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Rural Industries Research and Development Corporation

Investigation of the Cause of Miliary Hepatitis in Laying Chickens

A Model of the Disease

A report for the Rural Industries Research and Development Corporation

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Abbreviations

PBS –Phosphate Buffered Saline

Contents

Executive Summary

Miliary hepatitis is a disease of laying chickens which can cause mortalities and loss of egg production to broiler breeder and egg layer enterprises. Its cause is not known but is thought to be a bacterium and produces a characteristic lesion in the liver observed by histopathology. This trial attempted to establish a model of the disease.
Research is important to establish a causative organism so that preventative and control measures, such as vaccine development, can be undertaken.

Background
Miliary hepatitis is an ephemeral and sporadic cause of death in laying chickens. It is associated with rearing and housing of the birds on the floor but has been known to occur in cage-housed birds. There is an apparent seasonality, occurring in the warmer months in a number of states including Victoria, NSW and Queensland. Mortality rates can be up to 10% over a few weeks. The mortality appears to respond to treatment with antibiotics indicating a possible bacterial cause.

The disease itself is characterised by a short period of illness and rapid death in laying birds. Small white or yellow spots in the livers are consistently found in post mortem but bacteria are not observed or able to be cultured by conventional diagnostic means. Histopathology shows focal hepatocellular necrosis with a minimal infiltration of granulocytes. Special staining techniques for detection of microorganisms fail to reveal an infectious agent.

It is speculated that the cause may be a toxin released from organisms growing in the intestine and transported by the hepatic portal circulation to the liver where it produces necrosis of the hepatocytes. The physiological state of the liver in egg layers may induce the susceptibility of the bird to intoxication.

Objectives
The aim of this study was to determine whether the origin of the microorganism is the liver or the intestine. This was a starting point for further work to use more advanced cultural techniques such as strict anaerobiasis, egg inoculation and tissue culture. This trial would create a model for disease pathogenesis useful for further studies.
A second objective was to test *Campylobacter coli* and *Clostridium sordellii* cultured from natural disease cases of miliary hepatitis in this model to see if they can reproduce the disease.

Beneficiaries
People to benefit from this study were diagnostic laboratories and chicken producers who would have a better understanding of the management control measures necessary against miliary hepatitis. The results may lead to vaccine development by a biotechnology company.

Methods
Small groups of laying chickens were dosed at the peak of egg production with homogenates of liver and intestines from affected birds and organisms previously isolated from affected birds and thought to be causal agents. The birds were expected to die acutely and the disease confirmed histologically. Bacteria would then be cultured from the gut or liver and identified and stored for further study.

Results
No birds died of miliary hepatitis so the objective to produce a model of the disease was not fulfilled. Use of the *Campylobacter coli* and the *Clostridium sordellii* cultures in the trial has given evidence that these organisms are not primary aetiological agents.

Implications for relevant stakeholders
With this investigation neither a model nor a primary aetiological agent was defined. Two candidate organisms were shown not to have an effect on the development of miliary hepatitis in laying birds. The cause and a means of manipulating the disease under laboratory conditions were not identified.

Recommendations
The basis of this model should be used to investigate other organisms recovered from cases of miliary hepatitis. Manipulation of the environment to induce the disease could be applied eg by having wet litter with higher ambient temperatures.

Introduction

A disease has been recognised by the meat and egg poultry industries for more than a decade and has been called "spotty liver", "summer hepatitis" and "miliary hepatitis". It occurs on broiler breeding and free-range egg farms where the birds are floor reared and housed.. It has also been known to occur on farms where birds have been floor-reared and then placed in three tier cage systems.

The disease occurs most frequently in Summer and Autumn in Victoria but it has been known to occur in winter. It has a sporadic and ephemeral nature and there have been a number of years in Victoria where it has not been reported. The disease has been known to occur in Queensland, New South Wales and South Australia and is often reported in the warmer periods.

The losses from the disease can be significant and up to 10% of a flock can die over a few weeks before it is recognised. At about the same time, egg production drops have been reported. A cessation of mortality occurs following antibiotic therapy and this implies that the cause is bacterial.

