust samples were collected from 4-6 settle plates with a surface area of 520 cm^2 suspended at a height of approximately 1.4 m (**Figure 1**). Accumulated dust was scoped into individual zip lock bags on a weekly basis, weighed and a pooled sample was created using equal amounts of individual samples. Sample were stored at -20°C until further analysis.

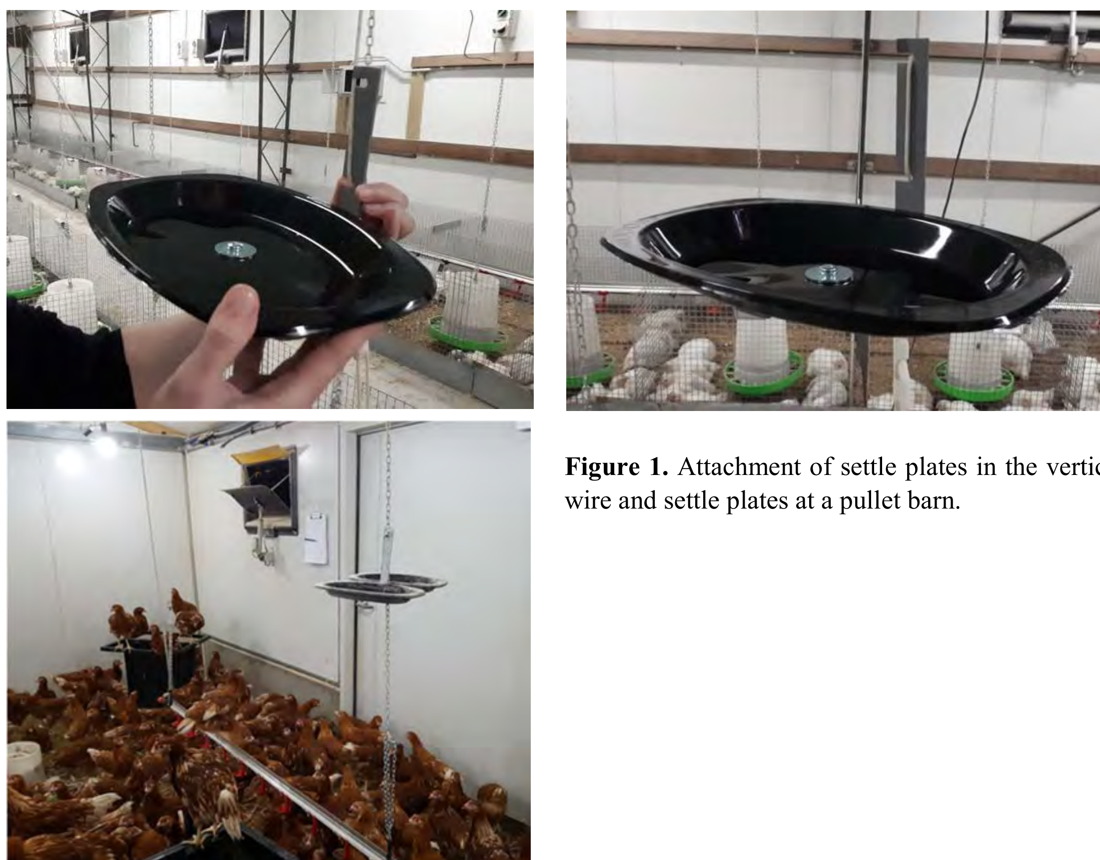

Figure 1. Attachment of settle plates in the vertical wire and settle plates at a pullet barn.

Scrape dust samples. Accumulated dust was scraped from surfaces in a barn and collected into zip lock bags. Samples were stored at -20°C until further analysis.

Tracheal swabs. Tracheal swabs were collected using a flocked swab (FLOQSwab 502CS01, Copan, supplied by Interpath Services, Melbourne Australia) and stored on ice until shipped to the laboratory. Samples were stored at -20°C until further analysis.

3.2 Nucleic acid extraction and PCR

Tracheal swabs were placed in 0.8 ml of sterile phosphate buffered solution and vortexed for 10 sec before nucleic acid 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 and extracts were (Roy et al., 2015). RNA was extracted from 200 µl of tracheal swab wash or approximately 5 mg of dust samples using the Bioline ISOLATE II RNA kit.

Except for the SYBR Green-based PCR targeting the *netB* toxin gene, all PCR assays described below are TaqMan probe-based. DNA extracts were tested for infectious laryngotracheitis (ILTV) (Callison et al., 2007) and Marek's disease virus (MDV) Rispens strain (Baigent et al., 2016) individually, and in a triplex reaction for fowl adenovirus (FAdV) (Gunes et al., 2012), *Mycoplasma synoviae*, and *M. gallisepticum* (Fraga et al., 2013). *Eimeria* species differentiation was carried out in a triplex PCR for *E. acervulina, E. brunetti,* and *E. maxima* (Vrba et al., 2010). *Clostridium perfringens* assay was performed as previously described (Wise and Siragusa, 2005). The *netB* toxin PCR was designed by A/Professor Shubiao Wu, UNE (Wu, unpublished) (netBNEF 5'-CCGCTTCACATAAAGGTTGG-3' and netBNER 5'-TCAGGCCATTTCATTTTTCC-3'). RNA extracts were tested for infectious bronchitis virus (IBV) (Callison et al., 2006), Newcastle disease virus (NDV) (Wise et al., 2004), and infectious bursal disease virus (IBDV) (Jayasundara et al., 2017) in a triplex reaction.

Results were reported in $\log_{10}$ genomic copies (GC) per milligram of dust or per tracheal swab reaction for ILTV, MDV, and in $\log_{10}$ GC/mg dust for *C. perfringens* and *Eimeria* spp.; and in pathogen concentration expressed as cycle threshold (Ct) of the sample minus a maximum Ct of 40 for IBV, NDV, IBDV, FAdV, *M. synoviae*, *M. gallisepticum* and *netB* toxin.

4 Detection of live vaccines in layer and broiler breeder flocks

This study aimed to determine the long-term pattern of detection of live vaccines in dust following vaccination. It also aimed to investigate the influence of the dust collection method (scraped from a surface or collected in a settle plate) on the detection of selected vaccines (MDV and ILTV). Some of the results presented here have been published (Nguyen et al., 2019).

To achieve this, dust samples were collected weekly from four or more settle plates per shed from two experimental layer flocks and five commercial flocks from week 1 up to week 50 after chick placement. Vaccination schedule and procedures were recorded for all farms. Subsets of the samples collected were tested for ILTV, MDV, IBV, NDV, IBDV, FAdV, *M. synoviae*, and *M. gallisepticum.*

4.1 Experimental flocks

4.1.1 Profile of detection of ILTV DNA in dust and tracheal swabs

The first experiment aimed to profile ILTV GC content in dust and tracheal swabs following vaccination and compare the sensitivity of detection of ILTV genome by the two methods as a proof of concept. This experiment 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 at the Zootechny Research Facility (Austral, NSW, University of Sydney Animal Ethics Committee approval no. 2017/1207). Each pen used for this study held 34 birds.

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 with strain SA2 (Poulvac Laryngo SA2, Zoetis, Australia) by eye-drop 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 at weeks 8.5, 9, 9.5, 12, 14, 15, 16, 18 and 30.

ILTV GC detection in dust samples and tracheal swabs has similar profile over a period of 10 weeks post-vaccination

Results are summarised in **Figure 2**. Overall, ILTV GC detection rates in tracheal swabs (48/66, 72%) and settle plate dust samples (17/22, 77%) were similar (P=0.35) and there was

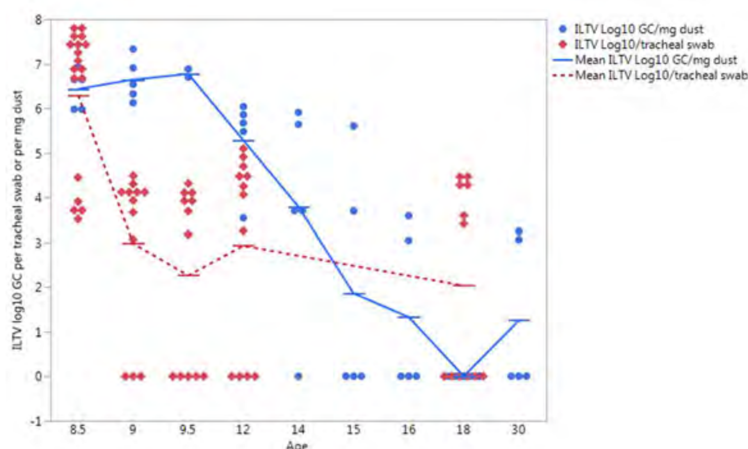

Figure 2. Experimental Layer Flock 1: ILTV DNA detection in trachea and dust. ILTV log_{10} GC over time in milligram of settle plate dust samples (blue circles) or per tracheal swabs (red rhombus) in individual samples. The blue solid line indicates the mean ILTV log_{10} GC/mg of dust and the red interrupted line indicates the mean ILTV log_{10} GC/tracheal swab. Birds were vaccinated with ILTV strain SA2 by eye-drop at 8 weeks of age.

a strong agreement (80%, 4/5) between sample types in the classification of a collection point as positive except on week 18, ILTV GC was detected in 6/12 tracheal swabs but in 0/5 dust samples. Location of dust sample collection had no effect in ILTV GC detection (P=0.46).

4.1.2 Longitudinal profile of ILTV and MDV DNA detection in dust samples

This experiment aimed to profile ILTV and MDV GC content in dust following vaccination and to evaluate methodologies for dust sample collection. The experiment was conducted at research facilities at the University of New England (University of New England Animal Ethics Committee AEC17-092) (**Figure 3**) and used 1700 Hy-Line Brown chickens from day old to 50 weeks of age.

