Attenuation of chicken anemia virus by site-directed mutagenesis of VP2

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Chicken anemia virus (CAV) is a significant immunosuppressive pathogen of chickens, but relatively little is known about the effect of specific mutations on its virulence. In order to study the virulence of CAV, an infection model was developed in embryos. Significant growth depression, measured as a reduction in mean body weight, was found for wild-type CAV infection. Infection with wild-type CAV resulted in a significant reduction in thymic and splenic weights and consistently produced severe lesions in the thymus, spleen and bone marrow, as well as haemorrhages. CAVs mutated in the VP2 gene were infectious for embryos, but were highly attenuated with respect to growth depression and CAV-specific pathology. Relative to wild-type infection, viruses Mut C86R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut R/K/K150/151/152G/A/A, Mut D/E161/162G/G and Mut E186G were highly attenuated, and viruses Mut L163P and Mut D169G were moderately attenuated. Attenuation of the ability to produce lesions was found consistently for the thymus, spleen and bone marrow, thymic and splenic weights, and for CAV-induced haemorrhage. There was no growth depression associated with infection by the group of highly attenuated mutant viruses and a moderate reduction in mean body weight was only found for virus Mut L163P. These findings show that mutations in the VP2 gene can reduce the virulence of CAV and these mutant viruses may have value as vaccine candidates.

INTRODUCTION

Chicken anemia virus (CAV) is a member of the genus Gyrovirus and the family Circoviridae and an immunosuppressive pathogen of chickens (Büchen-Osmond, 2006; Pringle, 1999). Clinical disease in chicks infected at 1 day of age is characterized by weakness, depression, stunting and anaemia (Farkas et al., 1996; McNulty et al., 1989; Rosenberger & Cloud, 1989). Affected chicks develop severe myelophthisis, atrophy of the thymus and spleen, and focal dermal haemorrhagic lesions. There is an increased incidence of secondary infections and vaccination failure due to immunosuppression. Subclinical disease in birds older than 21 days is associated with immunosuppression and growth depression. Experimental inoculation of 5-day-old chick embryos results in a high embryonic death rate, poor hatchability and reduced survival of hatched chicks (Lamichhane et al., 1991). The objective of this study was to examine the virulence of viruses mutated in the VP2 gene.

An experimental model for CAV-induced disease is required for the assessment of attenuation. Such a model does not initially need to represent the full spectrum of pathology observed in the field, but rather must enable assessment of attenuation under conditions that produce most severe pathology. We aimed to develop a model based on yolk sac inoculation of 7 day embryos and post-mortem at embryonic day 20. This parallels the vertical transmission of CAV to embryos in naïve breeder flocks infected at the point of lay, which has been recognized as resulting in the most severe naturally occurring form of disease induced by CAV. The model needed to consistently reproduce severe CAV-specific lesions. A further objective was to derive a standard grading system for lesion severity to apply to the model. Viruses demonstrating attenuation in this stringent model may have potential as vaccine candidates.

A full genome clone of virus CAU269/7 has been produced in the plasmid vector pGEM 4T (Brown et al., 2000). Previous studies have established equivalent infectivity in cell culture of virus recovered by transfection of the cloned genome and the parental virus. In this study, we aimed to compare the infectivity and pathogenicity of the parental and regenerated viruses in the embryonic model. In previous work, we produced mutated viruses from the genomic clone of CAU269/7 virus, and some CAVs
containing specific mutations in VP2 have been shown to grow to moderate titres in cell culture (Peters et al., 2005, 2006). The studies reported here investigated the ability of these viruses to cause disease in embryos.

**METHODS**

**CAVs.** The Australian CAU269/7 isolate of CAV was used in all experiments (Brown et al., 2000). CAU269/7 was recovered from MDCC-MSB1 cells transfected with the plasmid construct pCAU269/7 (Brown et al., 2000). CAVs containing the following VP2 mutations were constructed in our laboratory: Mut C86R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut R/K/K150/151, Mut D/E161/162G/G, Mut L163P, Mut D169G or Mut E186G. PCR mutagenesis of a full genome clone of CAV, the cloning of mutated constructs, the transfection of mutated cloned DNA into MDCC-MSB1 cells and the recovery of mutant viruses have been described previously (Peters et al., 2005, 2006).

