THE ROLE OF A DELETION IN THE GLYCOPROTEIN L (gL) GENE OF MAREK'S DISEASE VIRUS (MDV) ON MDV VIRULENCE

by

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ABSTRACT

Marek's Disease is a lymphoproliferative, immunosuppressive, and demyelinating disorder of chickens caused by a cell-associated alphaherpesvirus, Marek's Disease Virus (MDV). MD is considered to be the most expensive disease to control in poultry production, due to the cost of vaccination and the immunosuppressive nature of the causative agent. MD is mainly controlled through the use of avirulent vaccines and management practi. Vaccines can be comprised of three viruses, and are used in various combinations: attenuated MDV-1 strains (CV1988, Rispens), non-oncogeneic viruses MDV-3 and herpesvirus of turkeys (HVT). A bivalent combination of HVT and MDV-2 (strain SB-1) is the most commonly used vaccine for broiler chickens in the US. Due to the evolutionary pressures generated by extensive vaccine use and the selection of rapidly-growing chickens, field strains of MDV have continued to evolve in virulence. There have been no genetic mutations directly associated with the increased virulence of MDV strains that can explain the ability of virus to overcome vaccine protection.

We have previously identified a novel mutation in the glycoprotein L (gL) genes of highly virulent field isolates of MDV. This mutation is 12 nucleotide deletion within the coding sequence of gL and is found to be common to all the field isolates we have obtained from DE, MD, VA, PA and NC since 2005. In previous work, we proposed that the gL mutation directly affects bivalent vaccine efficacy in naturally-challenged chickens, when these chickens were exposed to vaccinated chickens infected with a gL mutation-containing MDV. Although these data suggested that this

mutation was responsible for this observation, it is unclear as to how this occurs, mechanistically. To confirm the observation using a genetically-identical background strain, and to test the mechanism of the observed effect, we have introduced this deletion in the gL gene of pRB1B BAC, an infectious clone of the RB1B strain of MDV. We introduced this mutation using a two-step recombination method resulting in markerless mutants. To confirm our analysis of this recombinant, we planned to generate a revertant virus using the same approach, but were unsuccessful. The parental (pRB1B) and mutant (pRB1BgLΔ) were compared with respect to: (1) their ability to replicate in cell culture, (2) differences in expression of gL, gH and the gH/gL complex on the surface of infected cells, (3) selection for the mutant at the level of cellular replication, (4) their ability to replicate, transmit, and cause disease in specific pathogen free (SPF) chickens, (5) their ability to overcome vaccine protection in contact-exposed chickens, and (6) their differences in their abilities to overcome antibody neutralization.

We found that the gL mutation in the context of pRB1B did not confer increased replication or competitive advantage at the level of replication in cell culture, although the mutant did show some increase in the plaque size in chicken embryo fibroblasts. In chickens, both parental and mutant viruses replicated to high titers and caused some mortality and tumor incidence, however, this was notably less than the level of disease caused by non-BAC-based RB1B. Due to the decrease in pathogenicity of the pRB1B-based viruses, we found no loss of vaccine protection in contact-exposed chickens, nor-did the gL mutation confer increased resistance to neutralization by maternal antibodies. Our data suggest that the gL mutation may

have arisen in the field after mutations arose that have more directly affected increased virulence, and that this mutation may confer advantage only in this context.

Chapter 1

INTRODUCTION

Marek's disease (MD) is a highly, contagious lymphoma of chickens caused by an acute- transforming, cell-associated avian alphaherpesvirus called Marek's disease virus (MDV) (73). Since its first description, the clinical disease has changed in its manifestation in commercially-raised poultry. The disease was first reported by a Hungarian veterinarian, Dr. Jozsef Marek, in 1907 and was associated with paralysis in laying hens (61). The disease became associated with the induction of lymphoid tumors after long-term infection of chickens in the 1920s and was termed, *Neurolymphomatosis gallinarum* (75, 76). By the mid-1960s, an "acute" form of MD was described that could cause lymphomas by ~12 weeks (8). This acute type Marek's disease quickly became predominant form of the disease in the poultry industry worldwide. The disease can manifest itself in a number of ways ranging from a mild form, mainly restricted to nervous tissue, to a severe form with visceral lymphomas and early mortality (120).

Clinical signs of MD include paralysis, skin leukosis, depression, and death. Lesions associated with MD are neural and visceral lymphomas, thymic and bursal atrophy, splenomegaly, and stunting (18). In poultry production worldwide, Marek's disease is considered to be the most expensive disease to control, due to the cost of vaccination, the direct effects of the disease on chickens, and the indirect effect of the immunosuppressive nature of the causative agent (10).

1.1 Marek's Disease Virus

MDV is a cell-associated virus of the Herpesviridae family, Alphaherpesvirinae sub-family, genus *Mardivirus* that causes malignant T-cell lymphomas in chickens (73). In infected cells, MDV is found in the nucleus as icosahedral viral nucleocapsids of about 100 nm in diameter, while enveloped viral particles are detected near the outer nuclear membrane, in the cytoplasm and on the cell surface (36). MDV is comprised of three antigenically-related viruses (MDV-1, MDV-2 and MDV-3 or herpesvirus of turkeys, HVT). MDV-1 strains include oncogenic viruses and their attenuated derivatives. MDV-2 strains are non-oncogenic viruses of chickens, and HVTs are apathogenic viruses initially isolated from turkeys (38).

Several studies revealed the similar morphology and morphogenesis of MDV and HVT (50, 80); in general, all the serotypes of MDV have characteristics typical of the other alphaherpesviruses. Even though the genome structure of MDV is similar to alphaherpesviruses such as herpes simplex (HSV) and varicella zoster virus (VZV), its biological properties suggest that MDV is more akin to gammaherpesviruses, such as Epstein-Barr virus (EBV) (47). The genomes of all the Herpesviridae family are double-stranded linear DNA molecules ranging from 108 to 230 kbp in size (60). There are a total of six different classes of genome organizations referred from A to F in the Herpesviridae family (86). In the Alphaherpesvirinae sub-family only class D and E genomes are found. MDV-1, MDV-2 and HVT belongs to Class E, which bear similarity to herpes simplex virus type 1 (HSV-1) (87).

1.1.1 The MDV Genome

MDV has a genome structure similar to that of herpes simplex virus (HSV), in that it has a unique long (UL) and short (US) regions that are flanked by terminal and internal repeat regions (TRL, IRL, TRS, and IRS) (30, 108). The US, UL, and repeats flanking the US regions of MDV primarily encode structural genes and genes involved in viral genome replication and assembly (59). Many of these genes are conserved with respect to homologs encoded by other alphaherpesviruses. Genes encoded in the repeat regions flanking the UL, however, differ significantly between MDV and herpes simplex, as well as in different serotypes of MDV (42, 52, 74). The RL regions of MDV-1 strains contain ~13 kbp of unique sequence encoding genes involved in oncogenicity and virulence. Transcription of the MDV genome in tumor cells and derived cell lines is largely confined to the repeat regions (89, 109, 110). Within these regions, there is a limited number of genes, including pp14 (39), viral IL-8 (vIL8) (78), viral telomerase RNA (29), pp38 (19, 22), RLORF4 (46), and Meq (**M**arek's **E**coRI-**Q**-encoded protein) (48).

1.1.2 MDV Pathogenesis

The pathogenesis of MDV has been characterized as occurring in four phases: (a) early cytolytic infection, (b) latent infection, (c) reactivation and late or secondary cytolytic infection, and (d) development of lymphomas or the transformation phase (15, 73).

1.1.2.1 Early cytolytic infection

MDV enters the host through inhalation of infectious dander (7). These dust particles are phagocytosed by lung epithelial cells and transferred to macrophages and B-cells, which disseminate the virus to lymphoid organs such as bursa of Fabricius,

thymus and spleen (1, 4, 83). Early infection occurs primarily in B- and T-lymphocytes. MDV infection of B- and T-cells is different as B-cells support lytic infection (productive/restrictive) and T-cells can either support lytic (CD8+, some CD4+) or latent (primarily CD4+) infection (106). Cytolytic infection of B-cells and T-cells is semiproductive and virus infectivity is strictly cell-associated.

1.1.2.2 Latency

An early innate host immune response to MDV lytic replication (i.e., induction of IFNs, NO, etc.) results in of the induction of latency, primarily in CD4+ T-cells (130, 133). During latency, the viral genome is present in cells without producing viral particles. Latently-infected T-cells persist for the life of the chicken leading to sporadic reactivation of MDV at peripheral sites, including the feather follicle epithelium (16). Latency is associated with a shift in viral genome expression, with expression limited primarily to the repeat sequences (9, 109).

1.1.2.3 Secondary cytolytic infection

Immunosuppression after latency can result in a secondary cytolytic infection in the lymphoid tissues, as well as at other sites (13). Secondary cytolytic infection also occurs in the feather follicle epithelium (FFE), the only known site of fully-productive viral replication, resulting in MDV shed to the environment (17).

1.1.2.4 Transformation

From three to four weeks post-infection, latently-infected CD4+ T-cells proliferate and become transformed (15). MDV has the capacity to transform other T-cell lineages (CD8+/ CD4-, CD8-/ CD4-, and CD4+/ CD8+) (96), although the transformed component of most MDV-induced lymphomas are CD4+ T-cells (11).