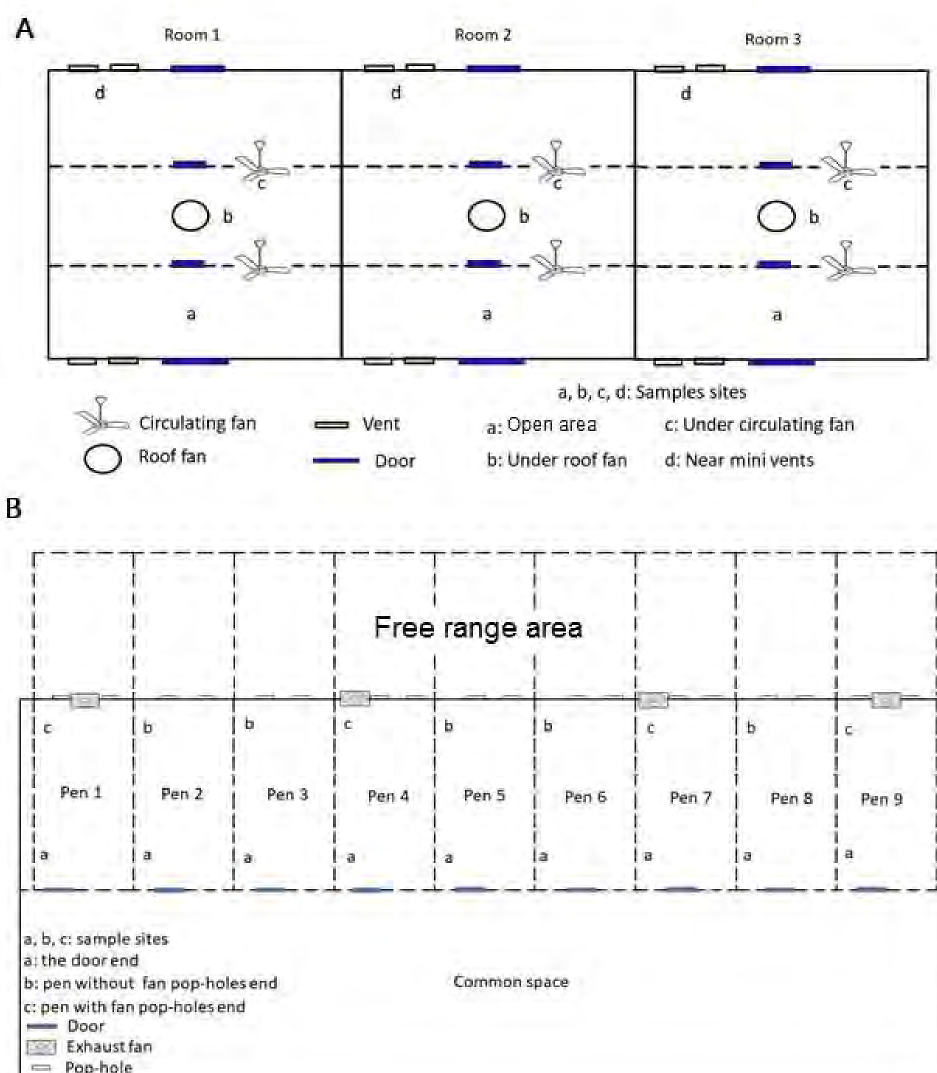

Figure 3. Experimental Layer Flock 2 facilities and location of sample collection.
Facilities and sample collection location in the shed during (A) phase 1 (0 to 16 weeks), and (B) phase 2 (17 to 50 weeks) for the MDV and ILTV longitudinal profiling in dust samples. In phase 1, birds were allocated to 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.

The experiment was divided into a pullet raising phase (phase 1, from day-old to 16 weeks of age at Kirby Poultry facility) and a laying phase (phase 2, from 17 to 50 weeks of age at

Laureldale Poultry facility). Day-old chicks were vaccinated against MDV-1 strain Rispens CVI988 (Vaxsafe RIS, Bioproperties) by subcutaneous injection at the hatchery and 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 (Poulvac Laryngo SA2) by eye drop at 12 weeks of age.

In phase 1, chickens were housed in 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. In phase 2, chickens were maintained in nine wire mesh and shade cloth sided pens sharing the same air space. Each pen had dimensions of 3.6 × 4.8 m and four of nine pens had an exhaust fan on the wall. Dust samples were collected weekly from 4-9 settle plates and by scraping dust from walls and horizontal surfaces at weeks 8.5, 9, 9.5, 12, 14, 15, 16, 18 and 30. One scraped and one settle plate pooled sample were created weekly by mixing individual samples. Dust samples were tested for ILTV and for MDV GC.

Longitudinal profiles of ILTV and MDV GC on pooled scraped and pooled settle plate dust samples

ILTV genomic copies (GC) were detected 4 weeks post water vaccination, peaked at weeks 12─14 and became mostly undetectable after week 18. MDV was detected on week 1, peaked at week 3 and was detectable at high levels until week 50. There was no difference between settle plate and scraped samples in ILTV GC load but higher MDV GC were found in scraped samples (**Figure 4**). 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.

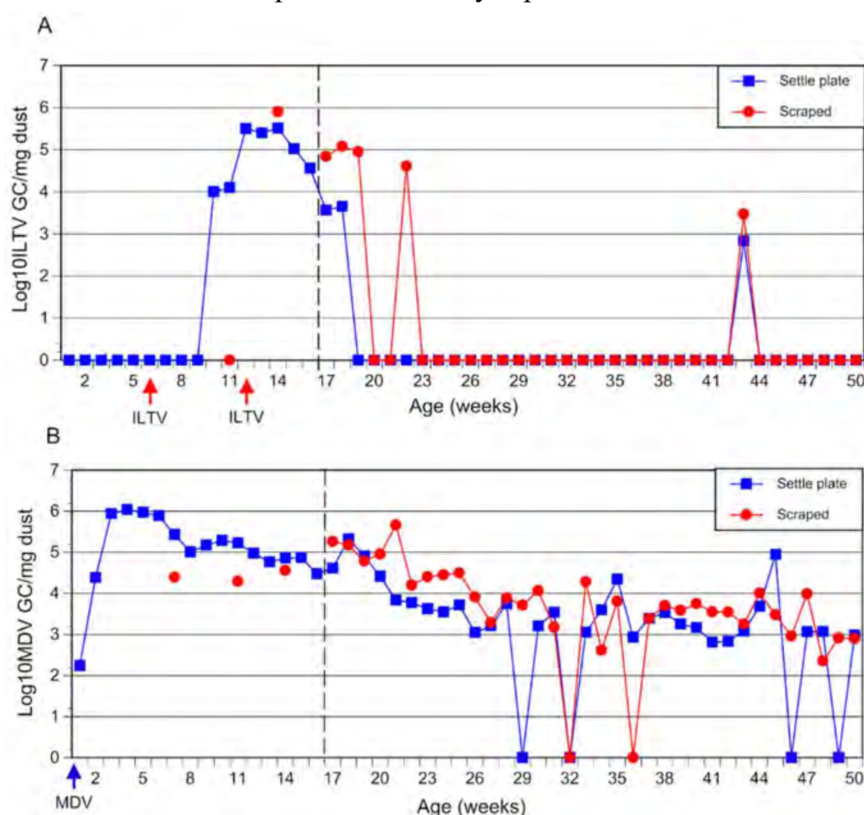

Figure 4. Experimental Layer Flock 2 ILTV and MDV profile in dust.
Log10 GC/mg of dust (LSM±SE) over time in pooled settle plate samples or pooled scraped dust samples for ILTV (A) MDV (B). The interrupted line indicates that birds were relocated from a pullet raising facility (weeks 1 to 16) to a laying facility (weeks 17 to 50). Birds were vaccinated against MDV at day old (blue arrow), and against ILTV at weeks 6 and 12 (red arrows).

Detection rates between pooled and individual samples varies with viral load

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). For MDV, there was a strong agreement 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. Pooled dust samples can be used to assess success of live vaccination when virus load is expected to be high e.g., in the following weeks after live vaccination or for viruses such as MDV that are shed at high rates but may decrease the chance of positive detection for viruses that are shed at lower levels such as ILTV.

Sampling location has no effect in ILTV and MDV GC detection

There was no difference in the detection rates of MDV and ILTV collected from settle plates in different locations of the shed, although there was a significant effect of location on dust deposition rates in settle plates (**Figure 5**). 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.

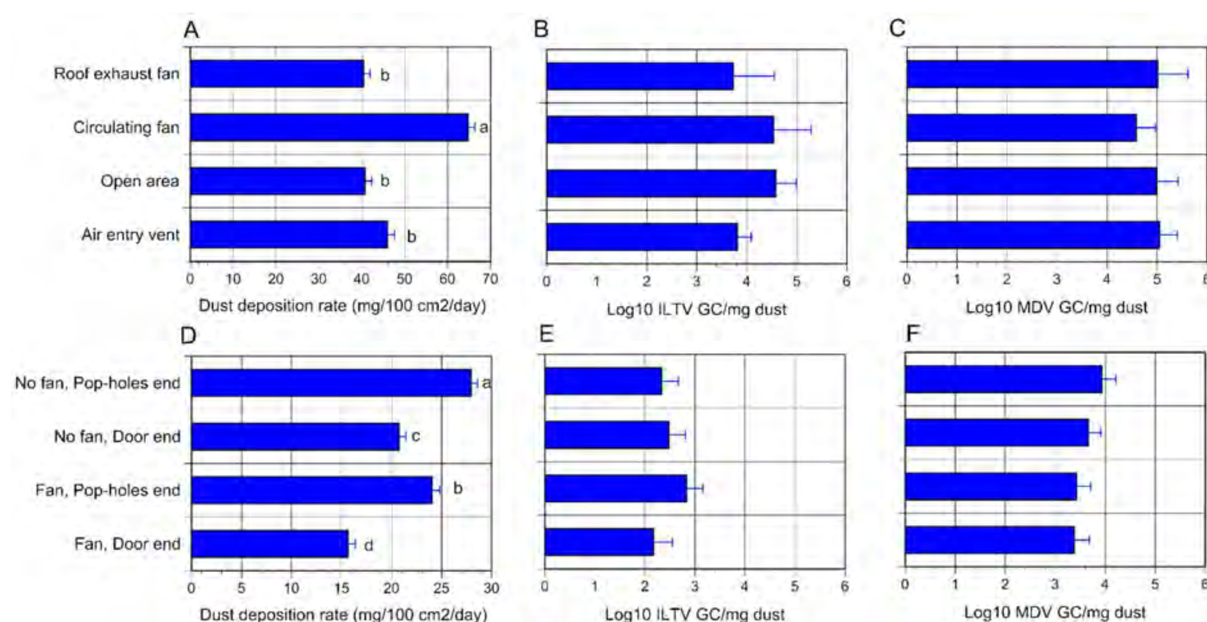

Figure 5. Experimental Layer Flock 2. Dust deposition in settle plates and viral detection in samples collected in different locations of the shed for the MDV and ILTV longitudinal profiling in dust samples. A) Dust deposition, B) ILTV GC levels and C) MDV GC levels in different locations of the rooms 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.

