# FURTHER DEVELOPMENT OF A LIVE ATTENUATED VACCINE FOR CHICKEN ANAEMIA VIRUS

**A report for the Australian Egg Corporation Limited**

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July 2005

AECL Publication No 05/09
AECL Project No UM-55A

ISBN 1 920835 60 1
ISSN 1440-6845

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*Publication No. 05/09*
*Project No. UM-55A*



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Published in July 2005

# Foreword

Chicken Anaemia Virus (CAV) is a virus whose role in immunosuppressive diseases has become more evident over recent years. It has now been found to be causally associated with high Marek's disease mortalities, with progeny from poorly CAV-immune parents showing exacerbated MDV mortalities both in Australia and overseas. CAV has frequently been isolated from affected birds in ultravirulent MDV outbreaks. Increasing attention is now being paid to declining immunity in donor flock dams which result in variable to poor progeny performance. The importance of maintaining a good level of immunity in donor flocks producing hatching eggs is similar to the situation seen in Infectious Bursal Disease Virus in the past. Currently in Australia, there is no attenuated CAV vaccine available. Fully virulent CAV has been used in a controlled exposure protocol, but while this has been successful, it perpetuates the presence of virulent CAV in the environment. The problem of declining immunity in donor flocks is not addressed by this approach because of the risk of exposing subpopulations of birds within donor flocks that are susceptible to reinfection with CAV and hence vertical transmission. The move to cage rearing is likely to increase the risk of clinical CAV infections as cage-reared birds are less likely to be uniformly exposed and thus less likely to have developed immunity by the time they commence production. The need for a safe immunogenic attenuated CAV vaccine is clearly evident. Improved control of CAV is also likely to result in reduced need for medication of flocks to control secondary diseases, and thus benefits in environmental sustainability and public health.

A previous RIRDC Egg Program-funded project (UM-37A) made considerable progress towards developing a defined attenuated CAV vaccine strain. The aim of this project was to perform a full assessment of the potential of the mutants developed thus far, determining their safety and efficacy as vaccines delivered in day old chicks.

This project was funded from industry revenue, which is matched by funds provided by the Federal Government.

This report forms part of the Australian Egg Corporation Limited R&D program, which aims to assist in developing the Australian egg industry and enhancing its export potential

**James Kellaway**
Managing Director
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# Executive Summary

Chicken Anaemia Virus (CAV) is a virus whose role in immunosuppressive diseases has become more evident over recent years. It has now been found to be causally associated with high Marek's disease mortalities, with progeny from poorly CAV-immune parents showing exacerbated MDV mortalities both in Australia and overseas. CAV has frequently been isolated from affected birds in ultravirulent MDV outbreaks. Increasing attention is now being paid to declining immunity in donor flock dams, which results in variable to poor progeny performance. The importance of maintaining a good level of immunity in donor flocks producing hatching eggs is similar to the situation seen in Infectious Bursal Disease Virus in the past. Currently in Australia there is no attenuated CAV vaccine available. Fully virulent CAV has been used in a controlled exposure protocol, but while this has been successful, it perpetuates the presence of virulent CAV in the environment and these vaccines have recently been withdrawn from the market. The problem of declining immunity in donor flocks is not addressed by this approach because of the risk of exposing susceptible subpopulations of birds to reinfection and hence vertical transmission. The move to cage rearing is likely to increase the risk of clinical CAV infections as cage-reared birds are less likely to be uniformly exposed and thus less likely to have developed immunity by the time they commence production. The need for a safe immunogenic attenuated CAV vaccine is clearly evident. Improved control of CAV is also likely to result in reduced need for medication of flocks to control secondary diseases, and thus benefits in environmental sustainability and public health.

The aim of this project is to extend a previous RIRDC Egg Program-funded project (UM-37A) to develop attenuated variants of Chicken Anaemia Virus. The development of such a vaccine will allow the development of protective immunity in all chickens, limiting the immunosuppressive effect of the virus and curtailing the associated affects on Marek's disease and other secondary diseases.

While the genome of CAV could be inoculated into embryos and generate infectious virus, and the genome in a single stranded positive sense form was the most infectious, the genome in double stranded form within a plasmid vector was not found to be infectious.

Of the seven mutant viruses assessed in day old chicks, five were found to be sufficiently attenuated to be regarded as safe and two were found to afford significant protection against challenge. These two mutant viruses are now suitable for assessment for commercial development as vaccines.