These MDV-transformed CD4+ T-cells express high levels of Meq, and surface antigens CD25, CD30, MHC-II, and one or more Marek's associated tumor surface antigens (MATSAs) (12, 56). Integration of the MDV genome is common to transformed T-cells, as well as cell lines established from lymphomas, and transformed cells typically contain 5-15 copies of the MDV genome (26, 27). Lymphomas are usually seen in visceral organs, but also at other sites including breast muscle, eyes, nerves, and skin. Since transformation is a multi-step process, the exact mechanism of transformation is not clear, but because of the high incidence and rapid induction of lymphomas, it appears that MDV encodes an oncogene or oncogenes.

Of the genes expressed in MDV-induced lymphomas, Meq fulfills most of the criteria for being a true oncogene. Meq is consistently expressed in all MDV-induced lymphomas and lymphoblastid cell lines (48), is absent from MDV-2 and HVT genomes, binds cell-cycle regulatory factors (p53, Rb, CDK-2, Skp-2, etc.), and transactivates known cellular proto-oncogenes (55, 57). Meq morphologically transforms both rat and chicken fibroblasts, induces proliferation, and blocks apoptosis (56, 58). In addition to Meq, other genes contributing to transformation and lymphoma progression are: RLORF4 (46), vTR (118), and a ubiquitin-specific protease (USP) activity of the major tegument protein encoded by the UL36 gene (43).

1.2 Control of MDV

Since the early 1970s, MD has been controlled in commercial poultry production through the widespread use of vaccines. The initial vaccine showing efficacy against acute MDV was an attenuated MDV-1 strain, HPRS-16att (20). In the US, an apathogenic herpesvirus of turkeys (HVT) was found to confer protection to

MDV-1 challenge became the most commonly used vaccine until the mid-1980s (121, 124).

1.2.1 Serotypes of MDV

MDV describes three antigenically-related members known as Marek's disease virus serotypes 1 and 2 (MDV1 and MDV2, or *Gallid herpesvirus* 2 and 3 or *Mardiviruses* 1 and 2) and herpesvirus of Turkeys (HVT) or serotype 3, or *Meleagrid herpesvirus* 1 (24).

All pathogenic MDVs belong to serotype 1, but may vary greatly in their relative pathogenicity. The other two species; MDV-2 and HVT are both non-oncogenic (54, 99). The sequence similarity between three viruses ranges from 50%-80% (52). MDV causes clinical disease in chickens whereas the other strains; MDV-2 (94), HVT (123) or attenuated MDV-1 strains (21) provide protection against the MD.

1.2.2 MDV Vaccines

MD vaccines were first introduced in 1970, when the first vaccine HPRS-16/att was used to protect against disease and mortality (20). This vaccine was derived by attenuation of a virulent strain and was replaced later by a herpesvirus of turkey (HVT) (123). In the US, HVT was used as the most predominant vaccine against MD (123). Due to the increased virulence of field strains of MDV in the mid-1980s very virulent (vv MDVs), HVT was replaced by use of a bivalent vaccine comprised of HVT along with serotype 2 strains such as SB-1 (123). The extensive use of monovalent (HVT) and bivalent vaccine (MDV-2 and HVT) lead to the evolution of more virulent strains of MDV-1 termed very virulent plus (vv+) MDV-1 (120).

In the early 1990's, the gold standard serotype MDV-1 vaccine, CVI988, also known as Rispens, was introduced to the poultry industry of the US (85). Rispens was a mildly virulent serotype virus 1 and was originally isolated in the Netherlands (85). To be used as a vaccine, Rispens was further attenuated by serial cell culture passage (85). Currently Rispens is used as the most prevalent vaccine worldwide, and provides superior protection against hyper virulent (vv+) pathotypes of MDV (85). Vaccines are used in either bivalent (Rispens + HVT/SB1, Rispens/ HVT) or in trivalent (Rispens + MDV-2 + HVT) combinations (125, 126)

MD vaccines are mainly of two types: cell-associated, which is administered as an infected cell suspension, and cell-free preparations, which can be lyophilized for transport to areas lacking storage capability for the cell-associated vaccine (41). The MD vaccine is administered intramuscularly or subcutaneously in chicks at 1 day-of-age (121). To expedite the process, vaccines are currently administered as cell-associated live virus to embryonated eggs around embryonation day 18 (E18) before hatching, or to neonatal chicks using automated head injectors (84). Vaccines are usually available in glass ampules containing 1000 or 2000 doses and are stored and transported in liquid nitrogen (-196°) (25). The proper handling of MD vaccine during its storage and transportation is an absolute necessity for the maintenance of the vaccine efficacy (25). The preparation of vaccine including thawing and dilution before administration should be done by using a clean and sterile adequate system. Improper handling of vaccine may result in failure of vaccine (25). The environmental contamination from highly virulent strains of MDV such as vv+ MDV may also contribute to vaccine failure. Therefore improved management practices and bio-security measure are an absolute necessity in order to reduce losses to producers (25).

1.2.3 Immunity elicited by MD vaccines

MD vaccination successfully protects against a naturally occurring virus-induced cancer. Although MD vaccination prevents tumorigenesis by targeting the viral replication during the first cytolytic phase of infection (100), it does not prevent initial infection, replication of virus, nor virus transmission (95).

Infection of chickens with MDV can stimulate the host innate immune responses including activation of macrophages, type I Interferons (IFNs), natural killer (NK) cells, and other cytokines (79). Macrophages play a central role in the innate immune response by phagocytosing viral pathogens and transporting antigens to primary lymphoid organs, such as bursa of Fabricius, from sites of infection (97). Through engagement of Toll-like receptors, macrophages release pro-inflammatory cytokines such as interleukins 1, 6, and 18, as well as nitric oxide (NO), chemokines and interferons (79). Macrophages are also antigen presenting cells (APCs) and present antigens to B and T lymphocytes (82). NO production by macrophages is essential for bactericidal activity and inhibition of virus replication in the host (131). MDV infection stimulates the production of pro-inflammatory cytokines such as IFN gamma (IFN-γ) that up-regulate the expression of inducible NO synthase (iNOS), which in turns increases the levels of NO and hence inhibits the virus replication (131).

Infection of chickens with HVT has shown to be related to the early strong expression of various cytokines such as IFN-γ, iNOS, and CC chemokines (28). In chickens, complete genomic sequencing of MDV revealed the involvement of certain genes such as TLRs and cytokines in immune responses (31). TLR ligands can increase the efficacy of MD vaccines by acting as adjuvants. Administration of MD vaccine along with TLR ligands presumably increases the maturation of immune

responses with respect to in *ovo* or at hatch vaccine programs (31). The exact mechanism of vaccine –induced immunity is poorly elucidated but is similar to that seen in response to pathogenic strains. This involves both development of virus – neutralizing antibodies (70), or virus –specific cytotoxic T lymphocyte (CTL) (68) .

Infection of chickens with MDV induces production of specific antibodies against variety of MDV glycoproteins such as gE, gI and gB. Among these neutralizing antibodies against gB exhibits protective immunity against MD by blocking MD entry into host cells (98). In the host, maternal antibodies reduce the clinical symptoms of MD, tumorigenesis, and mortality, but can interfere with vaccine response (14). In the host, non-neutralizing antibodies lyse MDV-infected cells by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) (98). Vaccination with non-oncogenic serotype 2 (SB-1) and serotype 3 (HVT) increases NK cell activity, but the exact mechanism is unclear (37).

In addition to innate immune responses and antibody-mediated defense mechanisms, the CD8+ CTL responses against various MDV envelope glycoproteins helps in controlling viral infection (62, 68). CD8+ T-cells play an essential role in anti-viral responses to MDV, as CD8+ T-cell-depletion of vaccinated chickens, results in decreased protection from challenge (64). After challenging birds with non-oncogenic MDV vaccine strains, the phenotype of CTL reported was CD3+CD4-/CD8+,TCR$\alpha\beta$1 T-cells (67). Vaccination of birds with a recombinant fowlpoxvirus expressing MDV gB (rFPV-gB) elicted CTL and neutralizing antibodies, as well as immune protection against a virulent MDV challenge of MDV (69). The up-regulation of perforin and granzyme A in MDV-infected chicken spleens after 4 and 7

days post-infection (dpi), also support the involvement of CTL in immunity to MD (92).

1.2.4 MDV evolution of virulence

Despite the effectiveness of MD vaccines in controlling Marek's disease, there has been a continuous evolution of increased MDV field strain virulence (120). There have been three major shifts observed in MDV virulence. The first shift was observed before vaccination but after the advent of high-density brooding practices in the 1960s. This first virulence increase was from "classical" strains, which caused nerve lesions with relatively few tumors, to "acute" strains, which caused a greater number of tumors within twelve weeks (8).

In the 1980s, a second shift was observed when very virulent (vv) strains of MDV were isolated which were found to cause profound immunosuppression, atrophy of the bursa, early morality, and a high incidence of visceral lymphomas (93, 120). These vvMDV strains arose after the near ubiquitous use of HVT as a vaccine to control MDV losses in broilers. To provide increased protection against vvMDV challenge in the field, a bivalent vaccine formulation, comprised of HVT in conjunction with MDV-2 (strain SB-1) came into practice (105). The most recent shift was observed in the early 1990s when very virulent plus (vv+) or hypervirulent strains of MDV were isolated from bivalent vaccinated flocks throughout the world (88, 120). These vv+MDVs have been associated with increased losses in bivalently-vaccinated chickens, rapid tumor formation, profound immunosuppression, increased neurological signs, and stunting (32-35, 88). These strains show an increased tropism for macrophages/monocytes and cause an acute paralysis due to high expression of cytokines in the brain (5, 6, 45).