Viral stocks for inoculation were prepared from 400 ml MDCC-MSB1 cells and the recovery of mutant viruses have been described previously (Peters et al., 2000). CAVs containing a previously described method (Goryo et al., 1987). CAV grows slowly in culture and thus the end point of titration can only be established after approximately seven passages. Duplicate 10-fold dilution series of viral stocks were prepared, ranging from a final dilution of 0.05 through to 0.5 x 10^{-6}, and inoculated onto cells. At intervals of 48 h cells were passed into fresh medium at a dilution of 1 in 4. Each well was scored for evidence of cytopathogenic effect (CPE), and cultures were serially passaged until no difference was detected between successive passages in the lowest dilution at which CPE was observed. The result was confirmed using an indirect immunofluorescence assay (IFA) on the end-point dilution with the anti-VP3 monoclonal antibody mAbVP3 (TropBio, James Cook University, Townsville, Qld, Australia). Viral stocks were resuspended at a final titre of 10^{4.5} TCID_{50} ml^{-1}.

**Infection model in embryonated eggs.** An infection model was developed in embryonated eggs in order to assess infectivity and the in vivo phenotype of wild-type and mutant CAV. The model was initially used to verify equivalence between virus regenerated from pCAU269/7 DNA transfection and CAU269/7 virus. Yolk sac inoculation of ten 7 day embryos (E7) with parental virus, virus regenerated from pCAU269/7 and clarified MDCC-MSB1 lysate was repeated on at least two separate occasions.

Chicken embryos were inoculated with viruses Mut C86R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut R/K/K150/151, Mut D/E161/162G/G, Mut L163P, Mut D169G or Mut E186G, wild-type CAU269/7 virus, RPMI 1640 medium supplemented with 10% fetal calf serum, or MDCC-MSB1 lysate (for n-values see Table 2) (Peters et al., 2005, 2006). Experimental treatments were repeated at least twice on separate occasions. The yolk sacs of specific-pathogen-free 7 day embryos (E7) (SPAFAS Australia) were inoculated with 0.5 ml of inoculum containing 10^5 TCID_{50} of virus. Embryos were monitored by candling to detect death before day E21. There was no significant difference in the rate of pre-hatch embryonic mortality between uninoculated control embryos and infected embryos, and embryonic mortality was in all cases less than 10% of embryos incubated.

Embyros were euthanized by cervical dislocation according to approved ethical standards at E21, weighed, and the entire thymic chains, spleen and Bursa of Fabricius dissected, weighed and scored for pathology. Blood was collected by cardiac puncture and the packed cell volume (PCV) was determined. The femoral bone marrow was scored for pathology and squash preparations were made. Haemorrhage was scored by gross examination, primarily of the fascial planes and the subcutaneous tissues. Sections of liver were removed for assessment by CAV-specific PCR.

**Assessment of viral infectivity.** The establishment of viral infection was assessed by CAV-specific PCR of liver homogenates and by IFA on bone marrow squash preparations to detect VP3. Cellular DNA was prepared from liver homogenates by proteinase K digestion and SDS lysis, and phenol/chloroform extraction. Cellular DNA was resuspended in 50 ml 0.1 M Tris/HCl (pH 8.0), 1 mM EDTA (pH 8.0). CAV DNA was amplified by PCR using the upstream primer CAV2, 5'-GGCGGACGCGGCGAGGCAAA-3', and the downstream primer CAV10, 5'-TGGTCATGTATGCTAGGTT-3'. A 100 ml reaction mixture was prepared containing 300 nM each of dATP, dCTP, dGTP and dTTP (Promega), 2 U Taq DNA polymerase (Promega) and 2 ml template DNA. The PCR reaction was incubated at 96 °C for 2 min, followed by 40 cycles at 96 °C for 4 s, 63 °C for 60 s and 72 °C for 60 s, with a final incubation at 72 °C for 5 min. The PCR products were analysed by agarose (1%) gel electrophoresis and infection verified on the basis of detection of a 677 bp band.