There is strong agreement in 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 **Figure 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. 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. 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.

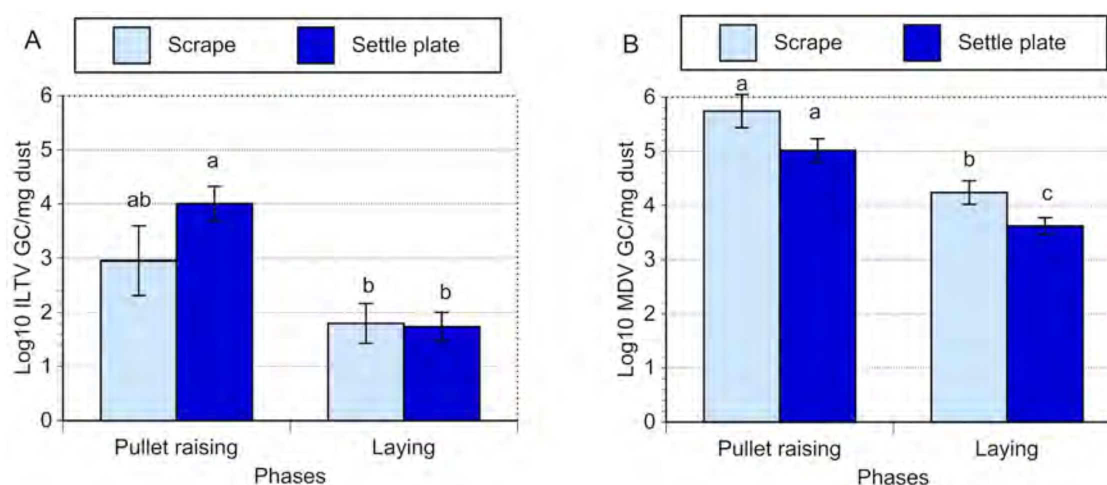

Figure 6. Experimental Layer Flock 2. Log10 GC/mg of dust (LSM±SE) in individual settle plate samples or individual scraped dust samples for ILTV (A) or MDV (B).
Pullet raising was from weeks 1 to 16 and laying phase from weeks 17 to 50. Individual settle plate samples (n = 12-18) and scraped samples (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.

Summary

There was no difference in the detection rates of ILTV and MDV collected from settle plates in different locations of the shed. 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.

4.2 Commercial flocks

Dust samples were weekly collected by settle plates placed in four locations from a single shed on three broiler breeder (Farms 1-3) and two layer (Farms 4 and 5) commercial farms (**Table 1**) from chick placement up to 25 weeks of age. The vaccination schedule was obtained for each farm. For each shed, a weekly pooled dust sample was tested by (RT)-PCR for IBV, NDV, IBDV, FAdV, ILTV, *M. synoviae*, and *M. gallisepticum*.

Vaccination schedule in each flock and the detection of nucleic acid live vaccine microorganisms in dust are summarised in **Figure 7**. Genomic material of all live vaccines could be detected in dust samples. For most farms and vaccines, the nucleic acid of the live vaccine organisms could be detected in dust between 1-2 weeks before or after the reported vaccine administration.

Table 1. Details of the commercial chicken houses used in the live vaccines detection in dust

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Operation type	Broiler breeder	Broiler breeder	Broiler breeder	Layer (free range)	Layer (cage)
Total n. houses on farm	3	6	5	4	4
Water source	Bore	Bore	Bore	Bore	Town water
Water sanitised before reaching the shed	Yes (chlorine)	No	Yes (chlorine)	No	Yes
Stabiliser for water vaccination	De-chlor and/or skim milk powder	Skim milk powder	De-chlor and/or skim milk powder	Vac-Pac Plus	Vac-Pac Plus
Studied flock					
Floor space	1440 m^2	N/A	1890 m^2	288 m^2	2175 m^2
Number of birds placed	11,920	N/A	15,520	3036	35,826
Shed ventilation	Tunnel, solid sides	Tunnel, curtain sides	Tunnel, solid sides	Conventional (natural ventilation)	Tunnel, solid sides
Litter type	Rice hulls	Rice hulls	Rice hulls	Sawdust	Multicube processed hay

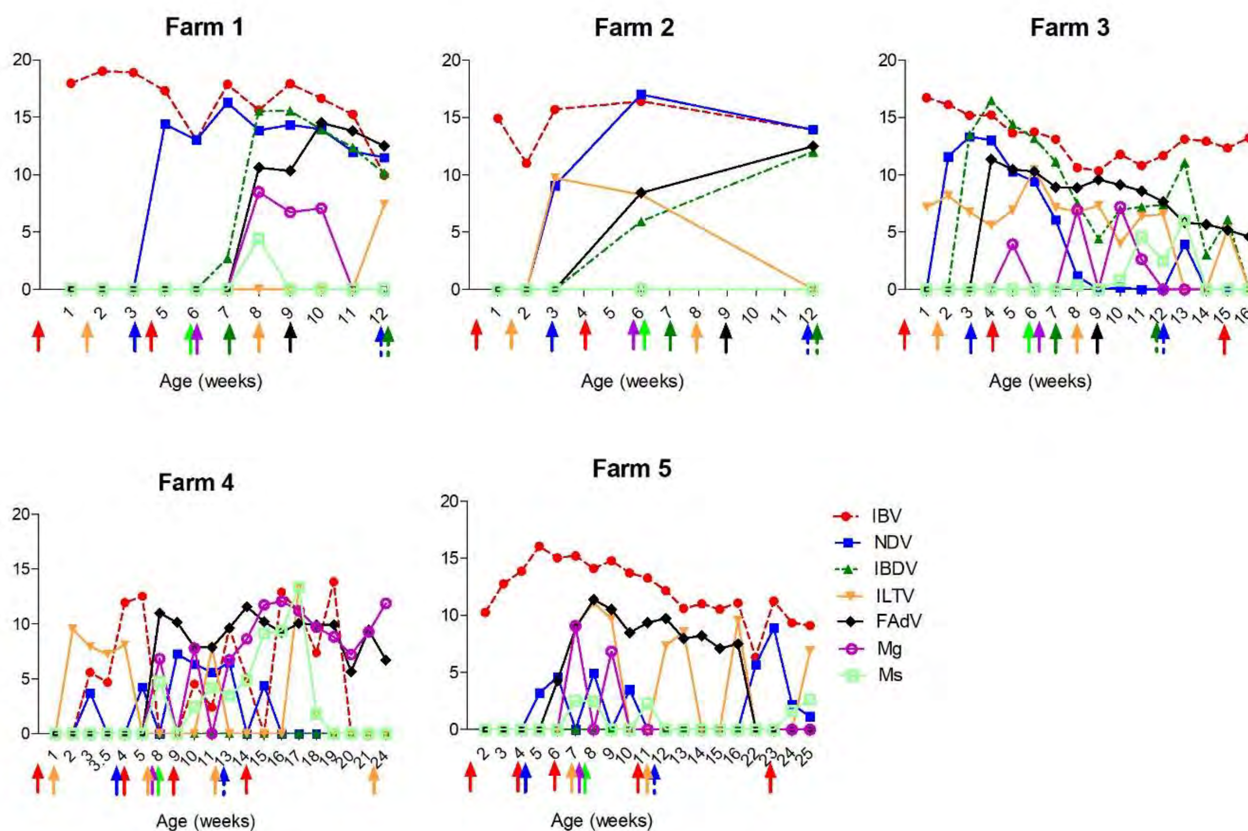

Figure 7. Levels of microorganisms (cycle threshold [Ct] - 40) over weeks post chick placement in pooled settle plate samples for IBV, NDV, IBDV, ILTV, FADV, *M. synoviae* (Ms) and *M. gallisepticum* (Mg). Full arrows are colour coded to indicate administration of live vaccines. Dashed arrows indicate vaccination of inactivated vaccines.

IBV. For all flocks, the levels of IBV RNA were high starting from the first week post-placement and remained high for the duration of the observation period, except for Farm 4. In this farm, the first IBV RNA detection in dust was on week 3, and the detection was intermittent afterwards, waning off after 3-4 weeks after each re-vaccination.

NDV. In most farms, NDV RNA was detected on the same week of vaccination (Farms 1, 2) or the week following vaccination (Farms 4, 5). In Farm 3, it was detected a week before the reported vaccination which suggests an error in the reporting of vaccine administration for that farm.

IBDV. Only the broiler breeder farms (Farms 1-3) vaccinated against IBDV and all samples from the layer farms were negative throughout the observation period. IBDV RNA could be detected on Farm 1 starting at the week of vaccination (week 7), and prior to vaccination on Farm 2 (week 6) and Farm 3 (week 2). Those inaccuracies may be due to an error in the recording of vaccine administration or in the identification of the sample. Alternatively, it may indicate incursion of wild-type viruses. The RT-PCR used does not distinguish between vaccine and wild-type strains.

ILTV. ILTV DNA could be detected on the same week of vaccination in all farms, except for Farm 1 in which ILTV DNA was first detected on week 12, indicating a failure in the vaccine administration (Ahaduzzaman et al., 2019; Groves et al., 2019). ILTV vaccine in Farms 1-3 was administered via drinking water and success in vaccine administration using this method has been reported to be low for ILTV (Groves et al., 2019). ILTV DNA was consistently detected on Farm 3 during the observation period and intermittently on Farms 2, 4 and 5.

FAdV. Only the broiler breeder operations (Farms 1-3) vaccinated against FAdV at week 9, however FAdV DNA was detected in dust samples from all farms, with first detection of the FAdV DNA on week 4 on Farm 3, and between weeks 6 to 8 on the other farms. There was a tendency of a decline on the load of FAdV over the observation time.

M. gallisepticum and *M. synoviae.* Both live vaccines had an intermittent detection from 1-2 weeks after vaccination, except on Farm 3 in which high levels of *M. gallisepticum* and *M. synoviae* DNA were detected for several weeks after vaccination.

Summary

The assessment of live vaccine nucleic acid in dust samples offer promise as a practical method of estimating successful vaccine administration. The usefulness of this method needs further investigation by correlating the levels of nucleic acid in dust to the vaccine uptake in individual birds.

5 Detection of selected pathogens in broiler chicken flocks

Clostridium perfringens is the causative agent of necrotic enteritis (NE) in chickens and the *netB* toxin is responsible for the disease. Coccidiosis, caused by *Eimeria* spp. is known to be a factor that predisposes occurrence of necrotic enteritis in the field.