The virulence of field strains of MDV has been determined by: protection indices and lesion scores in unvaccinated; HVT- or bivalently-vaccinated (HVT/SB-1) chickens (120), neuropathologies induced (34), and by direct comparison to strains of defined pathotype (122). The pathotypes are classified as a mild or classic, virulent (vMDVs), very virulent (vvMDVs) and vv+ or hyper virulent MDVs (vv+MDV, hvMDV). Comparison of genes encoded by different pathotype MDVs showed that the major glycoproteins were highly-conserved across pathotype, while Meq coding sequences showed pathotype-specific mutations that correlated with virulence (104). Genome-wide sequence comparisons across different pathotype MDVs showed similar results (111-113). Polymorphic regions were those encoding Meq and domains within the UL36 gene, while the glycoproteins of MDV were notably conserved across pathotype.

1.3 Herpesvirus glycoproteins

MDV and HSV-1 share genome structural and sequence similarities. MDV encodes numerous surface glycoproteins homologous to those of HSV-1 and other alphaherpesviruses. Like VZV, MDV infection *in vitro* and *in vivo* is strictly cell-associated (128). MDV spread from infected to uninfected cells takes place by formation of an intracellular bridge, which is mediated by viral glycoproteins on the infected cell surface (49). HSV-1 encodes 13 surface glycoproteins designated gB, gC, gD, gE/gI, gG, gH/gL, gJ, gK, gM/gN and UL43 (134). Five of these glycoproteins (gB, gC, gD, gH/gL) play roles in viral entry, with gB, gD and gH/gL being essential for this process (2). Glycoprotein complexes gE/gI (102), gH/gL (103), gM/gN (116), play essential roles in virus cell-to-cell spread. Deletion of

specific glycoprotein genes, notably UL44 (gC), UL49.5 (gK), and US7 and US8 (gI/gE) alter virus tropism and affect virulence. Glycoproteins C and the I/E complex have roles in immune evasion, through binding complement proteins (119) and forming an Fc receptor (65), respectively.

Glycoproteins B, C and H/L are highly conserved and found in all three sub-families of herpesvirus (53). For HSV-1, glycoproteins H and L form a hetero-oligomer, which has also been reported for the gH and gL proteins of Epstein Barr virus (EBV) (132), human herpesvirus 6 (HHV-6) (58), and human cytomegalovirus (HCMV) (51). The expression of both gH and gL is essential for the processing of both the proteins and their interaction is important for maturation, subcellular translocation, proper folding, and cell surface expression (40). Both gH and gL are important in HSV-1 replication (40). The sequence analysis of virulent (GA) and vv (MD-5) MDVs revealed 10 homologs of HSV-1 surface glycoproteins: gB, gC, gD, gE, gH, gI, gK, gL, gM and gN (72, 104). In addition, MDV encodes another surface glycoprotein, gp82 encoded by the UL32 homolog gene (127). The phylogenic analysis of gH homologs revealed that the gH of MDV is more similar to alphaherpesviruses than beta- or gammaherpesviruses (129). The amino acid sequence alignment of MDV gH homologs among the three serotypes of MDV revealed identities of 57.5% (MDV-1 vs MDV-2), 56.2% (MDV-1 vs HVT), and 50.1% (HVT vs MDV-2) (107). It has been shown that MDV-1 gL shares 18% identity with the HSV-1 (128).

Of the glycoproteins encoded by MDV, gB (101), gE/gI (102), gH/gL (103), and gM/gN (116) are essential for virus infection in cell culture. Glycoproteins C and D are nonessential for infection in cell culture, or in tumor formation (3, 44). The

expression of gD in fact appears to be very limited *in vivo* and its deletion has not been associated with any deficiency in infection, oncogenicity, or horizontal transmission. Loss of gC expression is associated with increased plaque size in cell culture and loss of horizontal transmission *in vivo* (115, 117). Expression of gC is somewhat complex in that spliced forms and secreted forms are expressed during infection (44). Co-expression of gH and gL homologs of MDV is essential for the proper processing and translocation of the gH/gL complex to the cell surface and for cell-to-cell spread of MDV infection (128).

1.3.1 The role of glycoproteins in the evolution of MDV virulence

The molecular basis for the increase in MDV virulence and pathogenicity is still unknown. In order to identify the common mutations associated with pathotype specific changes in MDV oncogenicity, our lab PCR-amplified, cloned and sequenced the genes encoding the major surface glycoproteins, as well as other regulatory genes (104). As a result, we identified no pathotype-specific mutations in glycoproteins B, C, D, E, H and I, whereas a novel mutation was identified in the glycoprotein L gene of three hyper virulent MDVs and one very virulent MDV. Our conclusion from this work was that the major glycoproteins (gB, gC, gE, gI), which are most homologous to those of vaccine strains (HVT, SB1) were not under direct selection in MDV-1 field strains. These results suggested that evolution of field strains of MDV was not directly tied to surface glycoprotein escape mutants (104).

The mutation observed in the gL gene of a select few strains of MDV suggested that a common selection was being applied to elicit the evolution of these strains. The mutation consisted of the deletion of 12-bp within the coding region of gL and containing the putative signal cleavage site (133), which is important for insertion

of protein into the lumen of endoplasmic reticulum during translation (104, 133). This mutation was found to be common to all submitted field isolates of MDV recently obtained from Delmarva Peninsula, Pennsylvania as well as North Carolina (114).

1.3.2 The Effect of the gL mutation on MD vaccine efficacy

In initial follow-up studies, we compared the pathogenecity of two strains of MDV (TK and RL, a.k.a. 615K and 615L), which were isolated from flocks at about the same time, had identical Meq genes, and differed at the gL locus (91). Each strain, TK (gLΔ) and RL (gL wt) was inoculated into SPF chickens with or without co-infection with HVT. It was observed that when TK strain was co-inoculated along with HVT, it was more pathogenic as compare to the TK strain alone, the RL strain alone, and the RL strain co-inoculated with HVT (91). The conclusion from this work was that the deletion observed in gL appeared to confer some increased virulence in the context of vaccinated (HVT co-infected) chickens. Increased virulence was seen in contact-exposed chickens and overcame age-associated resistance.

A second set of follow-up studies was performed to examine the effect of the gL deletion in combination with MDV vaccines in the context of naturally-exposed broiler chickens (114). In this study, TK-based strains were used that either had the gL deletion (TK1a) or did not (TK2a). The TK2a strain was isolated after serial passage through SPF chickens. TK2a was found to differ from TK1a at both the gL and Meq loci, however. By direct comparison, TK2a was more virulent in inoculated chickens than TK1a, causing more rapid mortality and higher incidence of MD. To assess the effect of the gL mutation on virus circulating in vaccinated chickens, embryonated broiler eggs were unvaccinated, HVT-, or HVT/SB1-vaccinated, *in ovo*. The chickens were then inoculated with TK1a, TK2a or a 1:1 mix of the two strains at

hatch. These were placed in separate rooms to shed virus into the environment. To assess the virulence of virus being shed from these chickens, we placed unvaccinated, HVT-, and HVT/SB1-vaccinated (*in ovo)* chickens in contact with the challenge virus-inoculated chickens.

Using this natural exposure model, we found that in contact-exposed vaccinated chickens, TK1a overcame bivalent vaccine (HVT/SB1) protection as compare to TK2a strain (114). It was also observed that in every treatment group where the bivalent vaccine failed to provide protection superior to HVT alone, the TK1a strain was the most prevalent. Our data suggested that there was a strong selection for this deletion, which occured in vaccinated chickens with the co-replicating deletion-containing virus. The study was repeated using quadruplicate treatment groups of commercially-vaccinated (bivalent) chickens and similar results were observed.

1.3.3 Effect of the 12 bp deletion on MDV glycoprotein L, gH and gH/L complex surface expression

After identifying the deletion in the gL genes of several hypervirulent MDVs, we hypothesized that this mutation may affect the surface expression or processing of this protein. Therefore, we tested the difference between the surface expression of gL, gH, or gH/gL in cells infected with the TK1a or TK2a using antibodies generated to MDV-1 gL, gH, and the gH/gL complex (103). As a result we observed that there was a marked decrease in the surface expression of gL, gH, and the gH/gL complex on TK-1a- compared to TK2a-infected CEF (data not shown).

In addition, we have found that antibodies to MDV-1 gL, gH and the gH/gL complex strongly cross-reacted to HVT- and SB-1-infected CEF (data not shown).

This cross-reaction and the downregulation of surface expression of gH and gL on TK-1a-infected cells suggests that vaccine strains may elicit potent immune responses to these proteins as antibody and perhaps T-cell epitopes. The signal peptide of glycoprotein K of HSV-1 and -2 is a potent CD4+ and CD8+ T-cell epitope, capable of binding both MHC-I and MHC-II (71). This decrease in the expression of gH/gL complex on the infected cell surface may therefore be functionally significant as gH and gL both are essential for MDV infection in culture (74).