# 1. Introduction

CAV is a significant cause of loss in the poultry industry, particularly due to its enhancement of disease caused by Marek's disease virus, but also through diminished efficacy of vaccination to control other diseases. The considerable resistance of the virus to heat and disinfectants, as well as its capacity to spread both horizontally and vertically, compounds the difficulties of controlling its effects. With the increasing requirement to improve biosecurity to control Marek's disease, the significance of CAV can be predicted to increase, as in the absence of suitable vaccines, more breeder flocks will come into lay without having encountered CAV, and hence their progeny will not be protected. Current control measures rely on infection of breeder birds with a virulent strain before they come into lay, or on natural exposure (encouraged by maintaining a "dirty system"), but have the major disadvantage that this perpetuates a high level of contamination of breeder operations with virulent virus. Furthermore the potential problems due to waning levels of immunity in older birds (and hence reduced passively acquired protection in their chicks) cannot be adequately addressed by reexposure to virulent virus without risking vertical transmission to some of the flock. It is clear that an attenuated live vaccine, with boosting of breeder flocks using an inactivated vaccine, would be a preferable alternative to the current measures. Optimally an attenuated strain that is also suitable for administration to layer chicks through drinking water would address control problems. The only attenuated strain of CAV available, produced by Loemann, is not available in Australia, and furthermore must be administered by injection into each bird as it is not able to establish infection after oral inoculation.

The cost of CAV infections to the egg industry in Australia have not been estimated. However studies overseas have estimated the losses due to clinical CAV infections to be 2% higher mortalities than in unaffected flocks. In addition to these losses, there is a significant contribution by CAV to the more severe outbreaks of Marek's disease, which are currently a major concern to the Australian industry. In addition to the well established potentiation of Marek's disease, CAV can also aggravate infections with infectious bursal disease, lentogenic Newcastle disease, reticuloendotheliosis virus and other bacterial and viral pathogens.

The expected result of this project, generation of an attenuated strain of CAV suitable for vaccination of chickens, will assist in the control of losses due to CAV and Marek’s disease in layer flocks. Current use of virulent strains of CAV, while effective against clinical disease, is ensuring continuing subclinical infection of birds and, on the basis of studies overseas, is probably resulting in ongoing significant losses. In addition, improved control of CAV will contribute to improved efficacy of vaccination against other infectious diseases and hence reduced losses due to these diseases.

Improved control of CAV will result in reduced levels of secondary infections with bacterial pathogens, and hence a reduced need for treatment of poultry with antimicrobial drugs. Reductions in antimicrobial usage have significant environmental and public health benefits, with reduced risk of development of antimicrobial resistance in bacterial populations that may transfer resistance into human pathogens.

In consultation with industry advisors, two issues became apparent. Firstly, current control measures are not universally available to the industry, and thus some producers are at a distinct disadvantage, and secondly, the virulent strains currently in use are likely to have a limited future. This advice suggests that improved CAV control is a significant issue for the whole industry. There also continues to be considerable industry concern over the reduced capacity of current Marek's disease vaccines to adequately control problems, necessitating a re-evaluation of approaches to control this disease. While improved Marek's vaccines are one goal, increased biosecurity and control of synergistic infections are likely to be just as important in achieving effective control. While these measures will assist in control of infection by Marek’s disease virus, they will heighten the risks of infection by the highly resistant CAV.

## 1.1 The Virus

Chicken anaemia virus was first isolated by Yuasa *et al* in 1979 in day old chicks (3). Subsequently the MDCC-MSB1 cell line, which is derived from Marek's disease virus induced lymphomas, was used for propagation (4). Other cell lines have been used, but all are cells transformed by either Marek's disease virus or avian leukosis virus. As a result, cell culture propagated virus is not suitable for use in vaccines due to contamination by these viruses. Currently, commercial vaccines need to be produced in chick embryos from CAV-free flocks. Australia is in a privileged position through the availability of CAV-free specific pathogen free birds for both experimental work and for the production of vaccines.

CAV is very resistant to inactivation by heat, surviving temperatures of 70°C for up to an hour (3). In addition it is resistant to many disinfectants, including quaternary ammonium compounds, iodophors and formalin, although it is destroyed by hypochlorite (1).

## 1.2 The Disease

The disease caused by CAV in experimental infections is characterised by infection and destruction of erythroblastoid (red cell lines) and cortical thymocyte (helper lymphocyte) cells, resulting in severe anaemia and immunodeficiency (3, 5, 6). Overt disease is only apparent in birds under 3 weeks of age, but older birds can still be infected, and significant losses in production have been observed in subclinically affected flocks (3, 5-7). Experimental studies have shown that, while anaemia is not apparent in orally infected 3 week old birds, they still have significant depression of their cell mediated immune function for at least 4 weeks after infection, thus potentially explaining the basis of the subclinical losses seen in flocks that are infected after maternal immunity wanes (8, 9). The disease seen in the field is more complex than that seen in experimental infections and includes higher rates of other diseases such as colibacillosis, more severe manifestations of Marek's disease, and a variety of opportunistic infections (5). The interaction between CAV and Marek's disease virus (10) is of particular concern given the increasing problems with control of Marek's disease in Australia.