The objectives of this study were 1) to provide proof-of-concept of detection of *C. perfringens* and selected *Eimeria* species in dust samples of experimentally inoculated broiler flocks, and 2) to evaluate the detection rates of *C. perfringens, Eimeria* species and *Mycoplasma synoviae* and *M. gallisepticum* in dust samples collected at the end of batch of commercial broiler flocks submitted to the Birling Avian Laboratories for routine monitoring of MDV.

5.1 Experimental flocks – proof of concept for detection of necrotic enteritis and coccidia

As a proof of concept of the suitability of using dust samples for monitoring *C. perfringens, netB* toxin gen, and *Eimeria* spp, dust samples were collected twice a week from broiler flocks experimentally inoculated with *Eimeria* spp and *C. perfringens* to produce subclinical or clinical necrotic enteritis (NE). Dust samples were collected weekly from an unchallenged flock and served as control. From all flocks, dust samples were convenience samples on ongoing trials carried out at UNE and no ethics approval was sought for the dust collection.

Sample collection

In both NE subclinical and clinical studies, 800 Ross-308 broiler chickens were housed in a single shed at Ring Road Poultry Facility at UNE. Birds were experimentally challenged with *E. acervulina, E. brunetti*, and *E. maxima* at day 9 and with pathogenic *C. perfringens* (expressing the *netB* toxin gene) at day 14 of age according to previously described challenge models (Wu et al., 2010). Dust samples were collected weekly from 3 locations in the shed.

In the unchallenged control study, 702 Ross-308 broiler chickens were housed in three sheds at Kirby Poultry Facility at UNE. Three dust samples per shed were collected weekly. Individual and pooled dust sample DNA extracts were tested by PCR for *E. acervulina, E. brunetti, and E. maxima* and *C. perfringens* and *netB* toxin gene.

Eimeria species nucleic acids were not detected in dust of unchallenged birds

The results on the detection of *Eimeria* spp. in dust are summarised on **Figure 8**. *Eimeria* spp. GC was only detected in samples collected after challenge, while none of the samples from the unchallenged flock was positive for *Eimeria* spp. Overall, in the *Eimeria* spp. challenged groups, 71/84 (84.52%) of settle dust samples were positive for *E. acervulina*, 48/84 (57.14%) were positive for *E. brunetti,* and 48/84 (57.14%) were positive for *E. maxima*.

There was no difference on the *E. acervulina* GC between groups with subclinical or clinical necrotic enteritis but the number of positive samples was higher for the group with clinical necrotic enteritis (P<0.001). There was no difference on the overall detection rates and load of *E. brunetti* and *E. maxima* in the challenged groups.

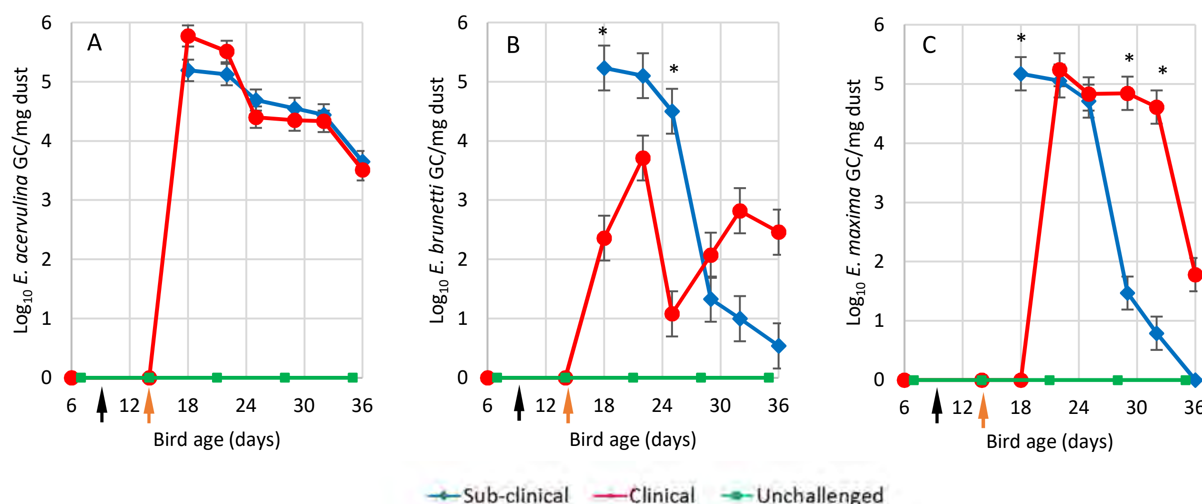

Figure 8. Genomic copies/mg of dust (log_{10}, LSM ± SEM) of A) *E. acervulina*, B) *E. brunetti* C), and *E. maxima* from samples collected from experimental broiler flocks.
Birds in the necrotic enteritis subclinical and clinical groups were challenged at 9 days of age with pathogenic strains of *E. acervulina, E. brunetti* and *E. maxima* (black arrow) and with *C. perfringens* (orange arrow) at 14 days of age. Birds in the unchallenged group did not receive *Eimeria* spp. nor *C. perfringens*. Asterisks indicate differences (p<0.05) on the *Eimeria* load between the necrotic enteritis subclinical and clinical groups.

Clostridium perfringens and netB toxin DNA were detected in challenged and unchallenged flocks

The results are summarised in

Figure 9. The bird age influenced the *C. perfringens* and netB DNA detection patterns in dust (P<0.001). In challenged groups, high levels of *C. perfringens* and *netB* DNA were detected in dust after challenge followed by a gradual decline until the end of the study at day 36. In the unchallenged group, low levels of *C. perfringens* DNA were detected on days 7 and 14, while dust samples collected on days 21 to 35 were negative.

In challenged birds, the highest *C. perfringens* load in dust was at day 18 (5.8-6.9 log_{10} GC/mg, 4 days post challenge), and the lowest (4.7-5 log_{10} GC/mg) at day 36. Interestingly, the *C. perfringens* load in dust was higher (P<0.0001) in the NE-subclinical group (6.42±0.16 log_{10} GC/mg) compared to the NE-clinical group (5.10 ±0.13 log_{10} GC/mg). In unchallenged birds, moderate to low levels of *C. perfringens* DNA (4-2.4 log_{10} GC/mg) were detected until day 14 and remained undetectable afterwards.

Surprisingly, the overall *netB* toxin load in dust from the NE-clinical group (25.88±1.43 40-Ct/reaction) was similar to the unchallenged group (24.54±1.70 40-Ct) and both were higher than the NE-subclinical group (13.75±1.75 40-Ct) (P<0.0001). Dust samples from the unchallenged group were positive for *netB* toxin DNA at moderate to low levels throughout the study period, despite being negative for *C. perfringens* DNA from days 21 to 36. For the NE-subclinical group, while 100% samples were *C. perfringens* DNA positive, only 41.6% were positive for *netB* toxin DNA.

Effect of sampling location in the detection of Eimeria and Clostridium DNA

The location of the settle plate within a shed had no effect on the number of positive samples or DNA load for any of the *Eimeria* species and *C. perfringens/netB* toxin in any of the studies. The data is summarised on **Figure 10**.

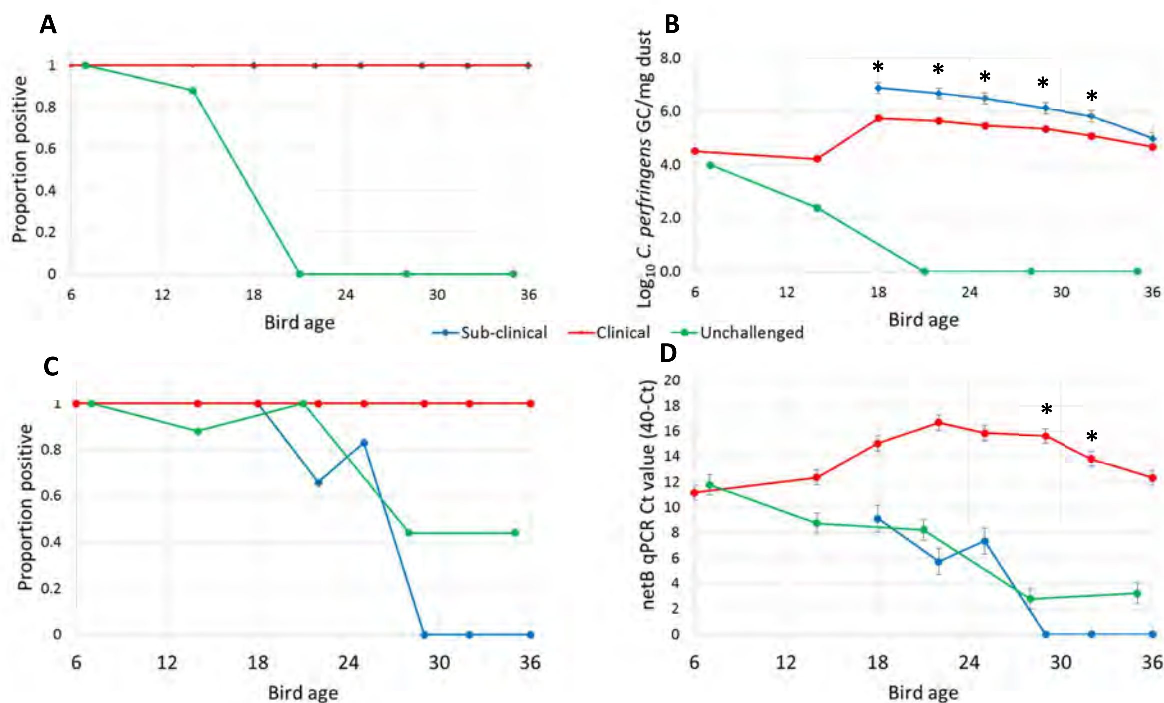

Figure 9. Proportion of DNA-positive dust samples for A) *C. perfringens* and C) *netB* toxin. B) *C. perfringens* $\log_{10}$ genomic copies/mg dust (LSM ± SEM) and D) qPCR Ct value (40-Ct).
Dust samples were collected using settle plates from experimental broiler flocks. Birds in the necrotic enteritis subclinical and clinical groups were challenged at 9 days of age with *E. acervulina, E. brunetti* and *E. maxima* and with *C. perfringens* at 14 days of age. Birds in the unchallenged group did not receive *Eimeria* spp. nor *C.*

perfringens. Asterisks indicate differences ($p<0.05$) on the *Eimeria* load between the necrotic enteritis sub-clinical and clinical groups.