The disease is characterised by a short period of illness followed by death in laying hens. It is often difficult to detect sick birds and they are found dead. At post mortem examination of birds, the consistent change that is seen are pale, white or yellowish spots, usually the size of millet seeds, scattered throughout the liver. They usually are neither depressed below the surface nor raised above the surface of the liver. Associated lesions are pinpoint haemorrhages in other internal tissues such as pericardial fat and on the surfaces of the heart. Bacteriological culturing by aerobic, microaerophilic or anaerobic techniques has failed to consistently isolate an organism from the livers. Histopathological examination of the liver lesions has shown multifocal areas of coagulative necrosis with usually a mild infiltration of heterophils, however, some birds die so quickly that the heterophils are absent. Searches for bacterial, fungal and protozoal causes of the lesions in the liver are unrewarding. Silver stain, PAS stain and gram stain fail to reveal organisms in the liver.

It has been speculated that the liver lesions may be due to the presence of a bacterial toxin produced in the intestines and carried by the liver's blood supply to be deposited in the liver causing the miliary necrosis of the hepatocytes and death of the bird. The physiological state of the chicken being in full production may be an important trigger to the disease. The metabolism of the hepatocytes of a bird in lay with high turnover of fat and active cells because of the production, may lay the foundations for the effects of the bacterial toxins.

Objectives

There were two objectives of this study. Primarily to establish if the originating cause of the lesions of miliary hepatitis is located in the liver or the intestine, whether it be bacteria or their toxins. This would enable an establishment of a model for future work and narrow the candidate causes. Secondly,

to see if *Campylobacter coli* and *Clostridium sordellii,* which are two organisms isolated from cases of affected chickens, can reproduce the disease.

Methodology

Birds were sourced from a flock of inbred white leghorns in which the line had been closed for 20 years. Bantam genes had been intergressed into white leghorns from the Australian commercial gene pool. They had been reared on wire and slats for all their life until the commencement of the trial which started with a moult. Inoculums were administered about 12 weeks later when they were laying at their peak of 60-66% eggs/day. They were 126 weeks old at the time of administration of the inoculums. No significant mortalities were previously recorded in the flock.
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Twenty five of theses commercial laying birds were housed on wood shavings on the floor, in a controlled climate in PC2 facilities and fed commercial layer rations. They were placed in pens (five per group) and each was identified by a wing band number.. The groups were designated;

- A - controls and were given PBS diluent only,
- B -were given an inoculum of homogenised intestines and contents from affected birds,
- C -were given homogenised liver from affected birds
- D - were given an inoculum of a candidate *Campylobacter coli* cultured from affected birds.
- E – were given an inoculum of a candidate *Clostridium sordellii* cultured from affected birds

The inoculums were prepared from a recent field case where the unopened intestines and livers were frozen at minus 80^{o} C as soon as possible after death and had been kept frozen for 20 weeks prior to the preparation of the inoculums. The frozen organs were thawed overnight, homogenised in a blender, suspended in PBS, centrifuged and the supernatants were dispensed in syringes with tubing suitable for gavaging the birds.
The *Clostridium sordellii* came from a diagnostic laboratory where it was isolated from the duodenum of affected birds.
The *Campylobacter coli* was sourced from a diagnostic laboratory where it was isolated from affected birds.

Intestinal and liver inoculums: 5 mLs were inoculated *per os* from a suspension of either intestine or liver from one bird.
Clostridium sordellii inoculum: 5 mLs of an 18 hour brain heart infusion broth of the clostridium was inoculated *per os* into each bird of the group
Campylobacter coli inoculum: 5 mLs of a log phase culture of Bain's sloppy agar was inoculated *per os* into each bird of the group.
Control inoculum: 5 mLs of the diluent PBS was inoculated *per os* into each bird of the group.

The birds were closely monitored and liver and intestine specimens were to be collected. The intention was to collect and store at minus 80^{o}C if a bird died and a sample of each was to be taken for bacterial culture and examined aerobically, microaerophilically and anaerobically. A sample of intestine and liver was to be fixed in formalin for histopathological examination

Bacteriology
Preparation of Inoculums:
The *Campylobacter coli* was received freeze dried and was resuscitated in 10 mL sloppy Bain's agar and incubated microaerophilically at 37^{o}C for 2 days, then subcultured onto a Skirrow's plate and incubated microaerophilically at 37^{o}C for 2 days. The organism was then confirmed as *Campylobacter coli* and inoculated into Bain's sloppy agar for the experimental inoculum.