Glycoprotein L (gL) does not have a transmembrane domain, but is essential for the surface expression of the gH/gL complex on infected cells (133). An objective of our work was to study the effect of the gL mutation on surface expression of the gH/gL complex of recent MDV field isolates. Our preliminary data suggest that the gL mutation decreases the surface expression of the gH/gL complex and further suggests that this complex is under genetic selection in vaccinated chickens.

1.4 Hypothesis and specific aims

The hypothesis of this research was that this mutation has evolved as a result of a widescale use of MD vaccination, and confers increased replication, pathogenesis, or immune evasion to MDV in vaccinated chickens. To address this hypothesis we have undertaken three specific aims employing a recombinant RB1B-based MDV-1 in which the mutation has been introduced (RB1BgLΔ). Using mutant and parental viruses, we have developed aims to examine the roles of the gL mutation in: (1) replication in cell culture, (2) immune evasion, and (3) replication and pathogenesis *in vivo*.

1.4.1 Aim 1: The role of the gL mutation on MDV replication in cell culture

Since the gH/gL complex functions in cell-to-cell spread, one possible role of the gL mutation would be at the level of cellular replication. If the mutation affects the level of gH/gL on the surface of infected cells, then virus may not transmit as efficiently or may transfer more efficiently, as a result of the mutation. To address these possibilities we have examined: (1) the surface expression of gL, gH, and the gH/gL complex on cells infected with RB1B or RB1BgLΔ viruses, (2) the replication kinetics and yield of parent and mutant viruses, (3) the average plaque sizes of each virus, and (4) the mean genome copy number of each virus per plaque.

1.4.2 Aim 2: The role of the gL mutation on immune escape

Since the deletion in gL likely affects some aspects of surface presentation or processing of the gH/gL complex, we assessed differences in surface expression of gL, gH and gH/gL complex in RB1B and RB1BgLΔ-infected cells. To assess the effect of the gL mutation on immune evasion, we: (1) Determined the change in antigenicity of MDV-infected cells using lysates from RB1B- and RB1BgLΔ-infected CEF and maternal antiserum from vaccinated hens.,and (2) Determined if the mutation in gL decreased the susceptibility of RB1BgLΔ-infected cells to neutralization by maternal antibodies from chicks from vaccinated hens.

1.4.3 Aim 3: The role of the gL mutation on MDV replication in vaccinated chickens

To assess the role of the gL mutation on MDV replication in vaccinated chickens, we plan to compare the replication of RB1B and RB1BgLΔ with and without HVT co-infection. Viremias will be compared for both RB1B-based and HVT viruses in spleen cells and PBMC isolated from chickens at 1, 2, 3 and 4 weeks

post-infection. To determine the role of the gL mutation on overcoming bivalent vaccine protection, we placed unvaccinated and bivalently-vaccinated chickens in contact with the virus-inoculated chickens. We performed virus reisolation on unvaccinated and bivalently-vaccinated chickens at 3 weeks post-contact. All chickens were monitored daily for 9 weeks (7 weeks for contact-exposed chickens) and assessed for mortality, MD, and tumor incidence. At the conclusion of the study, all chickens were euthanized, scored for MD lesions, and protective indices determined for bivalently-vaccinated, contact-exposed chickens.

Chapter 2

MATERIALS AND METHODS

2.1 Cells and viruses

For mutagenesis, pRB1B-BAC, an infectious clone of the RB1B strain of MDV, having the ability to transmit horizontally was obtained from Drs. Keith Jarosinski, Venugopal Nair, and Nikolaus Osterreider (44). To generate infectious virus, MDVs were transfected and propagated in secondary chicken embryo fibroblasts (CEF), prepared from specific-pathogen free (SPF) single comb white leghorn (SCWL) chickens (Sunrise Farms, Inc., Catskill, NY). Secondary CEF were grown in medium M199 supplemented with 3% bovine serum, 1X antimycotic (fungizone, Invitrogen), and 1X antibiotic mix PSN (penicillin g, streptomycin, neomycin, Invitorgen, Inc., Gathersburg, MD). The RB1B and RB1BgLΔ stocks were prepared by transfection of CEF using the calcium phosphate method of transfection (63). All stocks were titrated on secondary CEF prior to use.

2.2 Antibodies

For surface expression studies and flow cytometric analyses we have used rabbit polyclonal antibodies to Sindbis virus-expressed MDV gL, gH, and gH/gL complex antigens (provided by N. Osterrieder). All antibodies were pre-adsorbed using EtOH-fixed, uninfected CEF, (1:200) in the presence or absences of 0.2% saponin (Sigma). A goat-derived anti-rabbit Ig FITC conjugate (1:200, Sigma) was used as secondary antibody.

2.3 Recombinant virus construction

Mutagenesis of pRB1B-BAC was performed using the two-step Red-mediated recombination method (117). The mutagenesis strategy was first to replace the targeted gL gene in pRB1B with a kanamycin resistance gene flanked by the gL sequences having the 12 nucleotide deletion, and second to remove the Kan^r gene through resolution of the flanking sequence duplication as shown in Figure 1.

The Kan^r gene from plasmid pEPKan-S (a.k.a., pLay-2) was amplified using PCR primers listed in Table. 1. Oligonucleotides were custom-synthesized and purified via polyacrylamide gel electophoresis (PAGE) (Integrated DNA Technologies, Coralville, IA). The primers listed in Table 1 show the homology-targeting arms corresponding to sequences in the gL gene of pRB1B-BAC (lower case) and the 3' sequences show sequences for priming amplification of the I-*Sce* I-Kan cassette from pEPKan-S (upper case). PCR reactions were performed in 50 µl for 35 cycles of 94°C for 1 minute, 55˚C for 1 minute and 68˚C for 1.5 minutes with a final extension at 68˚C for 10 minutes using a high fidelity AccuprimeTM pfx DNA polymerase (Invitrogen). The PCR product was digested with *Dpn* I in order to remove template plasmid DNA, and the amplification product was purified from an agarose gel with the QIAquick gel extraction kit (QIAGEN, Inc.)

For preparation of competent cells, an L-arabinose-inducible RecE and RecT recombinase expression plasmid (pGET-Rec) was electroporated in DH10β cells. The electrocompetant DH10β cells harboring pGETrec plasmid were prepared as described (66). Electrocompetent cells harboring pGET-Rec were induced for 40 min with 1% L-arabinose prior to collection and were electroporated with the 100 ng of gel purified PCR product and 1 µg of pRB1B-BAC DNA. The electroporation was performed in a 1mm gap cuvette using a BTX PEP TM electroporator. The

parameters used were 15kV/cm, 25μ, and 200Ω. Following the pulse, cells were gently transferred to 1 ml of SOC medium and grown at 37˚C for 1 hr, after which, 100 μl of cells were plated on LB agar selective plates having 50 μg/ml of kanamycin (Kan), 30 μg/ml of chloramphenicol (Cam) and 50 μg/ml of amplicillin (Amp). The positive clones resistant for Kan and Cam were further analyzed by southern blotting as shown in Figure 2.

2.4 Southern blot analysis

For Southern blot analysis, 20 μg of mutant and parental RB1B-BAC DNAs were digested with *Bgl* II. Samples were loaded in duplicate and separated on a 1% agarose gel, and DNA fragments were transferred to a positively charged nylon membrane (Hybond N+, Amersham Scientific) using standard method (90). Blots were probed with gene-specific DNA probes for UL1 (gL) and Kan^r obtained as PCR amplicons from RB1B DNA and pEPKan-S plasmid, respectively. Primers for gene specific probes are listed in Table 1. Hybridization, washing, and exposure conditions were performed according to the manufacturer's instructions. Blots were visualized using chemiluminescent detection (Alpha Innotech Imaging System) and autoluminography and X-ray film (Kodak).

2.5 Second Red recombination

For removal of the Kan cassette from the gL locus, electrocompetent GS1783 *E. coli* cells, a line harboring an L-arabinose-inducible I-*Sce* I restriction enzyme and heat-inducible recombinase, were prepared as described (117). Recombinant clones were electroporated into GS1783 competant cells and were plated on LB agar containing Kan and Cam. Colonies were grown for 4 hrs at 32°C in 2 mL LB broth

with CAM for maintaining BAC. Once bacteria reached early logarithmic phase, 2 mls of warm LB medium containing CAM and 1% L-arabinose were added to induce the expression of I-*Sce* I enzyme. The culture was shaken an additional 60 min and transferred to a 42°C shaking water bath for 15 min. Cultures were then returned to 32°C and shaken for another 1-4 hr. Samples of 100 µl of 10^{-1} to 10^{-4} dilutions were plated on selective agar plates containing CAM and 1% arabinose (117). The recombinant colonies were screened by replica plating on LB + Cam + Kan and LB + Cam plates. Kan^{s} clones were further analyzed by Southern blotting and direct sequencing as shown in Figure. 3.

