AUSTRALIAN POULTRY CRC

FINAL REPORT

Program 3B

Project No: 09-34

PROJECT LEADER:

Prof. Steve Walkden-Brown

DATE OF COMPLETION: 12/2/2010

Project No: 09-34

Temperature inactivation of viral pathogens in litter

© 2010 Australian Poultry CRC Pty Ltd
All rights reserved.

ISBN 978 1 921890 27 7

Temperature inactivation of viral pathogens in litter
Project No. 09-34

The information contained in this publication is intended for general use to assist public knowledge and discussion and to help improve the development of sustainable industries. The information should not be relied upon for the purpose of a particular matter. Specialist and/or appropriate legal advice should be obtained before any action or decision is taken on the basis of any material in this document. The Australian Poultry CRC, the authors or contributors do not assume liability of any kind whatsoever resulting from any person's use or reliance upon the content of this document.

This publication is copyright. However, Australian Poultry CRC encourages wide dissemination of its research, providing the Centre is clearly acknowledged. For any other enquiries concerning reproduction, contact the Communications Officer on phone 02 6773 3767.

Researcher Contact Details
Stephen Walkden-Brown
School of Environmental and Rural Science
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 5152
Fax: -2 6773 3922
Email: swalkden@une.edu.au

In submitting this report, the researcher has agreed to the Australian Poultry CRC publishing this material in its edited form.

Australian Poultry CRC Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: info@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

Published in February 2010

Executive Summary

This short project had the following objectives:

1. To generate and obtain infective litter with a wider range of viral pathogens in it than we have found in project 06-15
2. To determine the effects of incubation of litter at temperatures of 50, 60 or 70˚C for 10 days on the level of viral infectivity of litter as determined in a chick bioasaay. These temperatures simulate the moderate, high and very high range of temperatures found in heaped or windrowed litter
3. To determine whether we can use quantitative molecular methods to detect virus in litter material and whether the viral load detected this way correlates with infectivity.

The project was successfully implemented in Nov-Dec 2009 and the experimental data have only just come out of the lab. The findings are generally positive with some negative findings.

On a positive note, we have shown that we can quantify Marek's disease virus directly from litter using real time PCR. This needs to be tested for other viruses as well but is a promising direct method of quantification. The experiment also clearly showed loss of infectivity over time at different temperatures for CAV and FAV, as measured in the chick bioassay. Both of these viruses completely lost infectivity by day 10 at all 3 temperatures loss of infectivity at day 5 being temperature dependant. The infectivity data for MDV and IBDV were less clear although there was an overall trend towards reduced infectivity with duration of heat treatment. There was no apparent transmission of IBV, NDV or ILT via litter in the experiment.

On a negative note, there was no association between MDV viral load measured in litter and the infectivity of that litter for chicks in the bioassay. However for the samples tested by qPCR (UNE samples) the MDV bioassay results were also odd, appearing to increase over time, so the association needs to be tested on a wider range of viruses, particularly CAV and FAV for which there was very good loss of infectivity with temperature and time in this experiment. *C. perfringens* appeared ubiquitously in all treatments including controls, whereas the reverse was true for coccidia, with zero counts even for chicks exposed to the day 0 fresh litter. This contrasts with the good results obtained on inactivation of coccidia in project 06-15 using cloacal contents at the end of the experiment.

In summary this set of results provides significant "proof of concept" for laboratory scale temperature treatment of litter to measure effects on viral inactivation rate and for quantitative measurement of pathogenic virus directly from litter using real time PCR.

It is recommended that future work in this area be supported, including the following specific details:

- qPCR measurement of virus in litter needs to be extended to a wider range of viruses. From the present experiment CAV and FAV are the priority as there was very good loss of infectivity with temperature and time for them, so a good test of association.
- Methods of litter preparation for DNA/RNA extraction and viral quantification need to optimised to maximise assay sensitivity and reproducibility.
- The end-point measures for *C. perfringens* and coccidia in the bioassay should be based on recovery from cloacal contents rather than from the accumulated faeces under the isolator floor.
- When deliberately contaminating litter with infected birds, virus shedding should be measured if possible, in addition to seroconversion in response to vaccination.
- Future experiments should examine some lower temperatures, such as those seen near the surface of litter heaps and windrows.

Table of Contents

AUSTRALIAN POULTRY CRC 1

FINAL REPORT 1

PROGRAM 3B 1

EXECUTIVE SUMMARY 1

INTRODUCTION 3

OBJECTIVES AND HYPOTHESES 3

METHODOLOGY 4

EXPERIMENTAL RESULTS 4

EXPERIMENT 1: LT09-C-CB9 "PRODUCTION OF INFECTIVE LITTER (POSITIVE CONTROL) FOR BIOASSAY OF LITTER INFECTIVITY" 4

Introduction *4*

Materials and Methods *4*

Summary and conclusions *5*

EXPERIMENT 2: LT09-C-CB8: "TEMPERATURE INACTIVATION OF PATHOGENS LITTER AND ASSOCIATION BETWEEN MOLECULAR DETECTION AND INFECTIVITY IN A BIOASSAY" 6

Introduction *6*

Materials and Methods *6*

Results *8*

Summary and conclusions *11*

PLAIN ENGLISH COMPENDIUM SUMMARY **13**

Introduction

Re-use of litter by broiler chickens can reduce the environmental impact and cost of chicken production but uptake of the practice is limited by risks of pathogen carryover. On Project 06-15 our research team has developed an effective chick bioassay to measure viral infectivity in litter, and has made detailed measurements of temperature, pH, moisture and litter chemistry on a wide range of litter treatments in the field involving partial composting of litter in heaps or windrows. One objective was to link the treatment effects with the temperature and other changes achieved during treatment.