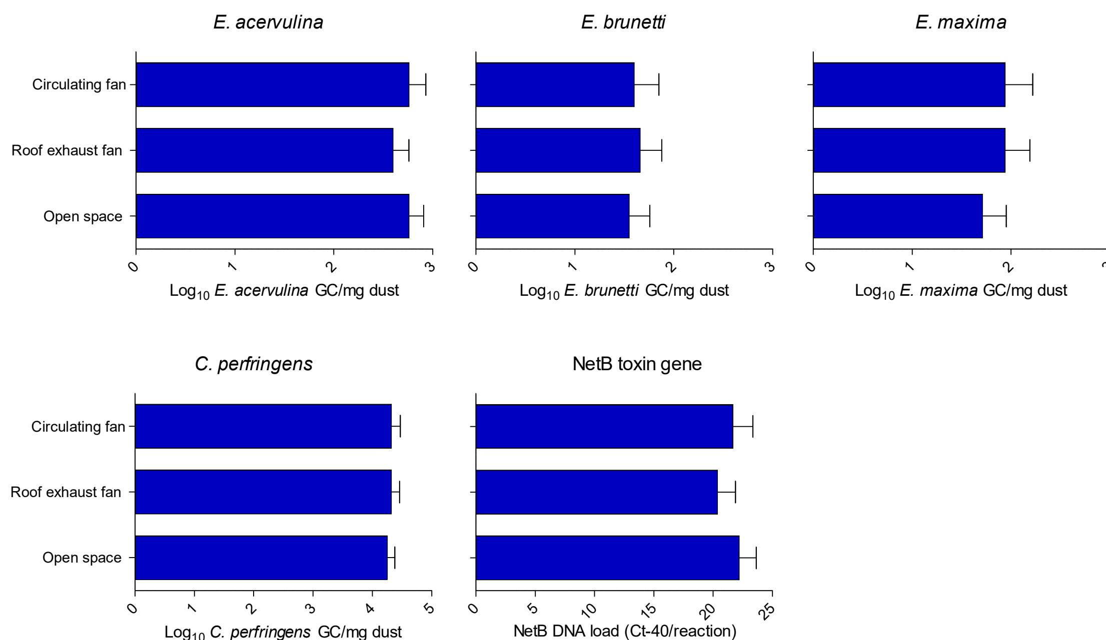

Figure 10. Microorganism levels in samples collected in different locations of a shed for *E. acervulina, E. brunetti, E. maxima, C. perfringens* and *netB* toxin gene.
Birds were challenged at day 9 with *E. acervulina, E. brunetti and E. maxima* and with *C. perfringens* containing *netB* toxin plasmid at 14 days of age. There was no difference in detection on the different locations of the shed for any of the tested targets.

Effect of dust collection method in the detection of Eimeria and Clostridium

There was no difference in the microbial load in scraped, individual settle plate and pooled settle plate samples for *C. perfringens*/*netB* and any of the tested *Eimeria* species. However, a higher number of scraped samples were positive for *E. maxima* (P=0.04) compared to individual settle plate samples. This discrepancy was mostly due to the scraped samples being positive for all tested *Eimeria* species prior to the *Eimeria* challenge in the NE-subclinical group **Figure 11**.

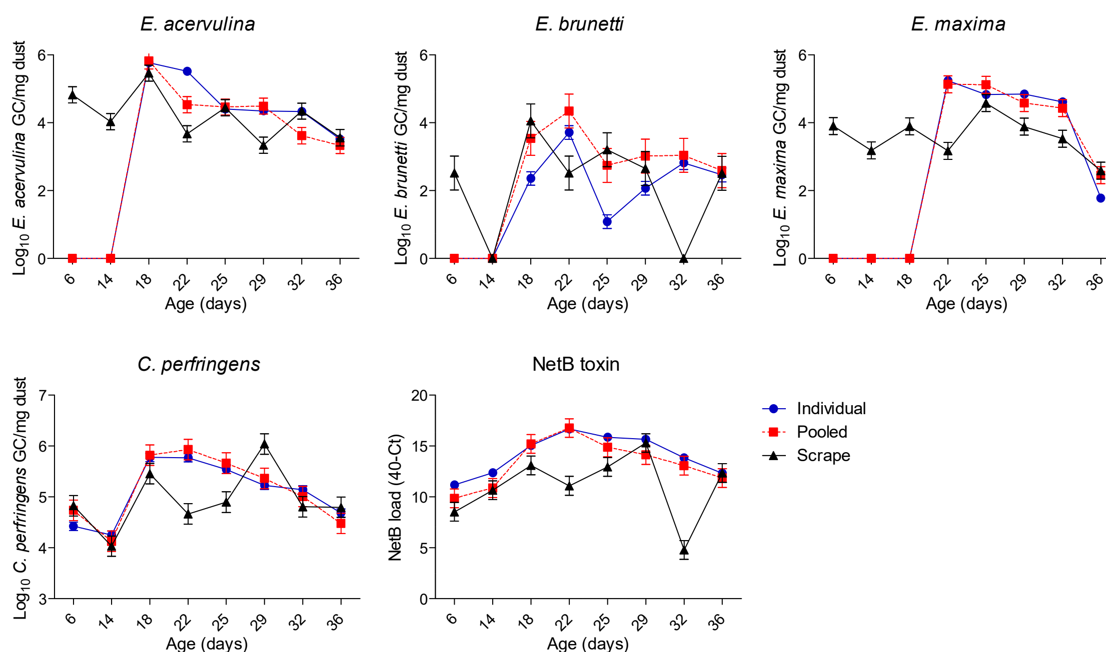

Figure 11. Genomic copies/mg of dust (log10, LSM ± SEM) of *E. acervulina, E. brunetti, E. maxima, C. perfringens* and *netB* toxin Ct (40-Ct) from samples collected from an experimental broiler flock.
At each collection day, four samples were collected by settle plates and tested individually and as a single pooled sample, or were scraped from barn surfaces in two arbitrarily selected places. Birds were challenged at 9 days of age with pathogenic strains of *E. acervulina, E. brunetti and E. maxima* and with *C. perfringens* containing *netB* toxin plasmid at 14 days of age to produce subclinical necrotic enteritis.

Summary

In challenged flocks, high levels of *C. perfringens* and *Eimeria* spp. were detected after challenge followed by a gradual decline over time. In unchallenged birds, *C. perfringens* and *netB* were detected in low levels, with levels tending to decline with age. There was no significant effect of sampling location within poultry houses on *C. perfringens* and *Eimeria* load. Scraped samples may overestimate the number of positive samples for *Eimeria* as they may reflect shedding from previous batches of birds. Results indicate that PCR detection of *C. perfringens* and *Eimeria* in dust samples may enable distinction between infected and healthy flocks.

5.2 Dust samples from the end of batch of broiler flocks

To evaluate the detection of selected microorganisms in commercial samples, 217 DNA extracts from dust samples collected at the end of batch of broiler flocks were acquired from Birling Avian Laboratories, NSW. Samples were collected from 1—7 batches of chickens housed in 116 sheds of 25 farms between 2015 and 2018. All DNA extracts were tested for *C. perfringens*, netB toxin, *M. synoviae* and *M. gallisepticum* by PCR. Samples from farms positive for *C. perfringens/netB* were also tested for *E. acervulina, E. brunetti* and *E. maxima*.

Out of 25 farms, four (6.2%) had at least one sample positive for *C. perfringens/netB* DNA with a very low overall detection of *C. perfringens* (2.7%, 6/217 samples) and *netB* toxin (1.3%, 3/217). All samples positive for *netB* toxin were also positive for *C. perfringens* DNA. The detection rates for *E. acervulina* DNA was 70.0% (21/30) and for *E. maxima* DNA was 33.3% (10/30) on samples from farms positive for *C. perfringens*. *E. brunetti* DNA was not detected in this sample set. These results are not unexpected as *E. acervulina* and *E. maxima* have been shown to be detected in more than 55% of commercial flocks in Australia while *E. brunetti* prevalence was very low (1.6%) (Godwin and Morgan, 2015).

Out of 217 samples, 2 (0.9%) tested positive for *M. synoviae,* and another 2 (0.9%) tested positive for *M. gallisepticum* at low levels. The lack of detection of mycoplasma in dust could be due to the absence of those pathogens in the studied flocks at the end of batch or could indicate the low sensitivity of dust samples for monitoring mycoplasma infections. DNA of both organisms could be intermittently detected in dust samples from layer and breeder flocks vaccinated with live vaccines.

6 Optimised guidelines for dust sample collection

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 scraped from barn surfaces or be collected from dust deposited by settling in a collection apparatus. For monitoring MDV incursion, 1 to 6 sampling locations per poultry barn have been used, and it has been suggested that a single dust sample per barn would allow detection of a positive result in infected flocks.

The influence of dust collection method in detection of different pathogens was investigated on the experimental layer and broiler flocks studied and detailed results are found in sections 4.1 and 5.1 of this report.

In summary:

- Settle plate dust can be collected at any location in the poultry barn for detection of the genome of all studied organisms MDV, ILTV, *E. acervulina, E. maxima, E. brunetti* and *C. perfringens* (**Figure 5** and **Figure 10**).
- A pooled settle plate sample per poultry barn can be used for detecting any of the microorganisms above when high microorganism load is expected (**Figure 11**)
- Scraped dust samples may have higher genome load compared to settle plate samples for microorganisms that are shed in high loads (**Figure 6** and **Figure 11**)
- Scraped dust may represent historical accumulation of dust from the same batch or from previous batches of bird (**Figure 6** and **Figure 11**).

7 What can be detected in dust: high-throughput sequencing

To have an overview of the microorganisms that can be detected in different production systems, 22 dust samples collected from different poultry production systems were subjected to high-throughput Illumina sequencing targeting DNA organisms.

7.1 Sample sets, laboratorial processing and analysis

Sample sets

1. *Broiler breeders* (n = 4). A cross-sectional study with a single sample collected per shed when chickens were 1, 15, 30 and 61 weeks of age to evaluate differences in the microbial population across the production cycle.
2. *Laying hens* (n = 10). A longitudinal study following a chicken population from week 1 of age until week 50. Samples were collected in duplicate on weeks 1 (placement), 17 (transfer to laying facility), 26 (peak of egg production), 30 and 50 to evaluate differences in the microbial population across the production cycle.
3. *Broilers* (n = 8). A single sample was collected at end of batch from 4 sheds of the same farm on two consecutive batches of birds to compare the microbial population between sheds (samples collected on the same date) and between batches reared on the same shed.