2.6 DNA sequencing

After construction of recombinant clones, all recombinant clones were further confirmed for gL deletion by direct sequencing. Glycoprotein L genes (771 bp) were amplified via PCR amplification using 50 ng/µl template DNA, gL pathotyping primers (2.5 pmol) as listed in Table 1, and high fidelity AccuprimeTM pfx DNA polymerase (Invitrogen). PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 53°C for 1 min and 68°C for 1 min. The final extension was at 68°C for 10 min. After amplification, 5 µl of PCR sample was loaded on a 1% gel to verify the 771 bp amplicon. The topo cloning reaction was performed on verified gL amplicons using TOPO TA Cloning® Kit from Invitrogen Inc. From the topo clone reaction, 4 verified clones were purified using Qiagen Midi Prep Kit (Qiagen) as per manufacturer's instruction and sequenced for confirmation at the Sequencing and Genotyping Center of the Delaware Biotechnology Institute (DBI). The sequences of all four clones were assembled using DNA STAR - Lasergene software for Sequence Analysis and Assembly , so as to confirm the 12 nucleotide deletion in the gL gene.

2.7 Flow cytometry

To determine the effect of the gL mutation on the surface expression of gL, gH and the gH/gL complex, we performed flow cytometric analysis using cells infected with parental RB1B and two mutant RB1BgLΔ strains (RB1BgLΔ1 and RB1BgLΔ2). Secondary CEF were plated in individual T75 flasks and infected with ~18,000 plaque-forming units (PFU) of RB1BgLΔ1, 20,000PFU/flask of RB1BgLΔ2 and 21,000 PFU of RB1B, separately. Secondary CEF were also plated in one T75 flask for uninfected CEF as a control. At 5 days post-infection, cells were trypsinized into a suspension using 0.05% trypsin and pelleted via centrifugation at 1,200 rpm for 6 min, at RT. Cells were washed with 15 mls of phosphate buffered saline (1X PBS, pH 7.4), pelleted at 1,200 rpm for 6 min at RT, and fixed with 2% paraformaldehyde in 1X PBS for 1 hour on ice. Cells were washed with 1X PBS, pelleted at 1500 rpm for 10 min at RT, and divided into two tubes treatment groups (+ and – detergent). One sample was resuspended in 1 ml of antibody diluent without detergent (diluent A, 1X PBS, pH 7.4, 3% goat serum, 1% BSA, 1% FBS, 0.1% sodium azide). The other tube was resuspended in 1 ml of antibody diluent with detergent (diluent A containing 0.2% saponin).

Non-specific binding of antibodies was blocked by incubation of cells in the antibody diluent A and solubilizing diluent B overnight at 4°C. The polyclonal antibodies to Sindbis virus-expressed MDV gL, gH, and gH/gL complex antigens (provided by N. Osterrieder) were diluted as 1:200 dilution in each antibody diluent A and B. To decrease the non-specific binding, the diluted antibodies were pre-adsorbed with ethanol-fixed, uninfected CEF monolayers (T75 flasks) using a rocker platform at 4°C for 2 hrs. Goat anti-rabbit Ig FITC conjugate was also diluted as 1:200 dilution in each antibody diluent A and B and similarly pre-adsorbed with ethanol-fixed,

uninfected CEF. All pre-adsorbed antibodies were filtered through 0.45 µm syringe filters.

RB1B, RB1BgLΔ1, and RB1BgLΔ2 infected cells were stained with primary antibodies and incubated on rotating platform at RT for 1 hr. Cells were washed thrice with their respective wash buffers (+ and – detergent), pelleted at 1,500 rpm for 5 min, and subsequently incubated with secondary antibodies preparations at RT for 30 min. Cells were washed (as above) and stained cells were finally resuspended in 300 µl of 1X PBS, 1% BSA and 0.1% sodium azide.

Samples (10,000 events/sample) were acquired using a FACScalibur flow cytometer and CellQuest Prosoftware (Becton-Dickinson). For negative controls, secondary antibody-only, and stained, uninfected CEF were used for gating positive cells.

2.8 Growth curves

To compare the replication of RB1BgLΔ1 to RB1B in cell culture, single-step growth curves were performed at 37°C and 41°C, the latter being the body temperature of the chicken (77). Secondary CEF (2.0 x 10^6 cells/dish) were plated in 24 identical 60 mm tissue culture dishes, for each virus. RB1B and RB1BgLΔ1 were then plated onto each dish at approximately 200 plaque-forming units (PFU) per dish. On 1, 2, 3, 5 and 7 days post-infection (p.i.), duplicate 60-mm dishes were harvested via trypsinization and titrated onto fresh CEF in three serial dilutions (1:10, 1:100 and 1:1,000) in duplicate. For days 3, 5 and 7, titration dishes were plated as 1:100, 1:1,000 and 1:10,000 dilutions. Titration dishes were likewise incubated at both 37°C and 41°C. All titration dishes were counted at 5-6 days postinfection and the mean plaque number per time point calculated.

2.9 Plaque area analysis

For plaque area determinations, we used RB1B- and RB1BΔgL-infected 60-mm dishes from growth curve titration dishes. Titration monolayers were fixed with 95% of cold ethanol (ETOH), and stored at -20°C until staining. Prior to staining, the monolayers were rehydrated with 0.45 μM-filtered 1X PBS, pH 7.4, and washed three times with the wash solution (1X PBS, pH 7.4, 3% goat serum, 1% BSA, 1% FBS, 0.1% sodium azide). Monolayers were then stained using the H.19.47 mAb (anti-pp38) (provided by Lucy F.Lee, United States Department of Agriculture, Avian Diseases Oncology laboratory, East Lansing, MI), as primary antibody and goat anti-mouse Ig FITC as the secondary antibody. Antibody dilutions were 1:1,000 for anti-pp38 and 1:200 dilution for goat anti-mouse Ig FITC. Dishes were stained for 2 hrs with primary antibody, washed three times with 5 mls wash buffer and incubated for 1 hr in secondary antibody. Finally, dishes were washed three times with wash buffer and 5 ml of 1X PBS was added to each dish. Virus plaques were examined using a Nikon TE2000 epifluorescence inverted-stage microscope (Nikon Inc., Tokyo, Japan), fitted with a filter for observing FITC fluorescence. For both viruses, plaque areas of 200 randomly- selected plaques were determined using NIS- Elements Imaging software (Nikon). Statistical analyses of plaque sizes were performed using an unpaired Student's t-test.

2.10 qPCR

DNA was extracted from RB1B-, and RB1BgLΔ1-infected CEF using standard methods (90). For determination of viral genome copy number, quantitative real-time PCR (qPCR) (Applied Biosystems 7500 Fast Real-Time PCR System) amplification of the MDV gB (UL27) gene was performed. Amplifications were

normalized to chicken to the ovotransferrin gene (courtesy to Milos Markis). All samples were run in triplicate. The mean genome copy number each sample was calculated. For all samples, the viral genome copy number per plaque was calculated by dividing the mean genome number by the number of plaques counted on that particular 60 mm dish. The mean for viral genome copy number was calculated for both the parental RB1B and mutant RB1BgLΔ1. The statistical analyses for viral genome copy number were performed using an unpaired Student's *t*-test.

2.11 Virus competition assays

To determine if the gL deletion conferred any replication advantage to RB1B, we performed competition assays using defined mixtures of RB1B and RB1BgLΔ1. Transfections were performed using mixed viral BAC DNAs at different ratios, and passaged three times in culture (Figure 10). Secondary CEF (2.0 X 10^6 cells/dish) were plated in (16) identical 60-mm dishes and cells were co-transfected by parental (P) RB1B and mutant (M) RB1BgLΔ1 DNA at the ratios (P:M): 10:1, 5:1, 1:1, 1:5 and 1:10. The parental (1μg) and mutant (1μg) and uninfected CEF DNAs (5μg) were also transfected individually as controls.

Prior to transfection, the DNA concentration was adjusted to 5 μg using uninfected CEF DNA and used to transfect duplicate dishes for each sample using the calcium phosphate ($CaPO_4$) method (63). At (4) days post-transfection, one dish from each duplicate was harvested and passaged onto fresh secondary CEF and were incubated for additional 5 days. At (5) days post-transfection, plaques were counted on the remaining dish. At each passage, DNA was extracted using standard methods (90) from one dish and quantified via spectrophotometry.

As a control for detecting mixtures of the virus genomes, we performed the PCR/RFLP assay for the gL mutation using parental RB1B- and mutant RB1BgLΔ-BAC DNAs at the following ratios (P:M) 100:1, 50:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:50, and 1:100. The PCR/ RFLP analyses for gL mutation were performed on the DNA samples as described in Shamblin, *et al.,* (104), and below (Section 2.12).

2.12 PCR/RFLP analysis

Virus-infected DNA samples from the competition assay of each passage were diluted with 1X TE, pH 7.5, to 50 ng/µl for PCR/RFLP analysis. Glycoprotein L genes (771 bp) were amplified via PCR amplification using 50 ng/µl template DNA, gL pathotyping primers (2.5 pmol) as listed in Table 1, and high fidelity AccuprimeTM pfx DNA polymerase (Invitrogen). PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 53°C for 1 min and 68°C for 1 min. The final extension was at 68°C for 10 min. After amplification, 5 µl of amplicons were loaded on 1% gel to verify 771 bp amplicon. PCR samples were then purified by ETOH-precipitation, centrifugation at 14,000 rpm for 20 min at RT, and pellets were washed with 70% ETOH, dried in a vacuum centrifuge at 45°C, and finally resuspended with 1X TE, pH 7.5. All the samples were subjected to *Dde* I enzyme digestion (FastDigest® Fermentas Life Sciences). The *Dde* I-digested and undigested samples were separated on 1.5% agarose gel by electrophoresis and visualized by ethidium bromide staining and UV transillumination.