IFAs were performed on squash preparations of bone marrow to detect cells expressing VP3. Cells were fixed in ice-cold methanol (90%) for 5 min and blocked for 1 h with a solution of 5% BSA in PBS containing 0.1% Tween 20 (PBST) at 37 °C in a humidified chamber. The samples were then incubated with mAbVP3 diluted 1/200 in 0.1% BSA/PBST for 1 h at 37 °C in a humidified chamber. Samples were then incubated with sheep anti-mouse antibody conjugated to fluorescein isothiocyanate (Dako) diluted 1/100 in 0.1% BSA/PBST for 1 h. The preparations were washed between all incubations with PBS containing 0.1% BSA and 0.5% Tween 20.

**Lesion scores.** A grading system was established to allow consistent classification of lesion severity associated with CAV infection. Gross lesions within the thymus, bone marrow and spleen were all scored on a scale of 1 to 4. Gross lesions within the bursa and haemorrhages were scored on a scale of 1 to 3. From this a cumulative lesion score was derived for the overall severity of pathology. Those lesions most central to the pathology of CAV such as thymic and bone marrow lesions were double weighted in the cumulative lesion score method to reflect their central role in CAV infection. This allowed us to include both minor lesions and significant lesions in our assessment. This method is supported by the findings in positive control-infected birds – where all birds will have severe thymic and bone marrow lesions, but not all birds have the minor lesions. The weighting of parameters according to their significance in determining an overall score for a trait is a common method in pathology studies and in statistical models in general, and is used in an attempt to arrive at broad definitions of disease that are not biased by over-representation of minor pathology.

In all cases, a score of 1 indicated no pathology. Table 1 outlines the scoring system used for gross pathology. Cumulative scores between 20 and 26 indicated severe CAV lesions, scores between 15 and 19 indicated moderate lesions, and mild CAV lesions scored between 7 and 14. Median lesion scores in each group of infected birds were compared using the Mann–Whitney test. Between trial variation in
All embryos infected with wild-type virus had lesions within the thymus, bone marrow and spleen, and severe fascial and subcutaneous haemorrhage, cumulatively categorized as severe CAV pathology. The parental virus and virus regenerated from transfection of the cloned construct pCAU269/7 were found to be equivalent in infectivity and virulence. There were no significant differences in the median lesion scores for the thymus ($P=0.2$), bone marrow ($P=0.1$), spleen ($P=0.2$), bursa ($P=0.1$), or for haemorrhage ($P=0.3$), or in cumulative lesion scores ($P=0.3$). Embryos infected with wild-type virus had median lesion scores significantly higher than those of uninfected embryos for lesions in the thymus ($P=0.01$), bone marrow ($P=0.02$), spleen ($P=0.01$), bursa ($P=0.02$), and for haemorrhage ($P=0.04$), and also had higher median cumulative lesion scores ($P=0.02$). No significant differences were found between the mean PCV of the uninfected embryos and the mean PCV of those infected with parental virus or regenerated virus. Mean body weight, mean thymus : body weight ratio, mean spleen : body weight ratio, and mean bursa : body weight ratio were all lower in CAU269/7-infected embryos than in uninfected embryos. All subsequent experiments were conducted with the virus regenerated from cloned CAU269/7 as the positive control.

Gross pathology after wild-type virus infection

Severe CAV-specific pathology was present in all birds treated with parental or regenerated CAU269/7 virus in five different experimental trials. Petechial haemorrhages of mild to moderate extent were found in the superficial and deep fascial and subcutaneous tissues. The spleen was reduced in size by between 50 and 80 % and, on sectioning, the parenchyma appeared diffusely pale and there were focal subcapsular haemorrhages. In all lobes of the thymic chains there was a severe loss of parenchyma and either lobar haemorrhages or a subcapsular, gelatinous, red fluid filling the lobes, consistent with acute cytolysis. A moderate to severe myelophthisis was evident in the medulla of longitudinally sectioned femurs, and the contents appeared either pale and fatty or red and liquefied, consistent with acute lysis. Paleness of the marrow and loss of cells evident as holes in the marrow is very obvious macroscopically in infected animals. In a minority of birds the Bursa of Fabricius was grossly reduced in size, the walls appeared thinner than normal, and the capsule and parenchymal folds had an appearance of increased convolutions and folding. No additional pathology was found in any organ systems beyond the CAV-specific lesions.
Infection with viruses mutated in VP2