## 1.3 Epidemiology

CAV occurs worldwide and the primary host appears to be the chicken. The prevalence of infection in older flocks is high (11-14). The virus is transmitted both vertically and horizontally, with most outbreaks linked to vertical transmission from an acutely infected breeder flock, followed by horizontal transmission among the progeny. Maternal antibody protects against CAV infection for about 3 weeks after hatch, but while most breeder flocks seroconvert between 8 and 12 weeks of age, some flocks do not seroconvert until they come into lay. Infection at this stage results in clinical disease in progeny for 3 to 6 weeks.

## 1.4 Experimental Reproduction of Disease

Experimental reproduction of disease due to CAV has been achieved by dosing day old chicks orally with $10^5$ TCID50 of virus, with 25% of birds developing anaemia (9, 15). Age resistance to disease develops by 2 weeks of age, although infection is still possible. Parenteral inoculation of day old chicks results in 100% of birds with anaemia (3, 5, 16). In addition, these birds have reduced bursa weights and body weights. More severe disease can be induced by infection of chick embryos via the yolk sac, with chicks dying at 10-15 days of age (17).

## 1.5 Control Measures

Current control measures for CAV rely on the use of virulent CAV to vaccinate breeder chickens before they come into lay. While this has been effective in eliminating most clinical disease, there are four areas of concern in relying on this approach. The first is the waning levels of maternal immunity provided by older flocks (18). Use of virulent strains of CAV for

boosting birds in lay carries the risk that some breeders will transmit the virus to their progeny, and thus cause outbreaks of clinical disease. The second concern is that ongoing use of virulent CAV perpetuates a cycle that ensures high levels of environmental challenge by CAV for all birds. Such high levels of challenge may well contribute to vaccine breakdowns, with CAV induced immunosuppression interfering with the efficacy of other vaccines, especially those against Marek's disease. Thirdly, the data from numerous sources suggests that subclinical CAV infection has adverse effects on production (7, 19). Where virulent vaccine strains are in use they are likely to be the major source of subclinical infections and hence a cause of significant loss. Finally, the synergism between CAV and Marek's disease virus suggests that ongoing high levels of challenge by virulent CAV is probably contributing to the increasing difficulties experienced in control of Marek's disease. There is a clear requirement for an attenuated vaccine strain of CAV to enhance control and reduce the losses inevitably associated with continuing use of virulent strains for control of clinical disease.

## 1.6 Problems in Producing a Vaccine

There have been attempts to produce attenuated strains of CAV using traditional approaches. Extended passage of CAV in cell culture reduced, but did not eliminate, the pathogenicity of CAV isolates. In addition, strains produced by such methods have rapidly reverted to virulence on repeated passage in chickens (20). The possibility of developing inactivated vaccines is restricted by the inability to produce high titred virus in either cell culture or chick embryos.

Two alternatives approaches to producing a more effective vaccine are possible. However the approach we have been taking thus far, the development of live attenuated strains, is likely to be the most commercially viable.

The first approach would be to produce subunit vaccines or virus-like particles by expressing CAV proteins in baculoviruses (viruses which infect insect cells and which can be used to engineer high levels of production of recombinant proteins). This approach would focus on the simultaneous production of virus protein 1 (VP1 - the capsid protein) and virus protein 2 (VP2) in the same cell, as this appears to be necessary to produce protein which can induce neutralising antibody (21). This approach may be suitable for production of a vaccine for administration to breeder birds, but would probably be too expensive for administration to layer pullets.

The second approach is to exploit the small genome size of CAV to generate mutants that are suitable for use as attenuated vaccines. These vaccines could be produced in chick embryos and administered in drinking water for immunisation of both breeders and pullets. These attenuated mutants of CAV could also be used in a more novel approach to vaccination. The full length genome of CAV is infectious in its own right. The genome alone can be transfected into cell lines to produce infectious virus (20, 22-25). This capacity could be exploited by adopting the relatively new technique of DNA vaccination. DNA alone could be used for *in ovo* vaccination, with uptake by the cells of the embryo resulting in the eventual production of the vaccinating virus. Given the increased virulence of CAV for embryos it is essential that the genome used be attenuated. However molecular biological techniques could be used to produce large quantities of genomic DNA very cheaply, thus avoiding the problems associated with contaminated cell lines and low titres of infectious virus. Such a DNA vaccine could also be used intramuscularly in older birds to boost breeders to prevent the occurrence of the old breeder syndrome, thus effectively acting as a killed vaccine.

