2 General Materials and Methods

For this PhD project, two animal experiments were conducted. The first experiment had duration of 56 days (20th July '09 – 23rd Sept '09) and was conducted in four climate controlled rooms of the UNE Animal house. It was designed to investigate the transmission rate of the Rispens/CVI988 vaccine virus in specific pathogen free chickens. The 2nd experiment was conducted in the UNE Isolator facility and was designed to investigate the effect of vaccination to challenge interval on the protection provided by the Rispens/CVI988 vaccine against MD and the kinetics of pathogenic MDV-1 and Rispens/CVI988 in coinfected chickens. This experiment started on 28/06/10 and ended on 02/09/10.

2.1 Animal Management

The chickens used in all experiments were maintained and treated according to the Australian code of Practice for the care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 2005, and the experiments were approved by the UNE Animal Ethics Committee.

2.1.1 Experimental Chickens

Day-old Specific Pathogen free (SPF) white leghorn chickens (SPAFAS, Melbourne Australia) were used in the first experiment. For the second experiment, day-old unvaccinated commercial female ISABROWN chickens from parent stock vaccinated with Rispens/CVI988 were used.

2.1.2 Animal House rooms

The chickens of the first experiment were reared in 4 climate controlled rooms. The climate control rooms measured 3.6 × 4 m and were supplied with temperature controlled filtered air. In each room, the chickens were placed in floor pens which were approximately 2.5 x 2.5 m and. Pine wood shavings at a depth of 5 - 10cm were used as bedding material (Figure 2.1).

Room temperature settings started at 35 °C at day -2 and reduced by 2 °C per day until 25 °C was reached.

Figure 2.1 Typical set up of the floor pens used in the experiment. For the first few days, ice cube trays were used as additional water source.

The chickens were offered feed (chicken starter and grower, Ridley Agricultural Products, Tamworth) and water *ad libitum* throughout the experiment. Lighting was initially 24 h light (days 1 - 2) followed by 12 h light : 12 h dark controlled by an automatic timer. Birds were inspected twice daily for general well being.

2.1.3 Isolator unit

Twenty four purpose-built soft body chicken isolation units kept in the UNE isolator facility (Building W33, Figure 2.2) were used in experiment 2. The isolator facility is under constant negative pressure and all outgoing air is HEPA filtered. Each isolator has a length of 2.05 m, width of 0.67 m and height of 0.86 m with a stainless steel frame. The floor is 2.5 mm stainless

steel (304 2b) with 12.7 mm holes punched out with centres 17.45 mm apart staggered providing a 49% open area. This is critical to enable housing of chickens from day-old to adult without faecal accumulation on the floor. Isolators are positive-pressure and soft-bodied with disposable plastic linings, gauntlets and gloves, disposed of after every experiment.

Figure 2.2 Interior of PC2 animal facility containing 24 isolators, also showing main air inlet duct which carries HEPA filtered, heated air to each isolator.

Isolators are provided with temperature-controlled HEPA-filtered air via a central air supply system and air is scavenged from each isolator via a series of scavenger ducts and HEPA filtered on exit. Both inlet and outlet airflow can be regulated manually to allow adjustment for isolator pressures. There are 12 – 23 airchanges/hour per isolator unit depending on fan settings.

Isolators are individually fitted with heat lamps under separate thermostatic control. The entire feed supply for each experiment was loaded into a large feed hopper for each isolator and sealed for the duration of the experiment. Four nipple drinkers were provided in each isolator connected with a low-pressure water supply. The entire facility was on an automated power backup via a 13KVA generator.

Chickens were offered feed (commercial layer starter then grower, Ridley Agricultural Products, Tamworth) and water *ad libitum* throughout the experiment. Isolator temperatures were set at 34 ˚C for the first two days and then decreased by 1 ˚C every second day until a temperature of 22 ˚C was reached. Lighting was initially 24 hr light (days 1 - 2) followed by 12L : 12D lighting set with an automatic timer.