Attenuation of CAV-specific lesions was seen in all embryos infected with VP2 mutated viruses (Table 2). Viruses could be categorized according to their degree of attenuation into two broad categories: highly attenuated viruses (Mut C86R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut R/K/K150/151/152G/A/A, Mut D/E161/162G/G and Mut E186G) and moderately attenuated viruses (Mut L163P and Mut D169G) (Peters et al., 2005, 2006). Cumulative lesion scores in embryos infected with any of the highly attenuated viruses were low (medians between 9 and 12) and were intermediate (median 15) for both the moderately attenuated viruses (Table 2). Median cumulative lesion scores in embryos infected with mutant viruses were significantly lower than in embryos infected with wild-type CAU269/7.

Mean body weights of embryos infected with mutant viruses were not significantly different from that of uninfected embryos, but were significantly greater than the mean body weight of embryos infected with wild-type virus (Table 3), indicating that the VP2 mutations attenuated the growth suppression induced by wild-type virus infection. Median scores for thymic, splenic and bone marrow lesions, and for haemorrhage, were all low in embryos infected with those mutant viruses inducing low median cumulative scores, with the exception of viruses Mut R129G and Mut D/E161/162G/G, for which median thymic lesion scores were intermediate (Table 2). 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 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 up to 30% compared with uninfected embryos, consistent with mild splenic atrophy, and the spleens appeared pale. In general, the bursae appeared normal.

For embryos infected with all VP2 mutated viruses, except Mut D169G, median thymic, splenic and bone marrow lesion scores were significantly lower than those of embryos infected with wild-type virus (Table 2). Median thymic and splenic lesion scores were intermediate in embryos infected with Mut D169G. Either median haemorrhage scores were low or there were no haemorrhages detected in embryos infected with mutant viruses. Median haemorrhage scores in all embryos infected with VP2 mutants were significantly lower than those in embryos infected with wild-type virus, with the exceptions of embryos infected with Mut R/K/K150/151/152G/A/A and Mut D169G.

Median bursal lesion scores were similar in embryos infected with either wild-type or VP2 mutant viruses, and the incidence of grossly apparent bursal lesions was sporadic (Table 2). However, there was a significant reduction in mean bursa : body weight ratio in embryos infected with CAU269/7 relative to uninfected embryos (Table 3). In embryos infected with Mut C86R, Mut R101G, Mut R129G, Mut Q131P, Mut L163P, Mut D169G and Mut E186G the mean bursa : body weight ratio was significantly greater than in embryos infected with wild-type virus.

There were no differences detected between the mean PCVs of embryos infected with wild-type virus, VP2 mutant viruses or uninfected embryos. The normal haematocrit cannot be attributed to an absence of CAV infection in the bone marrow as VP3-positive cells were identified by immunofluorescence in bone marrow smears taken from the femoral medullary cavity of infected embryos. It is more likely that normal haematocrits were observed because the rate of loss of infected precursor cells due to CAV infection did not exceed the rate of their maturation and release into the circulation. A reduction in haematocrit is a common finding in most reports of birds infected with CAV at 1 day of age. A haematocrit less than 27%, and typically between 9 and 23%, is found at 7–14 days post-infection for chicks infected at 1 day of age (normal 32–37.5%) (Connor et al., 1991; Imai et al., 1999; von Bulow & Fuchs, 1986). Reports of confirmed outbreaks of CAV in five flocks in New Zealand described the presence of typical CAV-induced lesions in lymphoid tissues, but no changes were observed in the haematocrit (Stanislawek & Howell, 1994). These reports indicate that anaemia is not always found in field CAV infections. The anaemia reported in birds infected at 1 day of age is a transient feature of the pathology due to the rapid maturation of erythroblastoid cells in the avian bone marrow. Birds infected at 1 day of age develop a peracute severe anaemia at 7 days post-infection, and the haematocrit rapidly returns to normal at 14 days. A normal haematocrit may have been observed in this study due to the 14 day time interval between infection and sampling. Haematocrit data has not been published previously for CAV infection in an embryonic model. The blood was watery on venepuncture due to primary coagulopathy arising from thrombocytopenia rather than due to low PCV. This distinction in the pathogenesis has been described in previous publications (Engstrom et al., 1988; Goryo et al., 1989; McNulty, 1991; McNulty et al., 1990; Taniguchi et al., 1982; Yuasa et al., 1983).