2.13 Virus neutralization assay

To determine differences in the susceptibility of RB1B and RB1BgLΔ1 to virus neutralization by maternal antibodies from chicks from vaccinated hens, we

performed virus neutralization (VN) assays using the design shown in Figure 12. For each virus, 200 PFU/ml was prepared in serum-free M199 medium supplemented with 1X antimycotic (fungizone, Invitrogen), and 1X antibiotic mix PSN (penicillin g, streptomycin, neomycin, Invitrogen, Inc., Gathersburg, MD). For the source of maternal antibodies to MDV, we used serum from unvaccinated broiler chickens hatched and raised in isolators. These broilers (Hubbard X Cobb) were provided as embryonated eggs from Allens Family Foods, Inc. (Seaford, DE), and blood samples from selected chickens were obtained at hatch, 7 and 14 days post-hatch. Serum was collected from coagulated blood stored at 4˚C overnight, aliquoted and stored at -20˚C until used. The dams for these chicks were trivalently-vaccinated (HVT/SB1 + Rispens) at the breeder facility in North Carolina (Mr. Frank Wills, hatchery manager, personal communication). The anti-MDV titers of the serum samples were determined by stepwise dilution and immunofluorescence analysis of HVT/SB1-infected CEF plated in 24-well dishes. All maternal antibody titers were determined to be greater than 1:320 for hatch, 7, and 14 days post-hatch (data not shown).

For VN assays, serum samples from 14 days post-hatch were diluted 1:5, 1:50, and 1:500 in serum-free M199 medium. The diluted serum samples were then mixed 1:1 with the virus preparation, for final dilutions of 1:10, 1:100 and 1:1000. As a control, RB1B and RB1BgLΔ1 viruses were mixed 1:1 with the serum-free M199 medium only. All samples were incubated at RT for 1 hour and then 1 ml of each sample was inoculated onto triplicates 60 mm dishes of secondary CEF. The plates were then incubated at 37°C for 5 days. Plaques were counted at 5 days of post-plating via immuno-fluorescence analysis using H.19.47 mAb (anti-pp38) staining (Dr. Lucy F. Lee, USDA-ARS, ADOL).

The VN assay was repeated using cryopreserved spleen cells (SPC) prepared from RB1B- and RB1BgLΔ1-infected chickens at two weeks post-infection. SPC were obtained during the *in vivo* analysis of RB1B and RB1BgLΔ1 and VNs were performed at the 1:10 dilution,. As a control for the use of chicken serum, serum from mock-infected SPF chickens (from a 2004 study) was similarly used at a 1:10 dilution for each virus. SPC were incubated with mock-infected SPF, or unvaccinated broiler serum for 1 hr at RT and then plated onto quadruplicate 60 mm dishes. Plaque numbers were counted at 5 days post-plating, as detailed above.

2.14 *In Vivo* comparison of RB1B and RB1BgLΔ1

In order to characterize the pathogenicity, oncogenicity, and immune evasion of RB1BgLΔ compared to its parent virus, we performed an *in vivo* study. This study was performed in the biosafety level 3 animal rooms of the Allen laboratory at the University of Delaware, (Figure 16). This study was conducted in combination with other recombinant viruses, in order to maximize isolator use and minimize repetition of control groups. For the study, 470 SPF, single-comb white leghorn chickens (SCWL) chickens were obtained as embryonated eggs (Sunrise Farms Inc., Catskill, NY). Chicks were hatched in Allen laboratory hatchery and were transferred to isolators after treatment. For this study, there were (8) treatment groups (Table 3). For each group, (20) one-day-old birds were inoculated with the viruses detailed in Table 3.

Briefly, the groups were: mock infected controls, bivalent vaccine control (for the generation of effector cells), each of the viruses by themselves (RB1B, RB1BgLΔ), each of the viruses with HVT co-infection (RB1B + HVT, RB1BgLΔ + HVT) and 1:1 mixes of the viruses with and without HVT co-infection. To evaluate

horizontal transmission and virulence of viruses being shed, freshly hatched unvaccinated and bivalently-vaccinated contacts were placed with all MDV-inoculated groups.

To evaluate the replication of MDVs in each of the groups, we performed virus reisolation from SPC and PBMC at 1, 2, 3 and 4 weeks post-inoculation. At 3 weeks post-placement, virus reisolation was performed from vaccinates and unvaccinated contact groups.

For all virus reisolations, chickens were bled via cardiac puncture for PBMC collection and were euthanized via cervical dislocation. Spleens from euthanized birds were removed aseptically, pooled, and homogenized using Tenbroek tissue grinders (Thermo Fischer Scientific, Pittsburgh, PA). The SPCs were then filtered through cheese-cloth and washed in M199 media supplemented antibiotics and antimycotics. For PBMC isolation, pooled blood samples were under-layered with 3 mls of histopaque 1119 (Sigma) and spun at 700 *x g* for 30 minutes at RT. After centrifugation, PBMC were removed and washed with M199 complete media. The spleen cells and PBMC were counted and adjusted to 2 X 10^7 cells/ml, and 100 µl were plated in triplicate 60-mm dishes of freshly plated CEFs. All the dishes were then incubated at 37°C for 5 days, fixed with cold 95% ethanol, and stained for plaque enumeration via IFA.

From all samples, 100 µl samples were transferred to eppendorf tube for DNA extraction via standard methods for PCR/RFLP analysis for the gL mutation (90). Similarly, during the course of the *in vivo* study, the feather tips (4-5) were removed from the birds showing positive MD lesions for PCR/RFLP analysis of the gL locus.

During virus reisolation, in addition to SPC and PBMC, bursa and thymus samples were collected in buffered formalin for histopathology. The remaining chickens were monitored daily for MD signs and mortality. Birds with severe symptoms of MD were culled for necropsy and tumor samples were collected for cell line establishment. At 7 wks post-inoculation, the remaining inoculate birds were euthanized via cervical dislocation and scored for MD lesions. Similarly, at 7 wks post-placement of vaccinates and contacts, remaining birds were euthanized and scored for MD lesions.

2.15 Statistical analysis

The statistical significance of differences observed in growth curves, plaque areas, and viral genome copy number, neutralization assays and viremia data from the *in vivo* study was assessed using an unpaired Student's t-test. The differences were considered significant at the level of $p < 0.05$.

Chapter 3

RESULTS

3.1 Construction of RB1BgLΔ

To determine the effect of a naturally-occurring gL mutation on bivalent vaccine efficacy, we introduced this mutation into pRB1B, an infectious BAC clone of the RB1B strain of MDV (81) via two-step Red-mediated mutagenesis (66) (Figure 1). We performed two rounds of mutagenesis in order to insert this mutation into the gL gene of the RB1B genome (Figures 2 and 3). After the first round of mutagenesis, we obtained 15 mutants having insertion of the Kan^r cassette, which was then confirmed via Southern blot analysis of four randomly-selected clones (Figure 2). Insertion of the cassette resulted in an increased size of the gL gene-containing *Bgl* II fragment to 2.3 kbp compared to the parental gL fragment of 1.2 kbp (Figure 2).

After the second step recombination, of more than 100 colonies screened for loss of the Kan^r cassette, we identified 22 individual Cam^r Kan^s colonies. Four of these were selected and screened by Southern blot analysis for deletion of the Kan cassette (Figure 3). These second stage recombinants were further screened for deletion of 12 nucleotides within the gL gene by PCR/RFLP assay (Figure 4), as described in (114) and by direct sequencing (Figure 5). All of the second-stage recombinant clones were found to have the 12 nt deletion within the gL coding sequence (Figure 5).

3.2 Flow cytometric analysis of gH/gL surface expression

We attempted to confirm our initial finding on the effect of the gL mutation on surface expression of the gH/gL complex on virus-infected cells. In our previous experiments, we found that TK1a-infected CEF showed decreased surface expression of the gH/gL complex as compared to TK2a-infected CEF (data not shown). We subsequently performed flow cytometric analyses for gH/gL expression pattern using parental RB1B-, mutant RB1BgLΔ1-, and mutant RB1BgLΔ2-infected CEFs.

We observed no difference between the surface expression of gL, gH or the gH/gL complex in cells infected with the parental RB1B, mutant RB1BgLΔ1 and RB1BgLΔ2 (Table. 2). Overall, the level of staining was very low, particularly for the anti-gL antibody. Since the staining with detergent should have stained both surface and internal proteins, the lack of an increase with the addition of detergent suggested that the titer of the antibodies had decreased. In addition to the flow cytometric analysis, we also used these antibodies for immunofluorescence analysis (data not shown), and found that these antibodies similarly had decreased in titer. We therefore plan to repeat these studies when new antibodies become available.

3.3 Replication of RB1BgLΔ1 in cell culture

To determine the role of the gL mutation on MDV replication in cell culture, we performed single-step growth curves for both RB1B parent and mutant RB1BgLΔ1 at 37°C and 41°C. RB1BgLΔ1 and parental RB1B strains showed essentially identical patterns of replication at both 37°C and 41°C (Figure 6). These growth curves indicated that the deletion of 12 nucleotides at the gL locus did not affect the replication of mutant RB1BgLΔ1 in cell culture at either temperature.