A problem with this approach has been that we can conduct large field experiments to measure effects of various treatments on pathogen survival, only to find a limited range of pathogens on the farm. We can deliberately infect chickens with pathogens at UNE, but it is difficult to generate enough litter for composting studies that will mimic the field situation.

This project aimed to overcome this problem by enabling us to simulate full composting using small litter samples containing a wide range of pathogens. We did this by

- generating infective litter at UNE,
- exposing it in climate chambers to various temperature regimens based on our field measurements and then
- conducting the chick bioassay, knowing that we will be evaluating effects on the full range of pathogens of interest, rather than the ones we obtain by chance in the field.

It will also undertake preliminary investigations into molecular quantification of viruses in litter material with a view to some time in the future replacing the need for a bioassay. This will be influenced by the extent to which non-infective virus is unlikely to persist and be detected by molecular methods, but there is a good probability that loss of molecular detection will correlate well with loss of infectivity.

Objectives and hypotheses

The project objectives are as follows:

1. To generate and obtain infective litter with a wider range of viral pathogens in it than we have found in project 06-15
2. To determine the effects of incubation of litter at temperatures of 50, 60 or 70˚C for 10 days on the level of viral infectivity of litter as determined in a chick bioasaay. These temperatures simulate the moderate, high and very high range of temperatures found in heaped or windrowed litter
3. To determine whether we can use quantitative molecular methods to detect virus in litter material and whether the viral load detected this way correlates with infectivity.

To meet these objectives we designed an experiment to test the following hypotheses:

1. By deliberately challenging or vaccinating chickens with a wide range of organisms we will successfully induce litter contamination and transmission for a wider range of viral pathogens than previously and from field litter tested concurrently.
2. Incubation for 10 days at 70˚C will completely remove viral infectivity in litter. In general inactivation will increase with increasing temperature and increasing duration of exposure to high temperature.
3. We will be able to detect and quantify viral DNA or RNA directly from litter

4. The amount of viral DNA or RNA we detect will correlate with the level of infectivity for that sample in the bioassay

Methodology

The broad methodological approach used is provided below. The exact details are described in the reports of each experiment.

Generation of infective litter. Broiler chicks were placed on wood shavings litter at UNE and deliberately infected with vaccinal or pathogenic strains of IBV, IBDV, CAV, MDV, ILT, Fav8, NDV, AE at appropriate times to ensure active shedding during in the period leading up to litter collection at day 35. In addition, field litters from end of batch commercial chickens were obtained and mixed to provide an alternative source of infective virus.

Litter heat treatment in incubators. 10 litre samples of litter were enclosed in semi-porous bags and placed in various temperature treatments in incubators with water trays in them to limit moisture loss. The treatment matrix involved fixed temperatures of 50, 60 and 70˚C with durations of exposure of 0, 5 and 10 days for each.

Bioassay of treated samples. The 18 samples were assayed for infectivity using the chick bioassay developed on Project 06-15 (Islam et al., 2009 Proc. Aust. Poult. Sci. Symp. 20, 176-179). The end point for infectivity was serology of 10 chickens per treatment for each virus involved. In the case of coccidia and *Clostridium perfringens* a pooled faecal sample from beneath the floor of the isolator was collected and used.

Molecular detection of virus in litter. We investigated molecular detection and quantification of a selected virus (MDV) from litter following filtration and centrifugation. Results were compared with the bioassay for sensitivity.

Experimental results

Experiment 1: LT09-C-CB9 "Production of infective litter (positive control) for bioassay of litter infectivity"

Start: 02/10/09 **Completion: 06/11/09** **AEC: UNE AEC09/125**

Introduction

One important issue arising from Project 06-15 was that the project could not test as wide a range of viral pathogens as desirable because probably they are either not present in the field litter or are not detectable by the bioassay. The objective of this experiment was therefore to generate known contaminated litter containing at least eight poultry pathogens, mainly viral.

Materials and Methods

Forty day-old broiler chickens were transported to UNE from a commercial hatchery and reared on a 3mx3m floor pen on wood shavings in a climate controlled room in the UNE animal house. Feed and water were provided *ad libitum* and commercial broiler rearing protocol was used. The live vaccines were given as shown in Table 1.

Table 1. Vaccine and challenge schedule to produce contaminated litter.