Nucleic acid extraction. Dust samples were extracted by weighing 10-50 mg of dust into 1.5 ml tubes and using DNeasy® Power Soil® Pro kit (Qiagen, cat # 47014) with an additional bead beating step for 5 min at maximum speed. Samples with high impurities as measured by Nanodrop were cleaned up using a commercial kit (Biobasic, cat # BS367). The extracted DNA was submitted to the Deakin's Bioinformatics Core Facility for sequencing.

Library preparation and quality control. The quantity and quality of the DNA were assessed using the Qubit BR dsDNA assay (ThermoFisher) and an Agilent DNA High Sensitivity series chip assay. Paired end libraries were prepared by standard Illumina methods and samples were analysed using a NovaSeq 600 Illumina System. For each library, read quality control was performed using FastQC v0.11.7.

Bioinformatics. The bioinformatics was performed in the first 10000 pair end sequences (paired reads) extracted from the original data. Read alignment against a protein reference database, NCBI NR (Benson et al., 2006) was done by DIAMOND (version 0.9.24) (Buchfink et al., 2015) in BLASTX mode. Blast2rma, from MEGAN6 community edition (Huson et al., 2016), was used to map TaxID to the alignment output. The relative abundance (percentage) of each taxon was produced using R (version 3.6.1). Barplots and heatmaps were produced using "barplot" and "pheatmap" packages of R. In order to display the differences of taxa composition in different samples, Principal Component Analysis (PCA) was used to construct 2-D graph to summarize variables that mainly contributed for this difference, similarity is high if two samples are closely located. The PCA of taxa at each major taxonomy level was done with the relative abundance of each taxon in each sample using "prcomp" method and "ggbiplot" package in R.

7.2 Microbial composition of poultry dust samples

Bacteria accounted for most of the readings in dust samples

Abundance of sequencing readings at phylum level is summarised in **Figure 12**. In all dust samples, chicken and human nucleic acid contributed to less than 1% of the readings while bacteria, mostly from the chicken gut and environment (*Firmicutes, Proteobacteria and Bacteroidetes*), accounted for the majority of readings in all sample sets. High readings of gut bacteria is expected as poultry dust is a combination of droppings, feed, litter, and dander from birds (Hartung and Saleh, 2007).

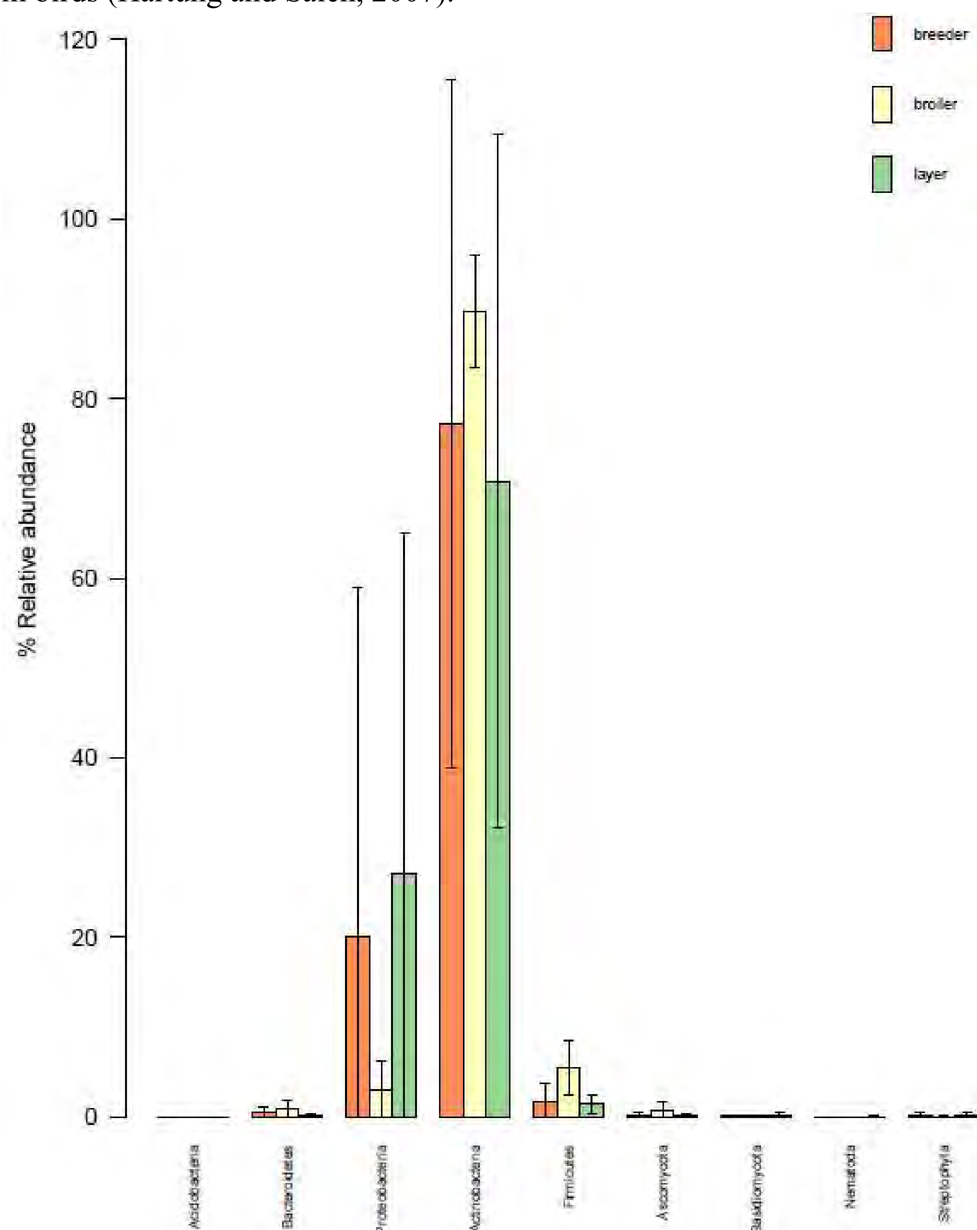

Figure 12. Microbial taxonomic profile of dust samples from broiler breeder (breeder), broiler and layer operations at phylum level.

Readings of feed components (e.g., *Liliopsidae* class, wheat), bedding, fungi, arthropods and nematodes were detected in all sample sets with relative abundance of organisms at a class level varying between data sets and within samples of the same data set (**Figure 13)**.

The readings for viruses were lower (0.1─2.5%) than a previous high throughput genomic sequencing report from dust scraped from a broiler house (25% of readings) (O'Brien et al.,

2016) due to the differences in the analysis of unclassified reads. The methods used in the report to treat unclassified reads likely overestimated the counts of viruses.

Across all samples, the most abundant virus reads were from the bacteriophage families *Siphoviridae*, *Myoviridae* and *Podoviridae*. Among viruses that infect poultry, fowl adenovirus readings were detected in few samples of the broiler and laying chicken sample sets. The lack of reads from viral families such as *Herpesviridae* (e.g., MDV and ILTV) on samples that were known to be positive for those viruses by qPCR on samples reflects the lower sensitivity of virus detection when complex samples are not enriched prior to sequencing (Paskey et al., 2019).

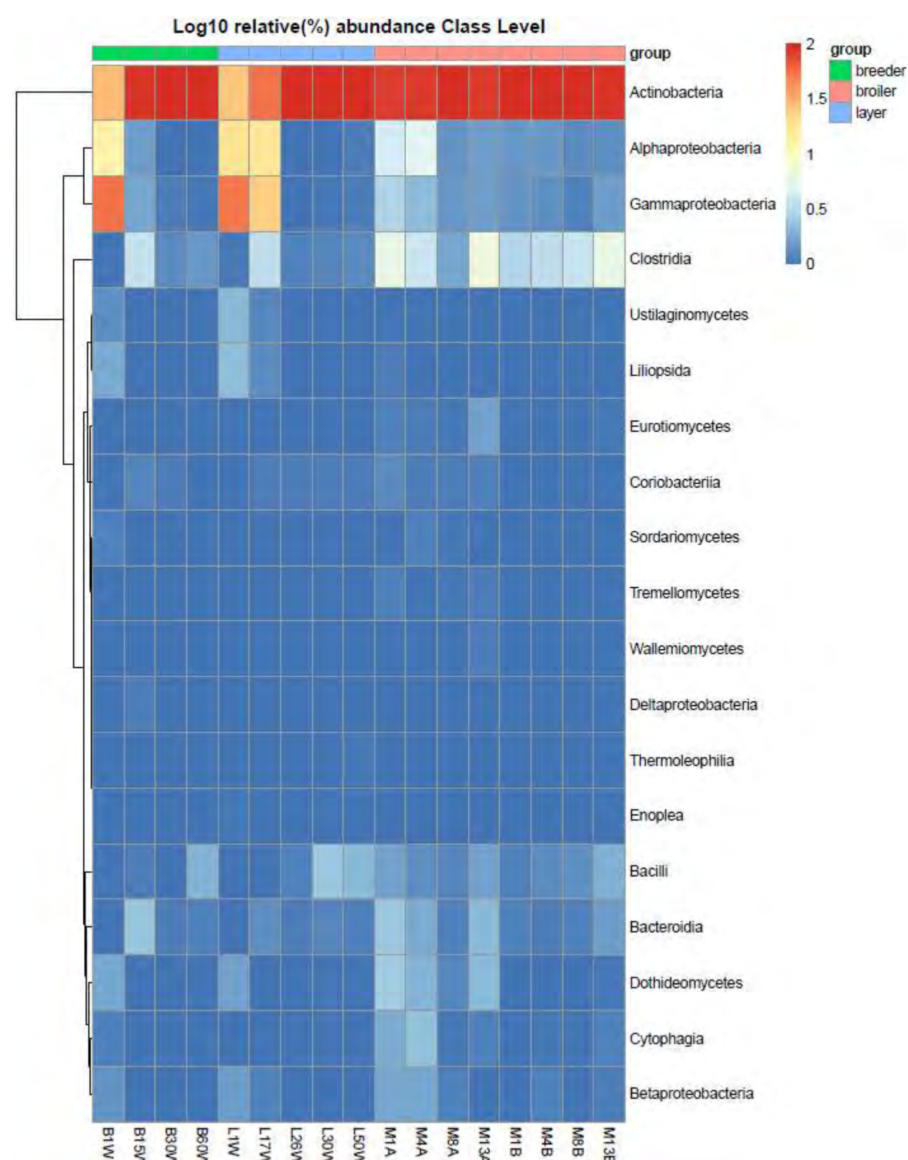

Figure 13. Heatmap of the microorganisms at class level grouped by the operation type.
Samples codes at the bottom of the figure are as: B indicates breeder broiler sample set; L indicates layer sample set and M indicates broiler sample set. For the breeder broiler and layer sample sets, "X"W indicates the age of the birds in weeks when the sample was taken. For the broiler sample set, the number indicates the shed in which the sample was taken and A/B indicate the time. Samples marked as "A" were collected from a contemporaneous batch of chickens in the same farm and samples marked "B" were from a subsequent batch of samples on the same shed.