To further compare the replication of these viruses in cell culture, we measured the plaque areas of both RB1B and RB1BgLΔ1 viruses at 5-6 days post-infection. For both the viruses, 200 randomly-selected plaques were examined under UV illumination and relative plaque area were measured using NIS-Elements imaging software. We observed a significant increase in plaque area for mutant RB1BgLΔ1 as compared to the parental RB1B (p value < 0.0001, Figure 7). This result was obtained using both user-designated plaque areas (traced by eye, Figure 7) as well as imaging software-designated (auto-feature detection) plaque areas (Figure 8). In each case, RB1BgLΔ1 showed plaques of greater size (p value < 0.0001), despite identical replication curves (Figure 6).

Since we saw a difference in plaque area, despite similar replication in cell culture, we surmised that the number of virus particles per plaque may be vary, as well (The absolute areas traced by eye was for RB1B plaque is 95330sq μm ± 47574, and RB1B gLΔ plaque is 135081 sq μm, ± 61489, the absolute areas measured automatically for RB1B plaque is 91612 sq μm ± 29401, and RB1B gLΔ plaque is 146773 sq μm, ± 53235).

We measured viral genome copy numbers for both the parental RB1B and mutant RB1BgLΔ1 viruses via qPCR. Surprisingly, we found no significant differences in the normalized viral genome copy number per plaque between the parental or mutant viruses (Figure 9). Consequently, the difference in plaque area is somewhat confusing, since both viruses replicate to the same titer, at the same rates, and their plaques contain roughly the same number of viral genome copies.

3.4 Competition of RB1B and RB1BgLΔ in cell culture

Since parental RB1B and RB1BgLΔ1 could be readily distinguished by a PCR/RFLP assay (104), we performed a direct competition assay to determine the relative fitness of parent and mutant viruses in cell culture (Figure. 10). This was to determine if the gL deletion was selected at the level of cellular replication with parental viruses. We observed that: (1) in mixtures of DNA, the mutant pattern could be seen as the predominant form up to a mix of 10:1 (P:M), and the parental gL form could not be readily detected at a ratio of 1:5 (P:M) or less, (3) during passage in cell culture, the mutant was seen as the predominant form in the 1:1 (P:M) mix, but not in the 5:1 (P:M) mix, and (4) mixtures remained constant in their composition with each passage. We observed no particular selection for parental or mutant virus at all the passage levels in this competition assay (Figure 11). Similar to our growth curve analysis, the competition assay suggested that there was no selection for the mutant virus during replication in cell culture.

3.5 Susceptibility of RB1B and RB1BgLΔ to Neutralization

In order to assess the effect of the gL mutation on immune evasion, we performed virus neutralization (VN) assays using RB1B and RB1BgLΔ1 as the test virus and antisera (from unvaccinated broiler chickens) drawn at 14 days of age (Figure 12). The main purpose of this experiment was to determine the change in susceptibility to virus neutralization by maternal antibodies. We observed that there was no significant neutralization of parental RB1B as well as mutant RB1BgLΔ1 viruses with all the dilutions of the sera as compare to the untreated control as shown in Figure 13. The assay was repeated using RB1B- and RB1BgLΔ-infected SPCs from the *in vivo* bird study in quadruplicate dishes. We observed that there was a significant

neutralization of parental RB1B virus at the 1:10 dilution of antiserum compared to the untreated control, but did not see any significant neutralization of mutant RB1BgLΔ for the same dilution of antiserum compared to the untreated control (Figure 14). So to confirm these results, we performed the virus neutralization using serum from mock-infected SPF chickens as a control group instead of medium-only control. In addition, antisera from three different unvaccinated broiler chickens were used as the source for maternal antibody. In this study, we found no significant neutralization of both parental and mutant virus at 1:10 dilution for all three maternal antisera compared to the mock-infected serum-treated group (Figure 15). These results were very surprising as the effect of the mock-infected serum was greater than the antisera containing maternal antibody to MDV.

3.6 Replication of recombinant virus *in vivo*

*Spleen cells***.** In spleen cells, both RB1B and pRB1BgLΔ viruses replicated in inoculated birds to comparable levels over the course of 4 weeks post-infection. (Figure15A, left panel). The absolute levels varied between the groups at weeks 3 and 4, however, these could be due to the onset of tumors in one or more of the birds sampled. Similar patterns were observed for each of the viruses that was co-replicating with HVT (Figure 15B, left panel), however the level were somewhat lower than the viruses in the absence of HVT co-infection. This was particularly true at 4 weeks post-infection, when the levels of virus were 2 – 4 fold less than the viruses without HVT.

The level of replication of RB1B and RB1BgLΔ decreased with HVT co-infection (Figure 17B, left panel). In the case of the mixture of RB1B and RB1BgLΔ,

the replication level increased at 3 weeks post-infection and then decreased at 4 weeks post-infection (Figure17C, left panel).

Peripheral blood mononuclear cells (PBMC). The patterns of RB1B and RB1BgLΔ replication in PBMC were very similar to those observed for spleen cells (compare Figure 17A, B, and C, left and right panels). Overall, the levels of PBMC infection were lower than the levels of spleen cell infection, and as observed for spleen cells, the co-replication of HVT decreased the overall levels of viremia (Figure 17B, right panel). The pattern of RB1B + RB1BgLΔ mixed infection was essentially superimposable to that observed for spleen cells, at a two-fold lower level (Figure 17C, right vs left panels). Overall, we found that the RB1BgLΔ was not attenuated for replication in chickens, and was comparable to RB1B.

3.7 Effect of RB1B and RB1BgLΔ infection on HVT replication *in vivo*

The replication of both RB1B and RB1BgLΔ was decreased by co-infection with HVT (Figure 17B). The level of HVT infection is usually quite low, compared to MDV1 strains (91). We similarly noted that the levels of both HVT and SB1 were lower than RB1B and RB1BgLΔ (Figure 18). In the spleen, the level of HVT dropped by week 4, particularly in MDV1-co-infected chickens (Figure 18A). In PBMC, the levels of infection similarly dropped and were overall lower than the level of spleen cell infection (Figure 18B). Interestingly, we did observe a minor increase in HVT in PBMC in the RB1BgLΔ co-infected group, however, given the low level of infection (8 PFU/10^6 cells), it is difficult to ascribe any functional significance to this increase.

3.8 Reisolation of RB1B, RB1BgLΔ, and HVT from contact-exposed chickens

Chickens exposed to mock-infected chickens showed no transmission of HVT, SB1, or MDV1 virus, indicating that our isolation system was effective at limiting exposure among the groups (Table 4). We observed limited horizontal transmission of HVT/SB-1 to unvaccinated contacts with SB1 transmitting at a somewhat higher level than HVT. In the case of RB1B, RB1BgLΔ, and the RB1B + RB1BgLΔ-mixed groups co-infected with HVT, we observed horizontal transmission of HVT to contact-exposed birds at a higher level than from HVT/SB-1 vaccinated itself. These data suggest that despite decreases in HVT replication in spleen cells and PBMC, there is a small increase in horizontal transmission of HVT with MDV1 co-infection. This result was not consistent with our previous study showing no horizontal spread of HVT in isolators (91), however, in those studies, we used an RB1B that did not spread horizontally.

The unvaccinated birds exposed to RB1BgLΔ and the RB1B + RB1BgLΔ mix showed higher levels of viremia compared to chickens exposed to RB1B alone (Table 4). These data are also reflected in the mortality and tumor incidences for these groups, in which 7% mortality and 29%-33% tumor incidence was observed for RB1BgLΔ and mixed infection contact-exposed chickens (Table 5). For the RB1B-exposed chickens, we observed 0% mortality and 21% tumor incidence. These differences however are minimal due to the small number of chickens used.

In the case of horizontal transmission to HVT/SB1 vaccinated chickens, we found that inoculate co-infection with HVT effectively blocked transmission of both RB1B and RB1BgLΔ, as measured by viremia at three weeks post-contact (Table 4). There was some level of transmission, however, as tumors were identified in a few of

these contact-exposed chickens at necropsy (Table 5). These data contrast our work with TK1a and TK2a viruses (114), in which we found HVT co-infection a mitigating factor to the loss of bivalent vaccine efficacy. We were not able to replicate this observation using the RB1B-based gL mutant.

3.9 PCR/RFLP analysis of tumors induced by RB1B and RB1BgLΔ mixed infection

During our *in vivo* study, we collected different tumor samples from the birds of different groups. The main purpose for collecting tumor samples was to identify the transforming virus in the mixed group by employing the gL mutation assay as described in our previous study (114). From the mixed-infection group, we isolated DNAs from feather tips using the method of Davidson *et al.*, (23), and from tumor cells using standard DNA isolation methods (90). We observed RB1B, RB1BgLΔ and mixed infection patterns in tumors caused suggesting that there was no selection for RB1BgLΔ in contact-exposed chickens (Figure 19). Our results are consistent with our cell culture comparison of RB1B and RB1BgLΔ, in that no discernable difference could be observed at the levels of replication. Our data extend this observation to show that no discernible difference could be observed *in vivo* with respect to replication, tumor incidence or mortality in inoculated or contact-exposed chickens.