Vaccine or virus*	Source	Strain	Dose	Age of chickens at inoculation	Route of infection
Vaxsafe IBD	Bioproperties	Strain V877	$>10^{2.4}$ EID50/dose	17 days	Eye drop
Vaxsafe ND	Bioproperties	Strain ND V4	$>10^{6.0}$ EID50/dose	21 days	Eye drop
Vaxsafe IB	Bioproperties	IBV Ingham's strain	$10^{3.0}$ EID50/dose	21 days	Eye drop
MDV	Another experiment	CVI 988	Two buckets of litter from vaccinated birds in another expt	17 days	Litter contamination
Steggles CAV vaccine	Intervet Australia	Strain 3711	$10^{1.575}$ CID50/dose	17 days	Oral, x10 dose used
Fowl pox vaccine	Fort Dodge	M Strain	Recommended	27 days	Wing-web scratching
ILT	Intervet Nobilis	ILT virus	Recommended	27 days	Oral
Eimeriavax 4m	Bioproperties	*E. acervulina, E. maxima, E. necatrix and E. tenella.*	Recommended	27 days	Eye drop
AE	Fort Dodge	I Strain	Recommended	28 days	Oral
Clostridium perfringens	CSIRO (Rob Moore)	Longford1	10^{9} cells/bird	28 days	Oral

* IBD (Infectious bursal disease), ND (Newcastle disease), IB (Infectious bronchitis), MDV (Marek's disease virus), CAV (Chicken infectious anaemia), AE (Avian encephalomyelitis), ILT (Infectious laryngotracheitis)

The experiment was terminated at day 35 and litter from these chickens were taken to infect chickens for the bioassay experiment either directly or subjected to temperature treatments at 50˚C, 60˚C or 70˚C up to ten days (detailed in Chapter 2).

At day 35 (before removing the chickens, blood sample was collected from the chickens and serum was separated to confirm the vaccination. The serological results were as follows (Table 2):

Table 2. Serological response to challenge/vaccination in shedder chickens used to produce contaminated litter.

Virus	Abbreviation	Response (+/total)	% Positive
Chicken anaemia virus	CAV	8/10	80
Newcastle disease virus	NDV	10/10	100
Marek's disease virus	MDV	10/10	100
Infectious bronchitis virus	IBV	5/10	50
Infectious bursal disease virus	IBDV	10/10	100
Infectious laryngotracheitis virus	ILTV	2/10	20
Avian encephalomyelitis virus	AEV	0/10	0
Fowl adenovirus (no vacc or challenge)	FAV	0/10	0
Reovirus (no vacc or challenge)	Reo	0/10	0

Summary and conclusions

The serological results confirmed that chickens were successfully vaccinated with CAV, ND, IBD, IB and ILT vaccines although the response to the latter was low. The infection with MDV was also successful. The low sero-conversion rate for ILT was probably because blood samples were collected only 8 days after vaccination. The chickens were not positive for AE antibody possibly because serum was collected only 7 days after vaccination. We don't have an assay to detect antibody against Fowlpox virus.

Experiment 2: LT09-C-CB8: “Temperature inactivation of pathogens litter and association between molecular detection and infectivity in a bioassay”

Start: 06/11/09 **Completion: 21/12/09** **AEC: UNE AEC09/130**

Introduction

The objectives and hypotheses for this experiment are enacapsulated in those provided for the overall project in the sections above. The aim of this experiment was to take the litter from Experiment 1, treat it with 3 different heat regimens in an incubator and examine the effects of heat treatment on pathogen survival using the chick bioassay to detect infective pathogens in the litter.

A second aim was to try and quantify a candidate virus (MDV in this case) directly from the litter and relate the viral load to infectivity as detected by the chick bioassay. While methods for the detection and quantification genomes of parasites and bacteria from litter are readily available this is not true for viruses. Filthy litter is not a suitable starting material for developing inoculates for cell culture. While detection and quantification of viral DNA/RNA in litter is theoretically possible, the level of genetic material may not necessarily reflect the level of infective virus (ie. inactivated virus may also be detected). However it is possible that once a virus the pathogen loses its infectivity in a litter environment, its genome will also lose its integrity and quickly degrade, meaning that molecular detection will correlate well with infectivity.

Ultimately, development and validation of such methods against a wide range of pathogens particularly for viruses would put monitoring of such pathogens on a similar footing to the bacteria and protozoa.

Materials and Methods

The design of the experiment was a 2 × 3 × 3 factorial design (+ 2 negative controls), providing a total of 18 treatment combinations plus the negative controls. Twenty isolators were used in the experiment, two for each heat x duration treatment combination.

- Two litter types (UNE and Field)
- Three temperatures (50° 60° and 70^oC)
- Three durations (0, 5 and 10 days) *plus*
- A negative control (new pine shavings)

The UNE litter was generated at UNE (Experiment 1) and the field litter was collected from a breeder farm aged 63 weeks and two broiler farms neat the end of batch, all close to Tamworth. The field litter was used an alternative contaminated litter considering that the vaccines used to generate UNE litter were attenuated vaccinal viruses, and for some pathogens (eg adenovirus, reovirus) no vaccinal treatment was used in Experiment 1. Field litters were collected fresh on day 0, the day of initial exposure to untreated litters.