2.1.4 Cleaning, disinfection and biosecurity

Physical cleaning was performed in each room and isolator units including all appliances immediately after every experiment. All appliances and materials used to build isolators were physically cleaned with detergent followed by high-pressure steam cleaning. A second cleaning was carried out with detergent and complete treatment with a virucide (0.5 – 1 % Virkon RS, Antec International Ltd, England, UK). The experimental rooms including isolation units were fumigated twice with formaldehyde before the start of experiment. All materials passed into isolators were placed into the access box, sprayed with Virkon S and left for 20 minutes before being introduced into the isolator. Staff changed into protective clothing and footwear on entering the facility, and wore disposable hairnets while in it.

2.1.5 Vaccination of birds

Upon arrival at UNE, experimental chickens were vaccinated subcutaneously (sc) under the loose skin on the dorsal aspect of the neck just below the head, using recommended doses of vaccine and diluent. Vaccines were thawed at 36 °C in a water bath and used within 30 minutes of thawing as they are cell associated and viability is lost with loss of cell integrity. Disposable sterile one ml syringes and 21 G needles were used throughout. In the first experiment vaccination was performed at hatch (day 0) and in the second experiment vaccination was performed sub-cutaneously at various days following experimental design. Three commercially available Rispens/CVI988 vaccines used in the first experiment were

obtained from Bioproperties Pty. Limited, Australia, Fort Dodge Australia and Intervet Australia Pty. In the second experiment, only the Rispens/CVI988 vaccine from Bioproperties Pty. Limited, Australia was used.

2.1.6 Challenge of birds with MDV-1

In experiment 2 chickens were challenged with the very virulent MDV-1 isolate 02LAR at a dose of 400pfu (plaque forming unit) per chicken in 0.2 ml via the sc route. The virus was grown and titrated in primary chicken embryo fibroblasts (CEF) at UNE. The protocol for the determination of MDV titre in CEF was as follows:

- On the day before titration, one or two six-well plates of CEF was placed
- 900 µl of CEF growth medium was added to a number of bijoux and labelled
- The virus was thawed at 37 °C in a water bath and mixed the content of the viral well
- 100 µl of viral content was taken and added to one prepared bijou following 10 fold serial dilutions in the remaining bijoux
- 200 µl of a range of dilutions added to duplicate wells of the CEF
- Then the plates are incubated at 38.5 °C, 5 % CO_2 as appropriate until plaques became visible
- The plaques were counted under an inverted microscope at the dilution that gave the easiest distinction between plaques
- The titre was calculated using the following equation:
  Count x 5 x dilution factor = titre (pfu per ml)

The virus stored in liquid nitrogen at -196 °C. Before use the virus was thawed at 37 °C in a water bath and diluted with M199 media containing 10 % fetal calf serum and antibiotics/antimycotics (all sourced from Invitrogen, Australia) and used within 30 minutes of thawing. Disposable sterile 1ml syringes and 21 G needles were used.

2.1.7 Euthanasia of chickens

Euthanasia was performed by AEC approved personnel following the method described by Zander *et al.* (1997). At the time of euthanasia the chicken was held in a fixed position by one hand. The thumb and the index finger of the other hand gripped the base of the skull and the

middle and ring fingers were held under the beak. Cervical dislocation was completed by the rapid extension of the arm holding the head with a concurrent dorsal flexion of the head.

2.1.8 MD lesion detection and scoring from birds

Standard *post-mortem* examination was carried out for all dead and euthanized chickens throughout the experiments (Bermudez and Stewart-Brown, 2003). Carcasses were wet in warm water containing water and detergent then checked for nodular lesions on the skin. Breast and thigh muscles were inspected for discrete lymphoid tumours or diffuse infiltration. The thymus was inspected for atrophy and scored 0 - 3 in ascending order of severity (0 = normal, 3 = complete or almost complete atrophy). After opening the carcass, the liver, spleen, gonads, kidney, proventriculus, mesenteries, gastro-intestinal tract, heart, lungs were examined for gross enlargement and discrete or diffuse MD lesions. Figure 2.3 shows some organs lesion found during *post-mortem* of the birds. The bursa of Fabricius was examined and scored for atrophy as for the thymus. Tumorous enlargement of the spleen was recorded as gross MD lesions. The sciatic nerve and plexus were examined for enlargement, change of colour or loss of striations, or asymmetry in size. Histopathological confirmation of lesions was not carried out. However unchallenged control chickens were always present for comparative purposes.