Mean thymus : body weight and mean spleen : body weight ratios of embryos infected with all VP2 mutant viruses were significantly greater than those of embryos infected with wild-type virus, and were not significantly different from the ratios in uninfected embryos (Table 3).

Gastrointestinal lesions were seen in 12 of 18 embryos infected with Mut 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, three had lesions in the crop, one in the proventriculus, one in the ventriculus, one in the duodenum, seven in the jejunum and two in the ileum. The majority of embryos infected with Mut R/K/K150/151/152G/A/A had mild CAV lesions, and only two had lesions graded as moderate (Table 2). Gross lesions in the crop,
Table 2. Lesion scores in the lymphoid tissues, haemorrhage scores and cumulative scores in embryos infected with CAV

S, Median score; R, range; P₁, probability that medians differ between CAU269/7-infected and treatment groups; P₂, probability that medians differ between uninfected and treatment groups; n, group size.

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<th>Treatment group*</th>
<th>Thymus</th>
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<th>Bursa</th>
<th>Bone marrow</th>
<th>Haemorrhage</th>
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<td>†</td>
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NA, Not applicable.

*Virus inoculated into E7 embryos.
†P<0.05; ‡P<0.005; §P<0.01; †P<0.001.
proventriculus, ventriculus and intestines consisted of thinning of the gastrointestinal wall, hyperaemia and serosal haemorrhages. Lesions were also found in the liver of six of the embryos, with a generalized pattern of irregular nodularity and purplish-red discoloration. In some there were subcapsular hepatic haemorrhages and focal or marginal infarcts in the hepatic lobes. In one embryo haemorrhage and irregular roughening was seen on the synovial surfaces of both femorotibial and tibiotarsal joints. Viral infection was detected by CAV-specific PCR on liver homogenates from embryos infected with all mutants and wild-type CAU269/7 (data not shown). PCR product was not amplified from uninfected embryos. Whilst it is possible that DNA from virus inoculated into the yolk sac at day E7 may be detected in the liver at day E21 simply by passive translocation, it is unlikely as the inoculating dose is 10⁵ TCID₅₀ and after 14 days it is therefore unlikely this would be detected in the liver without amplification by virus replication. Together with the IFA results, the PCR results confirm the establishment of virus infection. Bone marrow cells positive for VP3 expression by IFA were found in the femoral medulla of all infected embryos.

DISCUSSION

An embryonic infection model was established for CAV infection that consistently reproduced CAV-specific lesions. Yolk sac inoculation of 7 day embryos with high doses of virus is the most stringent virulence model available. This model best approximates the field situation for vertical transmission, in which naïve breeder birds at the point of lay are exposed to CAV and transmit virus transovarially. Chicks infected by vertical transmission have the highest rates of morbidity (100 %) and mortality (10–70 %) and the pathology is of greatest severity (Yuasa et al., 1983). Studies based on experimental infection of embryos have been reported previously in the literature (Rosenberger & Cloud, 1989; Yuasa et al., 1983), and as vaccine viruses could potentially infect naïve breeder birds at the point of lay, the virulence of mutant virus strains in embryos is of considerable interest. Severe CAV-specific lesions were consistently and uniformly produced in all embryos infected with wild-type virus. Cumulative lesion scores, mean body weight and mean thymus : body weight ratio were found to be representative of data obtained for all other parameters measured. Significant growth depression, measured as a reduction in mean body weight, was seen in embryos infected with wild-type CAV. Infection with wild-type CAV resulted in a significant reduction in thymic and splenic weights, and consistently produced severe lesions in the thymus, spleen and bone marrow, and haemorrhages. Gross bursal pathology was only seen sporadically, but there was a reduction in the mean bursa : body weight ratio. The bursa is not considered to be a target organ for CAV infection, but bursal lesions secondary to T-lymphocyte depletion in the thymus have been reported previously (Rosenberger & Cloud, 1989).