Litters were bagged (8 litres) in polypropylene green shopping bags, sealed with tape, and individually marked. Eighteen bags were prepared, 9 with Field litter and 9 with UNE litter. Six bags (three from each litter type) were placed immediately in six isolators (in two cat litter trays in each isolator) to expose chickens onto the litter in the bioassay (Islam et al., 2009). The remaining 12 litter bags were placed in a loosely closed autoclave bag to restrict evaporation, and placed the respective oven at a set temperature of 50, 60 or 70˚C. The temperature of the oven was set for a week before the start of the experiment and it was confirmed that the required temperature was being maintained.

Experimental animals: Two hundred and 20 day-old SPF chickens arrived from Melbourne (SPAFAS Australia) in the afternoon of the 6th of Nov (Day 0). Eleven chickens were placed in each of 20 positive pressure isolators in the main UNE isolator facility. The isolators have perforated floors so chickens have limited contact with their faeces. Isolators also have up to 20 air changes/hr. The chickens had *ad lib* access to feed (layer starter then grower) and water, 24hr light for the first 2 days and then 12L:12D thereafter. The chickens were exposed to litter materials at various days as shown in Table 3.

Exposure of chickens to litter: Day 0 litter had no heat treatment applied, coming straight from the animal house or field. Exposure to this litter occurred on the day of chicken arrival (Day 0). The day 5 and 10 exposure occurred subsequently with litter taken from the oven on these days. The litter exposure occurred in the various isolators as shown in Table 3.

A sub-sample of litter (approximately 50gm) was taken from each litter sample at the time of isolator exposure for later determination of dry matter and molecular detection of virus by PCR.

Table 3. Treatment allocations to isolators. Dates and sample collection.

Litter	Temperature	Duration of heat treatment	Isolator	Date litter placed in isolator	Sample for PCR collect
UNE	50°C	0	9	04/11/2009	Yes
		5	19	11/11/2009	Yes
		10	17	16/11/2009	Yes
	60°C	0	7	04/11/2009	Yes
		5	18	11/11/2009	Yes
		10	5	16/11/2009	Yes
	70°C	0	10	04/11/2009	Yes
		5	13	11/11/2009	Yes
		10	24	16/11/2009	Yes
Tamworth	50°C	0	20	04/11/2009	Yes
		5	15	11/11/2009	Yes
		10	8	16/11/2009	Yes
	60°C	0	6	04/11/2009	Yes
		5	11	11/11/2009	Yes
		10	14	16/11/2009	Yes
	70°C	0	23	04/11/2009	Yes
		5	16	11/11/2009	Yes
		10	21	16/11/2009	Yes
Neg Control	Ambient	0	22	03/11/2009	No
	Ambient	0	12	03/11/2009	No

Chickens were reared in isolators for 45 days with a blood sample collected at day 35 post-exposure to litter. Thus there were three bleeding days as well as litter exposure days (one for each of day 0, 5 and 10 days of heat treatment). At day 45 of experiment (21/12/2005), all chickens were weighed, humanly killed and disposed of.

Serology: Serum samples were serologically analysed for IB (ELISA), CAV (ELISA), IBD (ELISA) and ND (ELISA) and FAV8 (ELISA), ILT (ELISA), Reovirus, AE and MDV at UNE. Commercial kits were used ELISA from IDEXX Laboratories Inc. (Maine, USA) for IB, CAV, IBD, Reovirus, AE and ND. Kits from TropBio Pty Limited (JCU, Townsville, QLD) were used for FAV/IBH and ILT. The MDV ELISA was developed at UNE using a serotype 1 (Rispens CVI988) vaccinal virus based on the method described by Zelnik et al. (2004). Full validation and evaluation of the assay has not been completed at this point, but more than 1000 samples from a wide variety of experiments has been tested and the assay has proven to be very sensitive and specific for MDV although it is not serotype-specific. Sensitivity at this stage appears to be greater than for qPCR detection of MDV in spleen. Results from this assay are included in this report, but should be interpreted as indicative only at this stage.

DNA extraction and qPCR for MDV : DNA was extracted from the litter samples collected at the time exposure to the chickens, for qPCR detection of MDV1 by the method of Islam et al. (2006). A known amount of litter sample was taken and 4×volume of MilliQ water was added to each sample, mixed by shaking and sieved through 60mm and then 2mm metal sieve and the volume of the filtrate was recorded. The filtrate was centrifuge at 10,000rpm for 10 minutes. The supernatant was discarded and DNA was extracted from 5mg of the sludge using DnEasy DNA extraction kits (Qiagen, Pty Ltd.). The DNA was quantified and quality assessed using a NanoDrop spectrophotometer.