## 1.7 Molecular Biology of CAV

The reason a directed mutation approach is feasible in CAV is its small and relatively simple genome. At 2,300 base pairs it has one of the smallest of all viral genomes, and this genome can be amplified in its entirety using the polymerase chain reaction (PCR). The isolated

linear genome can be transfected into cell lines to reproduce infectious virus (20, 22-25). Thus manipulation of the genome to produce site specific mutations is easily achievable. The virus produces 3 known proteins VP1, VP2 and VP3. VP3 (also known as apoptin) has been shown to be capable of causing apoptosis (cell death) in cells by itself (26). VP1 appears to be the capsid protein. There is little information published about the third protein, and very limited understanding of how CAV controls its replication once it has infected cells.

# 2. Objectives

- To improve control of Chicken Anaemia Virus related disease in chickens.
- To extend studies on directed mutagenesis on the genome of chicken anaemia virus (CAV) conducted in a previous RIRDC Egg Program-funded project (UM-37A) to develop and assess attenuated mutants of CAV for their suitability as live vaccines for administration to layer breeder flocks and layer pullets. This application would obviously be extendable to other poultry.
- To assess the suitability of DNA vaccination with the genome of these attenuated mutants to control CAV related disease

# 3. Methodology

This project aims to extend the work performed in a previous RIRDC Egg Program-funded project (UM-37A) that established methods for introducing mutations into specific sites in the CAV genome and developed a number of candidate mutant viruses for assessment as vaccine candidates. The work performed in this project assessed the extent of the attenuation achieved in these mutants, and assessed their capacity to induce protection against virulent CAV.

## 3.1 Construction of Specific Mutants of CAV

In the previous project we amplified the entire 2.3 kb genome of an Australian strain of CAV in overlapping fragments and assembled into a complete genome in a plasmid vector. We confirmed that this mutated genome was infectious and established that it was virulent in 6 day old chicken embryos, essential steps in assessing the attenuation of mutated viruses. This cloned genome was used as the basis for site specific mutagenesis using PCR.

We derived a large series of mutants altered at specific sites in two areas of the CAV genome. The first series of four mutations were predicted to influence the efficiency of translation of the VP3 and VP2 proteins, which are thought to control viral virulence. The aim was to reduce the quantity of these proteins produced during infection. All 4 mutants were introduced into cells but only one was viable. This mutant only appeared to be viable for a limited time, with some viral replication initially, but eventual loss from the cells. It thus appeared unlikely that these mutations would produce an attenuated strain suitable for administration to chickens. It is possible that a mutant with limited capacity to replicate might make a suitable DNA vaccine for administration *in ovo*.

The second series of mutations were focussed on the VP2 gene. We generated 10 mutants in the coding region of the gene, attempting to disrupt specific structural features thought to be important in the function of the protein. All these mutants were transfected into cells and preliminary examination indicated that all were viable. At least two were capable of replication to sufficient titres to be viable for vaccine production. Examination of the phenotypes of these mutants *in vitro* showed that their patterns of replication were altered by the mutations, suggesting that they were likely to be attenuated *in vivo.*

## 3.2 Assessment of the Attenuation of CAV by Site Directed Mutation

As CAV is most pathogenic in embryos, we initially assessed the mutants by inoculation of virus obtained from transfected MDCC-MSB1 cells into 6 day old embryos. Our previous work had shown that the virulent parent genome causes severe lesions by the day of hatching and that it can induce an antibody response in embryos by this time as well. The mutants were inoculated into embryos at the same titre as the parent virus and the differences in weight, red cell volume, thymus weight and bursa weight of mutant and parent virus infected embryos compared. A series of 12 mutant viruses were initially assessed in embryos. Six mutants found to be attenuated in these experiments were further assessed. These attenuated strains were then assessed for virulence by parenteral inoculation of day old chicks. The birds were inoculated subcutaneously with 0.5 ml of CAV containing $10^4$ median tissue culture infective doses ($TCID_{50}$) of virus or with 0.5 ml of a lysate of uninfected MSB1 cells.

At day 14, birds were euthanased by exposure to halothane. At post mortem body weights were taken and all lymphoid organs, bone marrow, liver, spleen and dermus (for evidence of haemorrhage) examined for gross pathology. The thymic chain was dissected out and weighed.