<table>
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<th>Treatment group*</th>
<th>Body weight (g)</th>
<th>Thymus : body weight (mg g⁻¹)</th>
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NA, Not applicable.
*Virus inoculated into E7 embryos.
§P<0.05; ||P<0.01; †P<0.001.
In this study, the PCV was normal in all embryos infected with wild-type and VP2 mutant viruses, even though VP3-positive cells were found in bone marrow smears. Thus, the rate of loss of infected precursor cells due to CAV infection did not exceed the rate of their maturation and release into the circulation. Anaemia is a common finding in most reports of birds infected with CAV at 1 day of age, with the PCV reduced between 7 and 14 days after infection (Connor et al., 1991; Imai et al., 1999; von Bulow & Fuchs, 1986). However, there are reports of outbreaks of disease due to CAV in flocks with typical lesions in lymphoid tissues, but no signs of anaemia (Stanislawek & Howell, 1994). As anaemia in birds infected at 1 day of age is transient, it is possible that the lack of anaemia in infected birds in our study was due to the 14 day time interval between infection and sampling. PCV data have not been published previously for CAV infection of embryos.

CAVs mutated in VP2 were infectious in an embryonic infection model and highly attenuated with respect to growth depression and CAV-specific pathology. As for infection with wild-type virus, cumulative lesion scores and body weight were highly predictive of the pathological scoring and organ weights for all lymphoid tissues. Based on the degree of attenuation, the mutant viruses cluster into two groups: Mut C86R, Mut R101G, Mut H103Y, Mut R129G, Mut Q131P, Mut R/K/K150/151/152G/A/A, Mut D/E161/162G/G and Mut E186G were highly attenuated, and Mut L163P and Mut D169G were moderately attenuated. Attenuation was found consistently for lesions in the thymus, spleen, bone marrow, thymic and splenic weights, and for haemorrhage. There was no growth depression associated with infection by the group of highly attenuated mutant viruses and a moderate reduction in mean body weight was only found for Mut L163P. As these viruses are infectious in vivo and concurrently highly attenuated, they are of interest as potential vaccine candidates.

Atypical gastrointestinal lesions were observed in embryos infected with virus Mut R/K/K150/151/152G/A/A in two independent experiments. These lesions were not seen in embryos infected with other viruses or in uninfected embryos and were therefore a specific effect of this virus. Typical CAV-specific pathology was mild in all embryos infected with Mut R/K/K150/151/152G/A/A. However, severe haemorrhages were seen in the subcutaneous tissues and fascial sheets. The full thickness necrosis of the intestinal wall and serosal haemorrhages are not likely to be a result of severe anaemia and subsequent ischaemic injury to the gastrointestinal tract, as the PCV was normal and a more generalized pattern of injury would be expected with anoxia, with involvement of the myocardium and renal tissue rather than the intestine alone.

A change in viral tropism associated with mutagenesis is perhaps a more plausible explanation for the specific distribution of lesions in the gastrointestinal tract in embryos infected with Mut R/K/K150/151/152G/A/A. The virus contains three mutations that neutralize basic residues in a region of VP2 predicted to be basic amphipathic $\alpha$-helix. The mutations are also located in the genome at the junction of ORF2 and ORF3 in a segment of Alu repeat sequence. The mechanism by which translation of VP1 from the polycistrionic mRNA is initiated is unknown; however, the Alu repeat region could play a role in this process. As a change in tropism is more likely to involve an alteration in structural viral proteins such as VP1, the unique phenotype of this mutation, not seen with other mutations that similarly neutralize the basic charge in VP2, may be due to an influence on VP1 gene translation rather than due to substitutions in VP2.

This study has established that VP2 is an effective target for attenuation through site-directed mutagenesis and is the first study to demonstrate attenuation of viral effects in vivo through modification of VP2. This is in contrast to previous studies examining virus constructs mutated in VP3, which were not replication competent (Danen-Van Oorschot et al., 1997, 1999, 2000). The mechanism by which VP2 mutagenesis attenuates virulence remains unknown. It is probable that VP2 has multiple functions and that direct mutagenesis that maintains infectivity may maintain functions necessary to replication, whilst modifying functions associated with virulence.

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**REFERENCES**