Bacterial and coccidial counts: Litter samples (approx. 100 ml) were collected from under the floor of each isolator to enumerating *C. perfringens* and coccidial oocysts. For enumeration of *C. perfringens* 2 g of the sample were transferred into 15 mL MacCartney bottles containing 10 mL of anaerobic broth and the suspension was homogenized for 2 min in CO_2-flushed plastic bags using a bag mixer (Interscience, St. Norm, France). The suspension was then serially diluted in 10-fold increments in anaerobic broth Engberg *et al.* (2004). One millilitre of the homogenized suspension was then transferred into 9 mL of anaerobic broth and serially diluted from 10^{-1} to 10^{-6}. From the last three diluted samples, 0.1 mL each was plated on Tryptose-Sulfite-Cycloserine and Shahidi-Ferguson Perfringens agar base (TSC & SFP) (Oxoid, CM 0587 OPSP) mixed with egg yolk emulsion (Oxoid, SR0047) and incubated at 39oC for 7 days before enumeration of microbial populations. The bacterial numbers are expressed as $\log_{10}$ cfu per gram of sample. An attempt to count the coccidial oocysts from the litter samples was also made.

Results

There were five viruses for which there was no detectable seroconversion amongst chicks in the bioassay. These were IBV, NDV, ILTV, Reovirus and AEV. MDV and CAV infectivity was found in both types of litter whereas FAV and IBDV were positive only in the field litter. The results are presented in Table 4. All control chickens (20 from two isolators) were negative for all viruses except one chicken which was positive for MDV with a low titre.

Table 4. Proportion of bioassay chicks serologically positive to various viruses at day 35 days post exposure to putatively infective litter.

Litter	Temperature	Duration	MDV	CAV	FAV	IBDV
Field	50	0	6/10 (60%)	10/10 (100%)	10/10 (100%)	0/10 (0%)
		5	1/10 (10%)	2/10 (20%)	0/10 (0%)	0/10 (0%)
		10	6/10 (60%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
	60	0	10/10 (100%)	10/10 (100%)	9/10 (90%)	0/10 (0%)
		5	2/10 (20%)	0/10 (0%)	0/10 (0%)	1/10 (10%)
		10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
	70	0	7/10 (70%)	10/10 (100%)	10/10 (100%)	10/10 (100%)
		5	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
		10	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
UNE	50	0	4/10 (40%)	9/10 (90%)	0/10 (0%)	0/10 (0%)
		5	2/10 (20%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
		10	8/10 (80%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
	60	0	3/10 (30%)	1/10 (10%)	0/10 (0%)	0/10 (0%)
		5	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
		10	7/10 (70%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
	70	0	5/10 (50%)	10/10 (100%)	0/10 (0%)	0/10 (0%)
		5	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)
		10	8/10 (80%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

The CAV response (% serologically positive) showed that both litters (UNE and Field) were highly infective at day 0 with the exception of low infectivity in one of the day 0 UNE litters (10%). CAV lost infectivity by day 5 at 60 and 70˚C, but retained infectivity at 50°C up day 5 in the Field litter (Figure 1).

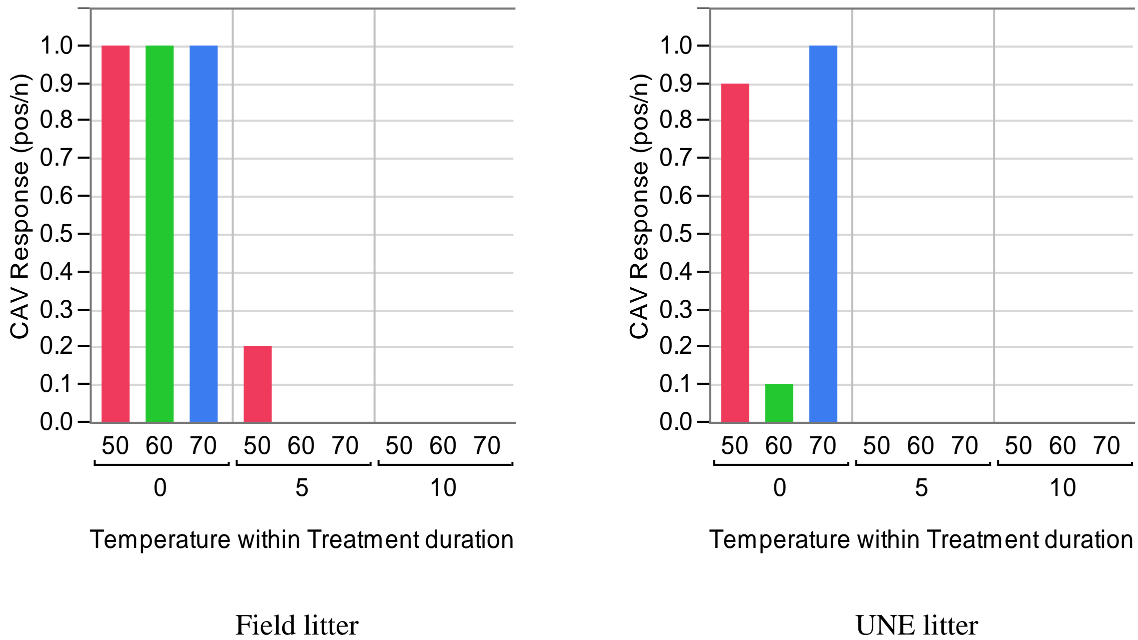

Figure 1. Ratio of chicks serologically positive for CAV by temperature and duration for Field and UNE litters.