Microbial profile changes with bird age and operation type

Interestingly, there was a significant shift in the composition of the dust relative bacterial populations after sexual maturity for long lived birds (**Figure 13**). Similar shifts have been reported in the composition of caecal and ileum microbiota in layer hens (reviewed by Kers et al., 2018; Ngunjiri et al., 2019). The relationship of dust and gut microbiota warrants further investigation.

The dust microbiota also differed based on production types and results are summarised in **Figure 14.**

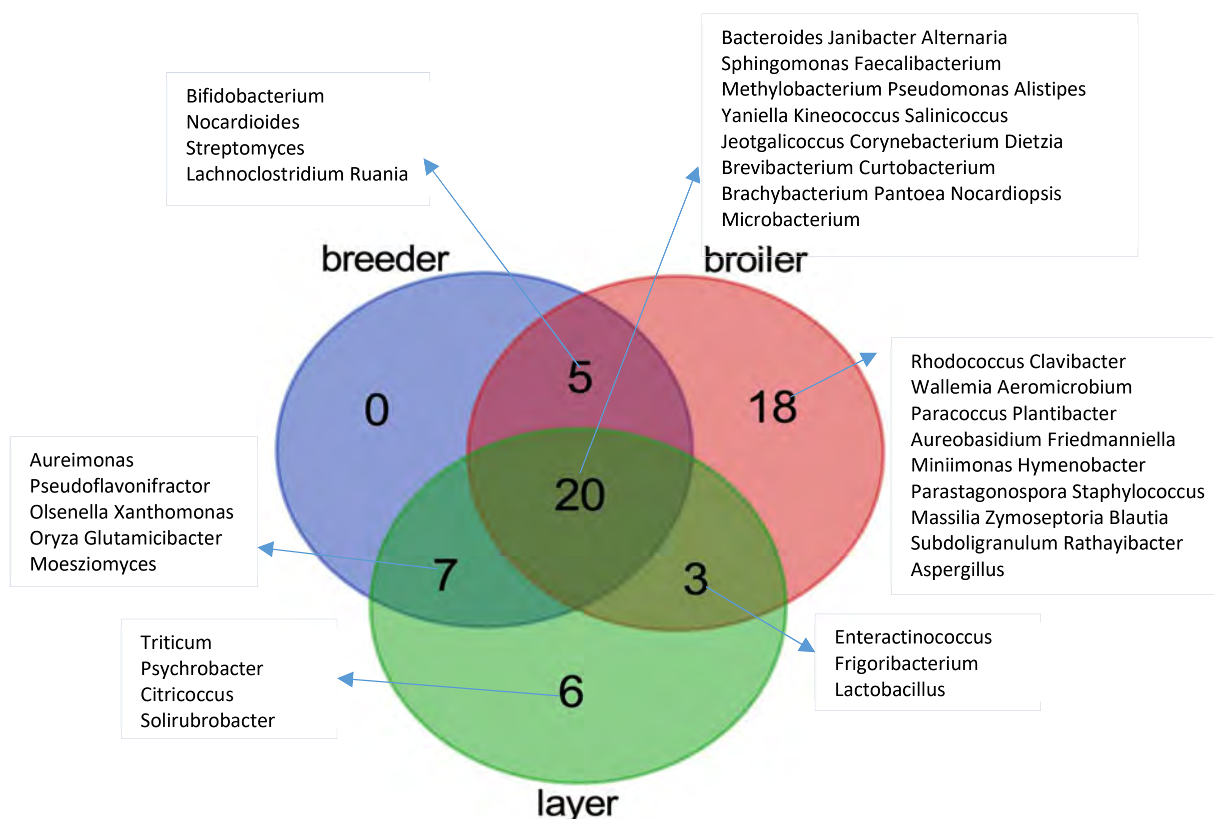

Figure 14. Venn diagram depicting the number of genus that were shared between chicken production operation types. Breeder, broiler breeder.

8 Optimisation of nucleic acid extraction methods of dust

8.1 High throughput DNA extraction at Birling Avian Laboratories

To enable high throughput DNA extraction from dust, an automated DNA extraction using the QIAsymphony instrument (Qiagen) was developed at Birling Avian Laboratories. Dust samples were prepared by weighing 20±2 mg into 1.5 ml tubes, adding 500 µl of ALT buffer (Qiagen) and the samples were homogenised by vortexing. The samples were then incubated as 56°C for 10 min, transferred to 2 ml tubes before being loaded onto the QIAsymphony instrument. Total nucleic acids (DNA and RNA) were extracted using the QIAsymphony DSP Virus/Pathogen Midi kit with the Complex 400_V6_DSP default IC protocol, the final elution volume is 60 µl.

To validate the successful extraction of viral DNA from dust, the quantity of MDV was investigated using a qPCR assay and compared to previous results of routine testing that used a manual DNA extraction protocol (QIAamp DNA mini kit, Qiagen).

In total, 20 samples were tested, with no difference between the number of positive and negative results between extraction methods (McNemar's test, P=0.38) and a strong agreement in the viral load (Intraclass Correlation Coefficient=0.77). This extraction method has been used for detection of ILTV DNA in dust samples at BAL.

8.2 Optimisation of total nucleic acid extraction methods at UNE

At UNE, two kits for total nucleic acids extraction were tested in 6 dust samples and 5 tracheal swab samples (GeneJET Viral DNA/RNA Purification kit, Thermo Scientific and EZNA universal pathogen kit, Omega) according to the manufacturer's recommendations or increasing the lysis incubation time to 3 h. Samples were tested by a qPCR for ILTV as a model DNA virus, and IBV as RNA virus model. Results were compared to extraction of DNA (Bioline Genomic DNA II) and RNA (Isolate II RNA mini kit, Bioline) using individual kits. EZNA universal pathogen kit has the lowest detection rate of all tested kits.

For swab samples, the viral load and detection rate of ILTV was similar using the Bioline or GeneJet kits, however, detection of ILTV DNA in dust samples was better using Bioline extraction kit. The detection rate for IBV RNA in dust was better using the Bioline kit while GeneJet yielded better results in swabs samples.

In summary, a single RNA/DNA extraction method using the GenJet extraction kit can be used for total nucleic acid extraction of swab samples, but higher detection rates of the viruses investigated were found when dust samples were extracted separately for DNA and RNA using the specific Bioline kits.

9 Dust profile in different operation types

Poultry dust is a mixture of bedding, feed, droppings, feathers, dander, and microorganisms. Dust in poultry barns is considered an important respiratory hazard for poultry workers and most of the studies on this area focus on microbiological and chemical contaminants for humans (Skora et al., 2016) while there is not much information on chicken pathogens.

Dust includes particulate matter, ranging from 0.001 to 100 µm in diameter. Dust particles can be deposited in various parts of the respiratory system depending on its size. In humans, particles with a diameter between 5 and 10 µm are mainly deposited in nasopharyngeal areas, particles between 1 and 5 µm in diameter are deposited in bronchi, bronchioles and alveoli whereas particles of less than 1 µm can pass into the lungs (Owen et al., 1992). The Andersen cascade impactor (ThermoFisher, USA) is an aerosol sampler that consists of a series of six stages (cascades) with orifices with diameters ranging from 1.81mm on the first stage to 0.25mm on the sixth stage (Andersen, 1958). Petri dishes are placed between the cascades and ambient air is drawn through the sampler by a vacuum pump. The accumulated dust is then tested by microbiological procedures to identify microorganisms of interest (King and McFarland, 2012).

The aims of this study were 1) to provide and estimation of size distribution of poultry dust using dust samples from layer and broiler poultry operations and 2) the dust fractions in which the selected live vaccine viruses (IBV, NDV, ILTV and FAdV) can be detected.

9.1 Optimisation of the Andersen cascade sampler for settled dust

This experiment assessed the feasibility of using the Andersen cascade sampler (**Figure 15**) with dust samples previously collected using settle plates. The variables evaluated were: dust pre-treatments (fresh and dry dust); collection tube (with and without); and use of Petri dishes in the cascades (with and without). Dry dust was obtained by drying it in an oven at 37°C for 48 h.

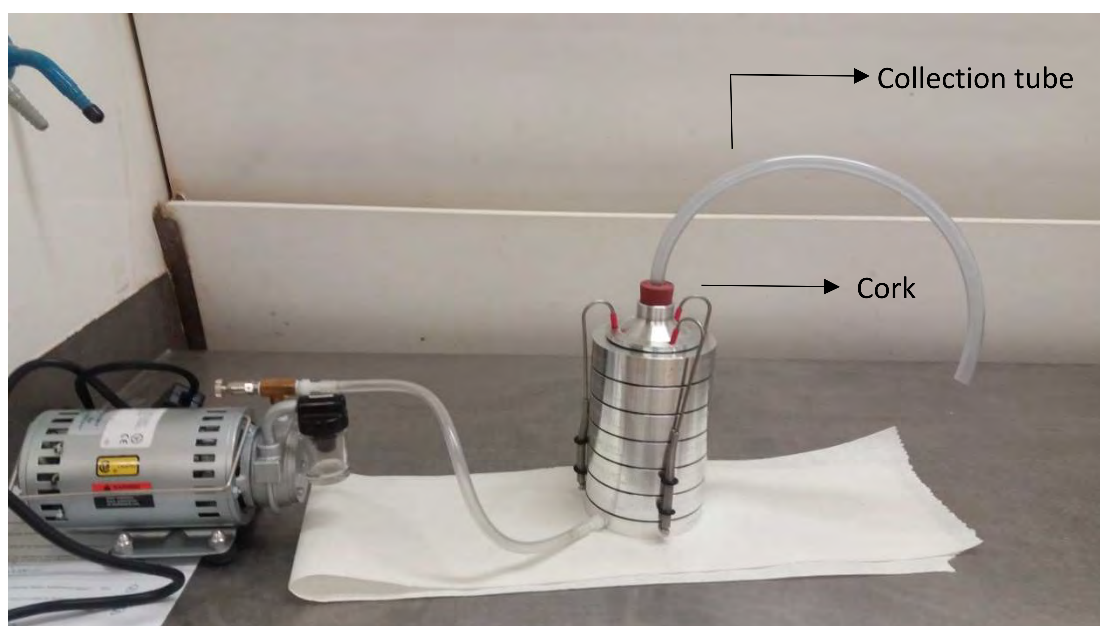

Figure 15. Modification of Andersen sampler by adding a tube to the first cascade with a piece of cork. The vacuum pump was run for 15 min for each sampling.