## 3.3 Assessment of the Protective Efficacy of Site Directed Mutants of CAV

The attenuated mutants of CAV were then assessed for their ability to produce effective immunity *in vivo*. Day old specific pathogen free chickens were vaccinated with the mutant strains and then challenged with virulent CAV at 21 days of age. The protective efficacy of vaccination with each mutant was assessed by comparing thymus weights and body weights 14 days after challenge.

## 3.4 Assessment of the Potential for DNA Vaccination with Mutant CAV

Purified CAV genomic DNA was generated from the cloned CAV genome as either double stranded genome, single stranded positive sense genome or single stranded negative sense genome. In addition the cloned genomic DNA within the plasmid was also used. These different forms of the egnomic DNA were inoculated into the yolk sacs of embryonated hens eggs and the eggs were examined at 18 days to assess whether viral replication had occurred.

# 4. Detailed Results

## 4.1 Attenuation of CAV Mutants for Embryos

Of the 12 mutants mutagenised within the VP2 gene, 10 were able to grow sufficiently well in MSB1 cells to allow them to be assessed for virulence by inoculation of 6-day-old embryos. Attenuation of CAV specific lesions was seen in all embryos infected with these mutated viruses. Viruses could be categorised according to their degree of attenuation into two broad categories: highly attenuated viruses (mutant C86R, mutant R101G, mutant H103Y, mutant R129G, mutant N131P, mutant R/K/K150/151/152G/A/A, mutant D/E161/162G/G and mutant E186G) and moderately attenuated viruses (mutant L163P and mutant D169G). Cumulative lesion scores in embryos infected with any of the highly attenuated viruses were low (median between 4 and 7) and were intermediate (median 9) for both the moderately attenuated

viruses. Cumulative lesion scores in embryos infected with wild type *CAU269/7* were high (median 13), and no lesions were found in uninfected embryos (median 1). Median cumulative lesion scores in embryos infected with mutant viruses were significantly lower than in embryos infected with wild type *CAU269/7*.

Mean bodyweight in embryos infected with mutant viruses was in the range 31.3 to 42.9 g, and was not significantly different from uninfected embryos (37.1 g) but was significantly greater than in embryos infected with wild type virus (24.8 g). These results indicate attenuation of the growth suppression induced by wild type virus infection by mutation of VP2.

Median thymic, splenic, bone marrow and haemorrhage lesion scores were all low in embryos infected with those mutant viruses classified as having low median cumulative scores, with the exception of viruses mutant R101G and mutant D/E161/162G/G, for which median thymic scores were intermediate. In embryos infected with mutant viruses, atrophy of the thymic parenchyma was limited to slight reductions in the diameter of typically between 1 and 3 lobes in a chain, and there were occasional, mild petechial haemorrhages or an inflammatory exudate within the thymic lobes. In a minority of embryos there were subcutaneous petechial haemorrhages on the flanks and thighs. In some embryos, the diameter of the spleen was reduced by at most 30% relative to uninfected embryos, consistent with mild splenic atrophy, and the spleens appeared pale. In general, the bursae appeared normal.

For all embryos infected with VP2 mutated viruses, except mutant D169G, median thymic, splenic and bone marrow lesion scores were significantly lower than for wild type infection. Median thymic and splenic lesion scores were intermediate in embryos infected with virus mutant D169G. Median haemorrhage scores were either low or there were no haemorrhages detected in embryos infected with all mutant and with wild type viruses. Median haemorrhage scores in all embryos infected with VP2 mutants were significantly lower than for scores in embryos infected with wild type virus, with the exceptions of embryos infected with viruses mutant R/K/K150/151/152G/A/A and mutant D169G.

Median bursal lesion scores were normal (median 1) in embryos infected with either wild type or VP2 mutant viruses, and the incidence of grossly apparent bursal lesions was sporadic. However, there was a significant reduction in mean bursa:bodyweight ratio in embryos infected with wild type *CAU269/7* relative to uninfected embryos. In embryos infected with viruses mutant C86R, mutant R101G, mutant R129G, mutant Q131P, mutant L163P, mutant D169G and mutant E186G the mean bursa: bodyweight ratio was significantly greater than in embryos infected with wild type virus.

There were no differences detected between the mean PCVs obtained from embryos infected with wild type virus, VP2 mutant viruses or uninfected embryos. For embryos infected with VP2 mutated viruses, the mean thymic weights were between 319 to 456 mg, and the mean splenic weights were between 8.3 to 15.5 mg. Mean thymus:bodyweight and mean spleen:bodyweight ratios for embryos infected with all VP2 mutant viruses were significantly greater than for embryos infected with wild type virus, and were not significantly different from the ratios in uninfected embryos (thymus: bodyweight ratio for mutant D 169 G was an exception).