The MDV response (% serologically positive) data revealed that both Field and UNE litters were positive for MDV. The Field litter retained MDV infectivity at 50° up to day 10 but only to day 5 at 60°C. At 70°C MDV was inactivated by day 5. However a very different result was found for the UNE litter, which remained infective up to 10 days in all three temperatures (Figure 2).

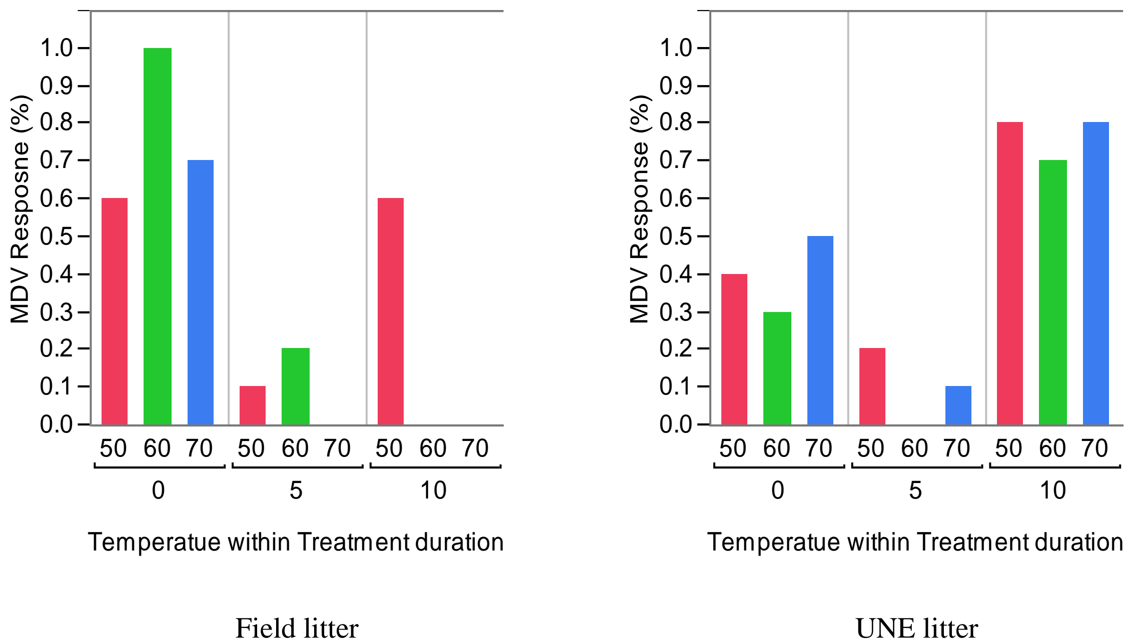

Figure 2. Ratio of chicks serologically positive for MDV by temperature and duration for Field and UNE litters.

For FAV only the field litter was positive, not the UNE litter. This is not surprising as that the shedder chickens that generated UNE litter were not vaccinated with FAV. The field litter was highly infective at day 0 but no infectivity was present by day 5 at all temperatures including the lowest 50°C (Figure 2.3).

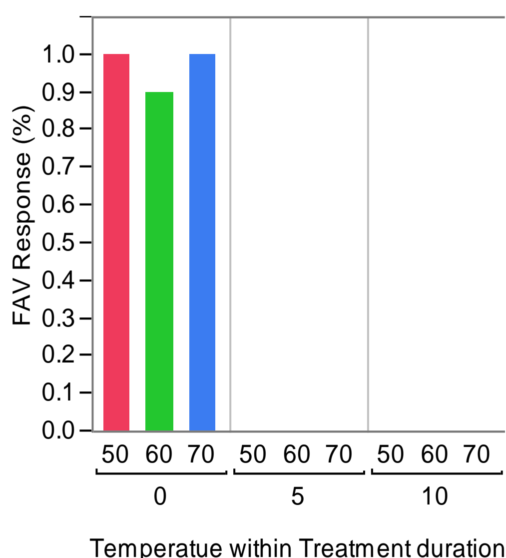

Figure 3. Ratio of chicks serologically positive for FAV by temperature and duration for Field litter.

The Clostridium spore count from the isolator litter revealed that all litters were positive for *Clostridium perfringens* including the negative controls. There was no significant effect of litter or temperature on the *C. perfringens* count, however there was a trend towards decreasing perfringens count with the increase of temperature in the UNE litter (Figure 4).