The *Clostridium sordellii* was received on beads and sub cultured onto sheep blood agar and incubated anaerobically at 37°C for 24 hrs. The organism was confirmed as *Clostridium sordellii* and subcultured onto sheep blood agar and then into Brain Heart Infusion broth (BHI) for the inoculum for the trial.

Cell counts of inoculums
Cell count for the *Campylobacter coli* inoculum = 3.25×10^6 CFU/mL
Cell count for *Clostridium sordellii* inoculum = 1.39×10^5 CFU/mL

Organ inoculums:
The liver and intestinal inoculums were cultured to check for *Campylobacter coli*, *Clostridium sordellii*, *Clostridium perfringens* and aerobes
From aerobic culture of the liver inoculum there was a moderate growth of a small grey colony identified as *Enterococcus durans*. A moderate anaerobic growth of small grey colonies was identified as *Clostridium perfringens*. No evidence of campylobacter was present in the liver inoculum.
The intestinal inoculum had a light growth of *E. coli* and pinpoint colonies identified as *Enterococcus faecalis* on aerobic culture. Anaerobic culture revealed a small grey colony that was not a clostridium. There was no evidence of Campylobacter.

Histopathology
Liver and intestines from affected birds would have been fixed in 10% buffered formalin and sectioned and stained with H&E.

Flow Chart

Day 1 minus 4

Prepare campylobacterial culture for inoculation minimum 25 mLs Bain's sloppy agar

Day 1 minus 1
Thaw frozen liver and intestine overnight at room temperature
Inoculate BHI broth with *Clostridium sordellii* and incubate over night.

Day 1
Prepare homoginised inoculums of liver and intestine of a mininimum of 25 mLs each
Prepare bacterial inoculums.
Inoculate:

Groups A	B	C	D	E
Control PBS	Homog intest	Homog liver	Campylobacter	Clostridium
5mL ea	5mL ea	5mL ea	5mL ea	5mL ea
p/os	p/os	p/os	p/os	p/os

Days 1 to 10

Observe and euthanase moribund birds exhibiting depression and weakness. Post mortem examinations to be performed on dead or euthanased birds.

Collect livers, intestines and affected organs for freezing at minus 80° C for further trials and examinations.
Collect intestines, livers and affected organs for histopathology.
Smear and culture intestines for clostridia from duodenal contents.
Smear and culture colon for Campylobacter.

Day 10
Euthanase birds – end of trial. Birds to be examined grossly for liver lesions

Results

No birds died during the trial period
Post mortem examination at the end of the trial did not detect any liver lesions consistent with miliary hepatitis.

Outcomes

A model for the production of the disease syndrome, miliary hepatitis in laying chickens, was not established.
Inoculation by mouth of *Campylobacter coli* and *Clostridium sordellii* cultured from cases of the disease does not appear to reproduce the disease in laying birds.
The inoculation by mouth of suspensions of homogenised liver and intestine did not reproduce the disease in laying birds.

Implications and Recommendations

Further investigations are recommended using bacteria isolated from natural cases. The two cultures from natural cases tried in this experiment (*Campylobacter coli* and *Clostridium sordellii)* inoculated by mouth did not reproduce the disease probably indicating that they are not primary pathogens and consequently others organisms isolated from field cases should be investigated.
Manipulations of conditions surrounding the environment of the experimental chickens should be included in future trials such as incorporating hotter, wet conditions in the pens. Stress on the birds in addition to laying stress may provoke the establishment of the disease.

Plain English Compendium Summary

Project Title:	Investigation of the cause of miliary hepatitis in laying chickens
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Objectives	To find a model of miliary hepatitis in laying chickens and to test two bacterial isolates in this model to see if they are a cause of miliary hepatitis
Background	This is a pilot trial to establish a model so that laboratory manipulation can establish the cause of the disease.
Research	Laying hens at their peak of production were inoculated by mouth, extracts of intestines and liver and two bacteria previously collected from affected birds and observed for 10 days in the hope that the disease miliary hepatitis could be reproduced. The disease has not been reproduced in this trial by the tissues or by the two bacterial inoculums given by mouth.
Outcomes	Understanding of the cause of miliary hepatitis has been advanced but the disease remains an enigma to the industry. The evidence is that two bacteria isolated in the course of investigation of natural disease appear not to be primary pathogens.
Implications	WM Forsyth, R Hodgeman, BS Oyay and A Howse
Publications	Investigation of the cause of miliary hepatitis in laying hens (Dec 2005 in preparation)