Gastrointestinal lesions were seen in 12 of 18 embryos infected with the virus mutant R/K/K150/151/152G/A/A in two independent experiments, and were not seen in embryos infected with any other virus. Of the 12 embryos with gastrointestinal lesions, 3 had lesions in the crop, 1 in the proventriculus, 1 in the ventriculus, 1 in the duodenum, 7 in the jejunum and 2 in the ileum. The majority of embryos infected with virus mutant R/K/K150/151/152G/A/A had mild CAV lesions, and only 2 had lesions graded as moderate. Gross lesions in the crop, proventriculus, ventriculus and intestines consisted of full thickness necrosis and thinning of the gastrointestinal wall, hyperaemia and serosal haemorrhages. Lesions were found in the liver in 6 of the embryos infected with virus mutant

R/K/K150/151/152G/A/A. On the surface and in transverse sections of the liver, there was a generalised pattern of irregular nodularity and purplish-red discolouration. In some cases there were sub-capsular hepatic haemorrhages and focal or marginal infarcts in the hepatic lobes. In one embryo infected with mutant R/K/K150/151/152G/A/A, haemorrhage and irregular roughening was seen on the synovial surfaces of both femorotibial and tibiotarsal joints.

## 4.2 Attenuation of Mutant CAV for Day Old Chicks

Day old chicks inoculated subcutaneously with $10^4$ $TCID_{50}$ of wild type CAV were examined at day 10 and 14. Maximal thymic lesions were seen at day 14, but no macroscopically apparent lesions were detected in any other organ and the mean PCV of birds inoculated with wild type CAV did not differ from that of uninfected birds.

Of the 10 mutants assessed in embryos six replicated sufficiently well within cell culture to be viable options as commercial vaccines and were thus assessed in day old chicks. In addition, a seventh mutant S77N was derived during passage of one of the mutants in cell culture and this mutant was also assessed in day old chicks. These 7 mutants were assessed in two separate experiments, the results of which are shown in Tables 1 and 2.

The results shown in Table 1 indicate that three of the mutant viruses that were attenuated for chick embryos were also attenuated for day old chicks, with the attenuation of mutant D169G somewhat intermediate compared to that of mutants R101G and D/E161/162G/G.

The results shown in Table 2 indicate that two of these mutant viruses were attenuated for day-old chicks with the attenuation of mutant E186G somewhat intermediate compared to that of mutant R/K/K150/151/152G/A/A. However, the dose of mutant R/K/K150/151/152G/A/A was $10^{1.4}$ $TCID_{50}$, much lower than that of the other mutants. Mutants S77N and Q131P did not appear to be attenuated.

**Table 1. Assessment of attenuation of three mutants in chicks 14 days after inoculation (Experiment 1).**

| Group | Treatment day 1 | Thymus Weight (g) | Thymus/Body Weight Ratio (mg/g) |
|---|---|---|---|
| 1 | Uninfected | $1.3 \pm 0.3^{a}$ | $8.8 \pm 1.6^{a}$ |
| 2 | Uninfected | $1.1 \pm 0.3^{ab}$ | $8.4 \pm 1.6^{a}$ |
| 3 | Wild type CAV | $0.8 \pm 0.3^{b}$ | $4.9 \pm 2.0^{b}$ |
| 4 | D169G | $1.1 \pm 0.3^{ab}$ | $7.1 \pm 2.1^{ab}$ |
| 5 | R101G | $1.3 \pm 0.4^{a}$ | $8.9 \pm 2.3^{a}$ |
| 6 | D/E161/162G/G | $1.3 \pm 0.3^{a}$ | $8.3 \pm 1.5^{a}$ |

Values in the same column with the same superscript letter are not significantly different

**Table 2. Assessment of attenuation of four mutants in chicks 14 days after inoculation (Experiment 2).**

| Group | Treatment day 1 | Thymus Weight (g) | Thymus/Body Weight Ratio (mg/g) |
|---|---|---|---|
| 1 | Media | $0.95 \pm 0.16^{a}$ | $7.78 \pm 1.24^{a}$ |
| 2 | Media | $0.77 \pm 0.16^{a}$ | $8.16 \pm 0.98^{a}$ |
| 3 | S77N | $0.48 \pm 0.14^{b}$ | $4.05 \pm 0.1^{b}$ |
| 4 | E186G | $0.70 \pm 0.19^{a}$ | $6.02 \pm 1.5^{b}$ |
| 5 | Q131P | $0.51 \pm 0.14^{b}$ | $4.36 \pm 0.85^{b}$ |
| 6 | R/K/K150/151/152G/A/A | $1.19 \pm 0.22^{c}$ | $9.28 \pm 1.6^{a}$ |
| 7 | Wild Type CAV | $0.58 \pm 0.12^{b}$ | $4.70 \pm 0.94^{b}$ |