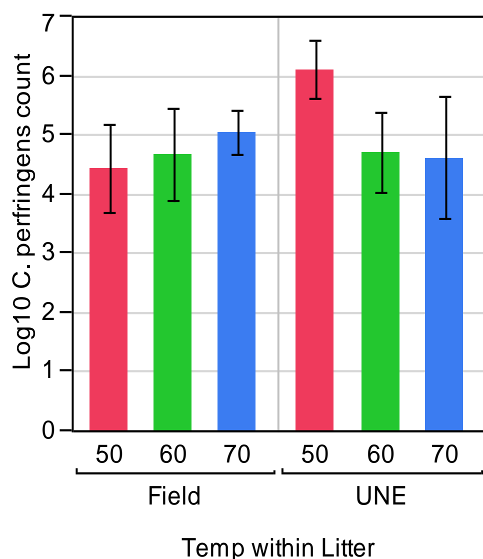

Figure 4. Clostridium perfringens count in isolator litters, effect of litter type and temperature.

Coccidial oocyst counts were negative.

DNA extraction and real time PCR was done for the quantification of MDV in the UNE litter as a test case. The DNA extraction produced reasonable amounts of DNA (20-60ng/µl) of variable quality (mean A260/280 = 1.36, range 0.62-2.15). Real-time quantitative PCR revealed that all 9 UNE litter samples were positive for MDV with a variable quantity of MDV genome in the litter (Table 5). There was no significant association between the MDV genome copy number in the litter and the MDV serological response of chickens exposed to the litter (Figure 5).

Table 5. MDV vial genome copy in UNE litters given different heat treatments.

Litter sample	Day	Temp	Litter Wt extracted (g)	Total MDV recovered (Viral genome copy number, VCN)	MDV load/g litter (VCN)
1	0	50	6.7	74,385	11,102
2	0	60	5.38	93,192	17,322
3	0	70	6.6	0	0
4	5	50	12.5	92,943	7,435
5	5	60	15.2	236,225	15,541
6	5	70	12.8	786,743	61,464
7	10	50	13.8	553,470	40,107
8	10	60	11.4	94,468	8,287
9	10	70	15.5	395,361	25,507

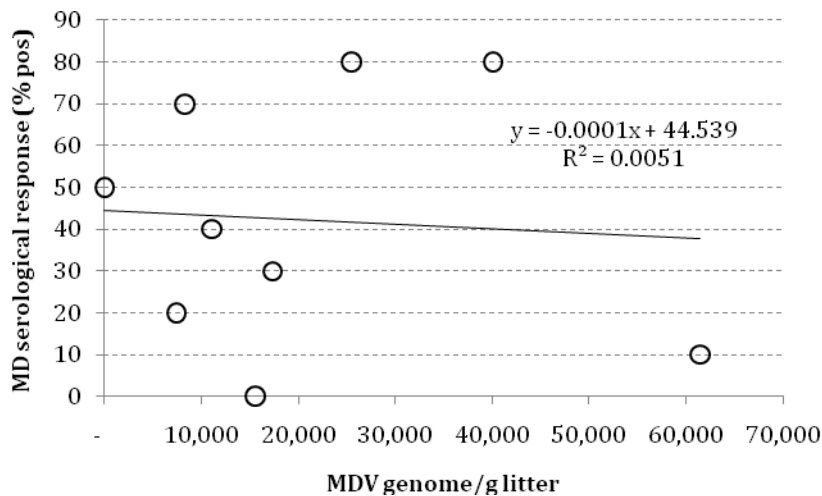

Figure 5. Linear association between of MDV copy number in litter and the MDV serological response (% MDV positive) of chickens exposed to the litter in the chick bioassay.

Summary and conclusions

Our findings with regard to the original hypotheses are summarised below.

Hypothesis 1. By deliberately challenging or vaccinating chickens with a wide range of organisms we will successfully induce litter contamination and transmission for a wider range of viral pathogens than previously and from field litter tested concurrently.

This hypothesis was partially supported. Shedder chicks (Expt 1) were serologically positive at day 35 for 5 of 6 vaccines administered for which it could be measured. The exception was AE. Seroconversion to fowl pox could not be measured, as we did not have an assay for it. Shedder chicken response to challenge with the Eimeria vaccine or clostridial challenge was not measured. On the other hand pooled field litter contained highly infectious FAV, for which we did not have challenge virus at UNE. Seropositive responses to challenge in the shedder chicks resulted in successful litter transmission of MDV and CAV only. There was no litter transmission of IBV, NDV, IBDV or ILTV from these chicks detected in the litter bioassay despite the chicks being seropositive. This may be due to lack of shedding of virus (wrong window of time, low shedding rates) or failure of the virus to transmit on litter. We know from Project 06-15 that IBV and NDV transmit poorly on litter but that that IBDV will, and in experiment 2 of the current project the field litter was also sporadically

infectious for IBDV. This suggests that our model for generating infectious litter requires refinement and measurement of actual viral shedding in addition to seroconversion.

Hypothesis 2. Incubation for 10 days at 70˚C will completely remove viral infectivity in litter. In general inactivation will increase with increasing temperature and increasing duration of exposure to high temperature.

This hypothesis was partially supported. FAV infectivity was lost by day 5 at all temperatures. CAV infectivity was lost by day 5 at 60 and 70˚C and by 10 days at 50˚C. Data for IBDV are inconclusive, however there was no infectivity detected by day 10. However significant MDV infectivity was detected at day 10, at all temperatures for the field litters at 50˚C for the UNE litter. The hypothesis could not be tested for IBV, NDV and ILT as there was no apparent transmission by litter at all.