Mean dust collection from all cascades was higher when dry dust and the collection tube (87.38%) were used. No dust (either dry or fresh) was passed through the stages of the Andersen sampler when the collection tube was not used. When Petri dishes were used between cascades dust was only deposited on the Petri dishes of the top three cascades.

9.2 Particle size distribution of poultry house dust

Dust samples collected at weeks 1 and 3 of chick placement from three broiler flocks and at weeks 8, 16 and 24 of chick placement on two layer flocks were weighed and 250 mg of each sample was transferred into a Petri dish and dried at 37 °C for 48 hours. Samples were weighed after drying to record the dry matter. Samples were then passed through the Andersen sampler.

The overall estimated dry matter of poultry dust was 94.15±0.68%, with higher ($P=0.05$) dry matter for broiler dust 95.45±0.82% than layer dust 92.85±0.82%. A summary of the dust deposition profile is shown in **Figure 16**. The dust deposition in the Andersen cascades depended on the cascade diameter ($P<0.0001$) and chicken operation type ($P=0.001$), but not on chicken age ($P=0.15$). As expected, the amount of dust deposited was higher in cascades with larger particle sizes (9 to 4.7 µm).

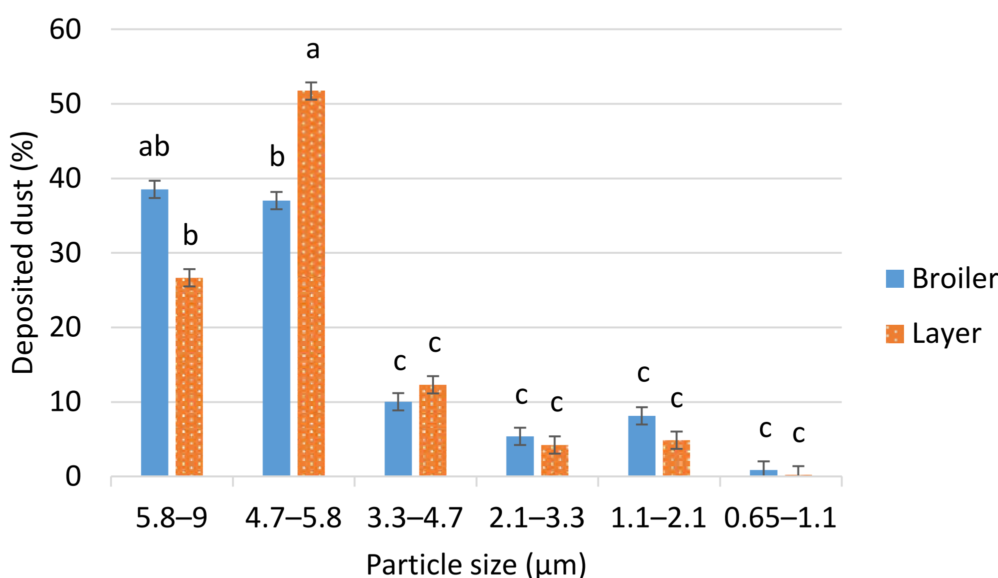

Figure 16. Particle size distribution of poultry dust using Andersen sampler by chicken operation type

9.3 Microbial load in different dust fractions

Samples were positive for all live vaccines investigated and the different fractions of dust ($P=0.21$), chicken operation type ($P=0.98$) and age of bird ($P=0.53$) had no effect in the microbial load detected. Mean PCR results of individually tested virus and their interaction with cascade diameter/stages are presented in **Figure 17**.

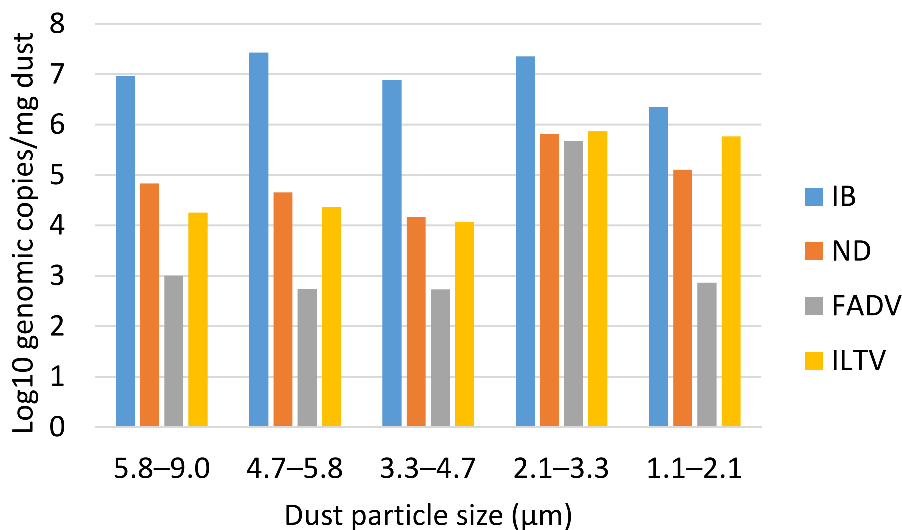

Figure 17. Mean vaccine virus load (log10 genomic copies/mg dust) in different fractions of poultry dust based on dust particle size fractionated by the Andersen sampler.

Summary

The particle size of poultry dust varies significantly based on the production system but not depending upon the age of the bird. The detection pattern and load of virus was detected in all fractions of dust using PCR, including fraction sizes deemed to enter deeply into the respiratory tract. This may have implications for transmission of respiratory diseases through dust.

10 Implications

This project provided proof of concept on the feasibility of detection of nucleic acid of a wide range of live vaccines and pathogens in poultry dust in experimental and commercial flocks:

- All live vaccines tested in this study could be detected in dust samples after vaccination by PCR, namely, MDV, ILTV, FAdV, IBV, IBDV, NDV, *M. synoviae*, *M. gallisepticum* indicating this method offers promise to monitor administration of a wide range of live vaccines
- There is good evidence that monitoring ILTV vaccine administration in dust is feasible while measurement of success of vaccine administration for other live vaccines evaluated here will require testing of individual birds to correlate with loads detected in dust samples.
- Nucleic acid of *C. perfringens*, *netB* toxin, and *Eimeria* species from experimentally challenged flocks were readily detected in dust. These findings will be further explored in commercial flocks.
- The differences in microbial communities in dust samples collected from different production systems and age groups may provide a biomarker to evaluate gut health in commercial flocks. These findings will be further explored in commercial flocks.

Guidelines for dust sampling for diagnostics were also optimised:

- Settled dust collected weekly reflects the current level of genome load while samples scraped from surfaces may reflect historical accumulation and lead to false-positives for detection of hardy microorganisms such as *Eimeria*.
- Settle plate dust collected at any place in the shed is representative of the population for detection of nucleic acids of MDV, ILTV, *C. perfringens*, *netB* toxin, and *Eimeria* spp, which means that there is no "best" place in a shed to collect dust samples.
- Testing of pooled samples can be used for detecting live vaccines shortly after vaccination when microbial load is high but testing of a single pooled sample may increase the chance of false negatives when microbial shedding is expected to be low.

This project has mapped live vaccines and some economically important pathogens that can be detected in dust. Further research is needed to relate microbial levels in dust and prevalence of positive results in individual birds using reference sampling methods.

Overall, this approach provides for an inexpensive, practical, and welfare friendly method to monitor chicken flocks. Availability of new sampling and testing methods will enable a more systematic approach for disease and live vaccine administration monitoring.

11 Recommendations

The main recommendations for dust sample collection from poultry houses for monitoring of live vaccine administration or pathogen incursion are:

- Poultry dust can be useful for routine monitoring of MDV and ILTV
- PCR-testing of dust samples offers promise for monitoring poultry flock health for
    - IBV, NDV, IBDV, FAdV, *M. gallisepticum*, *M. synoviae, Salmonella,* coccidia and pathogenic *C. perfringens* (necrotic enteritis)
    - Further validation work is needed to relate status in individual birds and detection loads in dust
- Dust can be collected from settle plates from the first week of chick placement
- Settled dust collected weekly is preferred over samples scraped from surfaces of the house as the may reflect historical microbial load accumulation from previous shedding of the same birds or previous batches of birds
- Settle plate dust can be collected at any location in the poultry house
- Collection of dust from at least two locations in the poultry house is recommended
- Samples can be pooled prior to test further reducing costs associated with diagnostic testing

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13 Media and Publications

Conference presentations

Nguyen TV, Ahaduzzaman M, Campbell D, Gerber PF, Walkden-Brown SW. Temporal variation of ILTV and MDV viral genome in dust samples after vaccination in a layer flock. Proceedings of the Australian Poultry Science Symposium. 2019;30:135-8.

Ahaduzzaman M, Gerber PF, Keerqin C, Musigwa S, Morgan N, Kheravii S, Wu S, Walkden-Brown S. Detection and quantification of Clostridium perfringens and *netB* toxin gene from poultry dust using real-time PCR. Proceedings of the 21st World Veterinary Poultry Association Congress, p. 161, Bangkok, Sept 16-20, 2019.

Journal paper (published)

Nguyen TV, Ahaduzzaman M, Campbell DLM, Groves PJ, Walkden-Brown SW, Gerber PF. Spatial and temporal variation of Marek's disease virus and infectious laryngotracheitis virus genome in dust samples following live vaccination of layer flocks. *Veterinary Microbiology*. 2019:108393. doi: 10.1016/j.vetmic.2019.108393.

14 Intellectual Property Arising

The project has not directly produced products of commercial value. However it has provided proof of concept of detection of many pathogens and live vaccines in dust samples.

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