Values in the same column with the same superscript letter are not significantly different

## 4.3 Protective Efficacy of Mutant CAV as Vaccines

The 7 mutants assessed for attenuation in day old chicks were also assessed for their capacity to induce protective immunity against subcutaneous challenge with $10^4$ $TCID_{50}$ of wild type CAV. These studies were performed in two separate experiments, with the results shown in Tables 3 and 4.

The results shown in Table 3 indicate that only mutant D169G induced significant protective immunity, while those in Table 4 indicate that mutants S77N, Q131P, E186G and R/K/K150/151/152G/A/A all induced significant protective immunity against the wild type challenge, giving a total of five mutants inducing protective immunity.

**Table 3. Protection afforded by vaccination with three mutant CAVs (Experiment 1).**

| Group | Treatment day 1 | Treatment day 21 | Thymus Weight (g) | Thymus/Body Weight Ratio (mg/g) |
|---|---|---|---|---|
| 1 | Uninfected | Uninfected | $4.1 \pm 0.8^{a}$ | $9.5 \pm 1.4^{a}$ |
| 2 | Uninfected | Wild type CAV | $1.3 \pm 0.3^{b}$ | $3.3 \pm 0.9^{b}$ |
| 3 | Wild type CAV | Wild type CAV | $5.1 \pm 1.2^{a}$ | $10.5 \pm 1.9^{a}$ |
| 4 | D169G | Wild type CAV | $3.5 \pm 0.4^{a}$ | $9.0 \pm 0.8^{a}$ |
| 5 | R101G | Wild type CAV | $2.2 \pm 0.4^{c}$ | $5.0 \pm 1.4^{b}$ |
| 6 | D/E161/162G/G | Wild type CAV | $2.0 \pm 1.0^{bc}$ | $5.2 \pm 2.1^{b}$ |

Values in the same column with the same superscript letter are not significantly different

**Table 4. Protection afforded by vaccination with four mutant CAVs (Experiment 2).**

| Group | Treatment day 1 | Treatment day 21 | Thymus Weight (g) | Thymus/Body Weight Ratio (mg/g) |
|---|---|---|---|---|
| 1 | Uninfected | Uninfected | $2.58 \pm 0.77^{a}$ | $7.8 \pm 2.02^{a}$ |
| 2 | Uninfected | Wild type CAV | $1.08 \pm 0.26^{b}$ | $3.7 \pm 0.90^{b}$ |
| 3 | S77N | Wild type CAV | $2.23 \pm 0.29^{a}$ | $5.8 \pm 1.40^{a}$ |
| 4 | Q131P | Wild type CAV | $2.12 \pm 0.65^{ac}$ | $6.7 \pm 1.30^{a}$ |
| 5 | E186G | Wild type CAV | $2.38 \pm 0.61^{ac}$ | $7.4 \pm 1.90^{a}$ |
| 6 | R/K/K150/151/152G/A/A | Wild type CAV | $2.34 \pm 0.89^{ac}$ | $6.1 \pm 2.43^{a}$ |
| 7 | Wild Type CAV | Wild type CAV | $1.66 \pm 0.27^{ac}$ | $6.8 \pm 1.18^{a}$ |

Values in the same column with the same superscript letter are not significantly different

## 4.4 Potential for DNA Vaccination

Only genomic DNA that was cleaved from the plasmid was able to generate infectious virus in inoculated eggs, with single stranded positive sense genomic DNA the most infectious.

# 5. Discussion of Results

The work conducted in this project has shown that mutant CAV viruses created by site directed mutagenesis are attenuated in chick embryos and that five of these mutants that replicate reasonably well in cell culture are also attenuated in day old chicks. Of these five viruses two, mutants D169G and E186G, induce significant protective immunity against challenge. These two mutants are clearly appropriate candidates for assessment as vaccines for commercial development.

The approach used to develop these vaccine strains can be applied to further attenuate these strains should they prove to be too virulent in subsequent field trials. Furthermore, studies initiated in the preceding project were able to establish an *in vitro* assay for assessment of the effect of the mutations. This *in vitro* assay would enable preliminary screening of future mutants should they be needed.

Cloned genomic DNA containing attenuating mutations has potential for *in ovo* vaccination, but only after the CAV genome has been cleaved from the plasmid vector.