Hypothesis 3. We will be able to detect and quantify viral DNA directly from litter.

This hypothesis was supported. For the test virus (MDV) we were able to directly measure viral load using a simple protocol on our 1st attempt. Refinement of the method may lead to increases in sensitivity.

Hypothesis 4. The amount of viral DNA we detect will correlate with the level of infectivity for that sample in the bioassay

This hypothesis was not supported for the test virus (MDV) and the samples tested (UNE samples). Unfortunately the pattern of infectivity for MDV for the UNE samples was odd (Figure 2) appearing to increase with exposure to temperature. For this reason, and because methods of viral quantification from litter have not been optimised, the hypothesis needs to be tested with a wider range of samples following validation of the viral quantification method. CAV and FAV gave good results in this experiment, and these two are good candidates for such testing. Time and money precluded this being done on the current short project.

With regards coccidia and *Clostridium perfringens* it is suggested that future experiments use recovery from cloacal contents at the end of the experiment, rather than from the accumulated faeces under the isolator floor. *C. perfringens* appeared ubiquitously in all treatments including controls, whereas the reverse was true for coccidia, with zero counts even for chicks exposed to the day 0 fresh litter. This contrasts with the good results obtained on inactivation of coccidia in project 06-15 using cloacal contents at the end of the experiment.

In broad terms the aims of the project were met and proof of concept confirmed for

- *in vitro* testing for inactivation of virus in litter by heat
- direct detection and quantification of viral pathogens in litter.

References.

Engberg, R.M., Hedemann, M.S., Steenfeldt, S., Jensen, B.B., 2004, Influence of whole wheat and xylanase on broiler performance and microbial composition and activity in the digestive tract. Poult Sci 83, 925-938.

Islam, A., Cheetham, B.F., Mahony, T.J., Young, P.L., Walkden-Brown, S.W., 2006, Absolute quantification of Marek's disease virus and herpesvirus of turkeys in PBL, feather tip and dust samples using real-time PCR. Journal of Virological Methods 132, 127-134.

Islam, A.M.F.M., Walkden-Brown, S.W., Groves, P.J., Wells, B., 2009, Determination of infectivity of viral pathogens in poultry litter using a bio-assay: effect of chicken type and age of exposure. Proc. Aust. Poult. Sci. Symp. 20, 176-179.

Plain English Compendium Summary

Project Title:	
Project No.:	09-34
Researcher:	Prof. Stephen Walkden-Brown
Organisation:	University of New England
Phone:	02-6773 5152
Fax:	02-6773 3922
Email:	swalkden@une.edu.au
Objectives	Overall: To improve our ability to measure viral pathogen load in end of batch broiler litter and to determine the effects of temperature and duration on inactivation of infective virus in litter. Specifically: 1. To determine the effects of incubation of litter at temperatures of 50, 60 or 70˚C for 10 days on the level of viral infectivity of litter; and 2. To use quantitative molecular methods to measure virus levels in litter material and see whether this correlates with infectivity.
Background	Re-use of litter by broiler chickens can reduce the environmental impact and cost of chicken production but uptake of the practice is limited by risks of pathogen carryover. On Project 06-15 our research team has developed an effective chick bioassay to measure viral infectivity in litter, and has made detailed measurements of temperature during partial composting of litter in heaps or windrows. However the large field trials and the bioassay are expensive. Therefore this project was proposed to test proof of concept of: • use of laboratory incubators to simulate different litter heap temperatures to provide more detailed information on temperatures and durations required to inactivate viral pathogens • use of real-time PCR (qPCR) methods to directly quantify viral pathogen load in litter and relate this to changes in infectivity
Research	Two experiments were conducted, one to generate infective litter containing vaccine strains of many viral pathogens and one to test the effects of heating at 50, 60 or 70 ˚C for 0, 5 or 10 days on the level of viral infectivity in litter as determined in a chick bioassay using 220 SPF chicks in 20 isolators. DNA extraction was carried out for 9 litters and this tested for Marek's disease virus (MDV) using qPCR.
Outcomes	Regarding objective 1, litter containing CAV and FAV lost infectivity by day 10 at all 3 temperatures, with loss of infectivity at day 5 being temperature dependant. The infectivity data for MDV and IBDV were less clear although there was an overall trend towards reduced infectivity with duration of heat treatment. There was no apparent transmission of IBV, NDV or ILT via litter in the experiment. Regarding objective 2, the amount of MDV present in litter was successfully measured using qPCR but there was no association between MDV viral load measured in litter and the infectivity of that litter for chicks in the bioassay.
Implications	These results provide significant "proof of concept" for laboratory scale temperature treatment of litter to measure effects on viral inactivation rate, and for quantitative measurement of pathogenic virus directly from litter using real time PCR. However considerable additional work is required to bring these methods to full application in industry.
Publications	Short project only. Results will be presented at the Australian Veterinary Poultry Association meeting in May 2010 and also PIX.