# 6. Implications

This project has established that two mutants of chicken anaemia virus constructed *in vitro* using PCR mutagenesis have potential for development as vaccines. These mutants have been shown to replicate well in cell culture, to be attenuated in both embryos and day old chicks and to induce protective immunity against challenge. These mutants are suitable for assessment in pre-registration trials.

# 7. Dissemination/Adoption

Findings from this project have been presented annually at Australian Veterinary Poultry Association meetings. The two mutants with greatest potential as vaccines will enter pre-registration testing through the Australian Poultry Cooperative Research Centre.

# 8. Publications

## 8.1 Papers


Peters, M. A., Jackson, D. C., Crabb, B. S., and Browning, G. F. (2002). Chicken Anemia virus VP2 is a novel dual specificity protein phosphatase. *Journal of Biological Chemistry*, **277**: 39566-73.

Peters, M. A., Browning, G. F., Washington, E. A., Crabb, B. S., and Kaiser, P. (2003). Embryonic age influences the capacity for cytokine induction in chicken thymocytes. *Immunology*, **110**: 358-367.

Peters, M. A., Browning, G. F., Crabb, B. S., Washington, E. A., and Kaiser, P. Chicken Anemia Virus infection modulates cytokine and MHC class I transcription in lymphoid tissues. Submitted.

Peters, M. A., Jackson, D. C., Crabb, B. S., and Browning, G. F. Mutation of Chicken Anemia Virus VP2 differentially affects serine/threonine and tyrosine protein phosphatase activities. Submitted.

Peters, M. A., Washington, E. A., Crabb, B. S., and Browning, G. F. Site-directed mutagenesis of the VP2 gene of Chicken Anaemia Virus affects viral replication, cytopathology and host cell MHC Class I expression. Submitted.
Peters, M. A., Tivendale, K. A., Crabb, B. S., and Browning, G. F. Attenuation of Chicken Anaemia Virus by site-directed mutagenesis of VP2. In preparation.
Peters, M. A., Washington, E. A., Crabb, B. S., and Browning, G. F. Global depletion of the immune repertoire in Chicken Anaemia Virus is attenuated through mutation of VP2. In preparation.
Peters, M. A., Browning, G. F., Crabb, B. S., and Kaiser, P. Chicken Anaemia Viruses attenuated through mutations in VP2 have different patterns of cytokine induction relative to wild type virus. In preparation.
Tivendale, K. A., Brown, H. K., Peters, M. A., Browning, G. F., and Crabb, B. S. Translational initiation signals in Chicken Anaemia Virus. In preparation.
Tivendale, K. A., Shrestha, S., Peters, M. A., Crabb, B. S., and Browning, G. F. Safety and efficacy of attenuated Chicken Anaemia Viruses. In preparation.
Peters, M. A., Crabb, B. S., and Browning, G. F. Inoculation of embryos with Chicken Anaemia Virus genome. In preparation.

## 8.2 Presentations

Molecular biology of Chicken Anaemia Virus – Australian Veterinary Poultry Association, Melbourne, November 2000.
Chicken Anaemia Viruses with specific mutations are replication competent – 2nd International Veterinary Vaccines and Diagnostics Conference, Oxford, UK, July 2000.
Chicken Anaemia Virus VP2 is a novel protein tyrosine phosphatase – 2nd International Symposium on Infectious Bursal Disease and Chicken Anaemia Virus, Rauischholzhausen, Germany, June 2001.
Viral Protein 2 as a target for attenuation of Chicken Anaemia Virus, Australian Veterinary Poultry Association, Gold Coast, April 2002.

## 8.3 Patent Applications

Browning, G. F., Peters, M. A., Scott, P. C., Brown, H. K., Tivendale, K. A. and Crabb, B. S. – Circovirus Vaccines. Australian Patent Application No. PR5674, International Patent Application No. PCT/AU02/00787, Japanese Patent Application No. 2003-506453, Chinese Patent Application No. 02815146.1, European Patent App No. 02740122.3, US Patent Application No. 20-528-5829.

# 9. Recommendations

Ongoing studies will further define the most appropriate methods to use for control of high risk areas on studs. In addition these ongoing studies will evaluate the diagnostic significance of detection of virulent *R. equi* in the expired breath of foals.

# 10. Intellectual Property

The intellectual property generated in this project has been protected by a patent application.

# 11. Communications Strategy

Investigators working on the project have presented findings on an ongoing basis to the Australian Veterinary Poultry Association. Once the vaccine strains developed have passed through pre-registration testing, the results of these studies will also be communicated through this forum.

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