Chapter 4

DISCUSSION

In commercial poultry production, Marek's disease in controlled largely by vaccination. Despite the great success of MD vaccination in preventing losses due to disease, MDV field strains have continued to evolve in greater virulence from decades. No genetic mutations have been directly linked to these virulence changes until recently, and these mutations have not been confirmed experimentally, therefore the molecular basis for these virulence changes in MDVs need to be addressed.

In an attempt to identify the common mutations contributing to these virulence changes in MDV, our lab has identified a unique mutation in the glycoprotein L (gL) genes of vv and vv+ strains of MDV. This mutation was found to be common in all the field isolates obtained from DE, MD, NC, PA, and VA (104, 114). In our previous study, we have shown that a vv+MDV having this mutation overcame bivalent (HVT/SB1) vaccine protection in contact-exposed chickens (114).

The main goal our research was to confirm the results from our previous study using a genetically-identical background strain (RB1B) in order to separate the effect of the gL mutation from any other genomic differences. Our hypothesis was that the gL mutation was essential to overcoming bivalent vaccine protection in contact-exposed chickens, and hence conferred one aspect of increased virulence to MDV strains. Our scientific approach was to construct the recombinant virus with the backbone of a modified infectious bacterial artificial chromosome (BAC) clone of pRB1B harboring this mutation and to characterize it in cell culture, as well as *in vivo*.

4.1 The effect of the gL mutation on MDV replication

We have characterized RB1B and RB1BΔgL with respect to their surface expression of gL and gH, their replication in CEF, their relative plaque areas, their viral genome copy number per plaque, and their ability to compete with each other during replication in culture. We were unable to reproduce results showing that the gL mutation affected the level of gH/gL surface expression, but this was likely due to degradation of the antibodies being used. Our growth curve results showed that there was no significant difference in the replication kinetics of the viruses in CEF. This result was consistent with our finding that the viral genome copy number per plaque was likewise comparable between the two viruses (RB1B 1563 viral genomes per plaque, ± 850, RB1B gLΔ 1973 viral genomes per plaque, ± 425). Although RB1BgLΔ was numerically greater in viral genome copy number, this difference was less than significant (p value = 0.43). Despite this similarity in replication and viral genome accumulation in cells, the plaque size induced by RB1BgLΔ was significantly larger than those induced by parental RB1B in cell culture ($p \leq 0.0001$). These data were independent of how the plaques were traced; either by eye or automatically, the data were essentially identical. Thus, there does seem to be increased ability in spreading cell-to-cell for RB1BgLΔ.

In direct competition, we did not observe any particular selection for either mutant or parent virus in cell culture after repeated passage. However, we did observe that the mutant gL pattern was discernable even when present at $1/5^{th}$ to $1/10^{th}$ the concentration of the parent virus, suggesting that the mutant virus is detectable even in minute concentration.

4.2 The role of the gL mutation in RB1B pathogenesis

In vivo, we found no evidence that the gL mutation was responsible for decreased bivalent vaccine protection, or that the mutation conferred increased pathogenicity to the virus when co-infected with HVT. We did observe increased transmission of HVT from co-infected chickens, compared to vaccinated chickens, but this did not correlate with the presence of the gL mutation. These results are not consistent with our previous studies (91), which employed TK-strain based viruses. One key aspect of our work is that the pRB1B strain itself did not cause significant disease in unvaccinated contact-exposed chickens (0% mortality, 21% tumor incidence). Consequently, the genetic background strain may not be of sufficient virulence to observe subtle changes in phenotype. Clearly, the gL mutation was unable to confer increased virulence in vaccinated chickens in and of itself.

4.3 Role of the gL mutation in MDV immune escape

In conjuction with the *in vivo* experiment, we also assessed the effect of the gL mutation on immune-evasion by performing virus neutraliztion assays to determine if the gL mutation has been selected through escape of the antibody response to vaccination. However, we did not see a consistent significant difference in the the ability to overcome maternal antibody virus neutralization between pRB1B and pRB1BgLΔ viruses.

4.4 Conclusions

During our course of study, we have been able to address the main hypothesis of our research regarding the role of the gL mutation in decreased bivalent vaccine efficacy, in pRB1B pathogenesis, and its role in MDV immune evasion. Our data show that this mutation itself is unable to confer increased pathogenicity to a vvMDV,

and that other mutation in vv+MDV are likely to be essential for the phenotype conferred by the gL mutation to be observed. As one future goal, we will delete the BAC sequences from pRB1B and pRB1BgLΔ in order to increase their virulence and pathogenicity *in vivo*. Alternatively we will pursue constructing infectious clones of the TK strain in order to provide an identical vv+MDV genetic background from which to generate the gL mutation.

Table 1 PCR and mutagenesis primers

Site of Amplification	Direction	Sequence
A. Kan^r gene	gL mut kan Forward	5'atagagtactcgtgcatctttccttcgtgctaggtatgtttacggttc tcgcatggtcgaaatacgacTAGGGATAACAGGGTA ATCGATTT-3'
	gL mut Kan Reverse	5'ctcataaagccgtgtaccaggtcgtatttcgaccatgcgagaac cgtaaacatacctagcacgaaggaGCCAGTGTTACAA CCAATTAACC-3'
Expected Size		1.1Kbp
B. UL1	gL Forward	5'-GTAAAGAGATGTCTCAGGAGCCA-3'
	gL Reverse	5'-GGAGGCCATCGGGAGATTATC-3'
Expected Size		0.7Kbp
C. Kan^r gene	Kan ORF Forward	5'-GTTATGAGCCATATTCAACGG-3'
	Kan ORF Reverse	5'-CTGATTAGAAAAACTCATCGAGC-3'
Expected Size		1.0 Kbp
D. UL1	gL Forward	
	gL Reverse	
Expected Size		2.0 Kbp

Table 2 Surface expression study of gH/gL

Virus	Localization	Anti-gL	Anti-gH	Anti-gH/gL
pRB1B	Surface	2%	3%	3%
	Total (+ det)	2%	11%	5%
	Surface/Total	1	0.3	0.6
pRB1BgLΔ 1	Surface	1%	2%	2%
	Total (+ det)	1%	4%	3%
	Surface/Total	1	0.5	0.7%
pRB1BgLΔ 2	Surface	1%	2%	2%
	Total (+ det)	1%	7%	3%
	Surface/Total	1	0.3	0.7
pRB1B/ pRB1BgLΔ 1	Surface/Total	1	1	1
pRB1B/ pRB1BgLΔ 2	Surface/Total	1	1	1

Table 3 Treatment Groups for viral pathogenesis study

Isolator	Virus	Dose [A]	Route	In ovo Vaccine Dose [B] HVT/SB 1	Inoculates	Unvaccinated contacts	HVT/SB-1 contacts
1	Mock	-	IM[1]	5000/2500 PFU	20	20	0
2	HVT/SB1	5000/2500 PFU/bird	IM[1]	5000/2500 PFU	20	20	0
4	pRB1B parent	1000 PFU/bird	IM[1]	5000/2500 PFU	20	15	15
5	pRB1BgLΔ1 mutant	1000 PFU/bird	IM[1]	5000/2500 PFU	20	15	15
6	pRB1B + HVT	1000/5000 PFU/bird	IM[1]	5000/2500 PFU	20	15	15
7	pRB1BgLΔ + HVT	1000/5000 PFU/bird	IM[1]	5000/2500 PFU	20	15	15
8	pRB1B + pRB1BgLΔ1	500 PFU each	IM[1]	5000/2500 PFU	20	15	15
9	pRB1B + pRB1BgLΔ1 + HVT	500 PFU each + 5000 PFU HVT	IM[1]	5000/2500 PFU	20	15	15

IM[1]: via intramuscular injection (thigh), [A] Virus dose in PFU, [B] Target vaccine doses in PFU

Table 4 Virus reisolation from contact-exposed unvaccinated and vaccinated chickens.

Virus	Unvaccinated Contacts SPCs	HVT/SB-1 vaccinated contacts SPCs
Mock	0	-
HVT/SB-1	0.3 ± 1 / 5 ± 1	-
pRB1B	68 ± 12	1 ± 1
pRB1BgLΔ1	206 ± 26	3 ± 1
pRB1B/HVT	8 ± 1 / 10 ± 3	0 / 8 ± 1
pRB1BgLΔ1/HVT	33 ± 19 / 35 ± 7	0 / 13 ± 1
pRB1B + pRB1BgLΔ1	259 ± 19	11 ± 1
pRB1B + pRB1BgLΔ1/ HVT	0 / 12 ± 1	0 / 16 ± 4

Table 5 Virus-specific mortality and tumor incidences

Virus Group	Inoculate Mortality	Inoculate Tumor	Contact Mortality	Contact Tumor	Vaccinate Mortality	Vaccinate Tumor
Mock	0	0	0	0	-	-
HVT/SB1	0	0	0	0	-	-
pRB1B	18%	82%	0	21%	0	8%
pRB1BgLΔ1	35%	88%	7%	33%	8%	8%
pRB1B + HVT	0	35%	0	13%	0	0
pRB1BgLΔ1 + HVT	6%	25%	0	13%	0	0
pRB1B +pRB1BgLΔ1	47%	73%	7%	29%	0	0
pRB1B +pRB1BgLΔ1 +HVT	ND*	ND*	0	7%	0	0

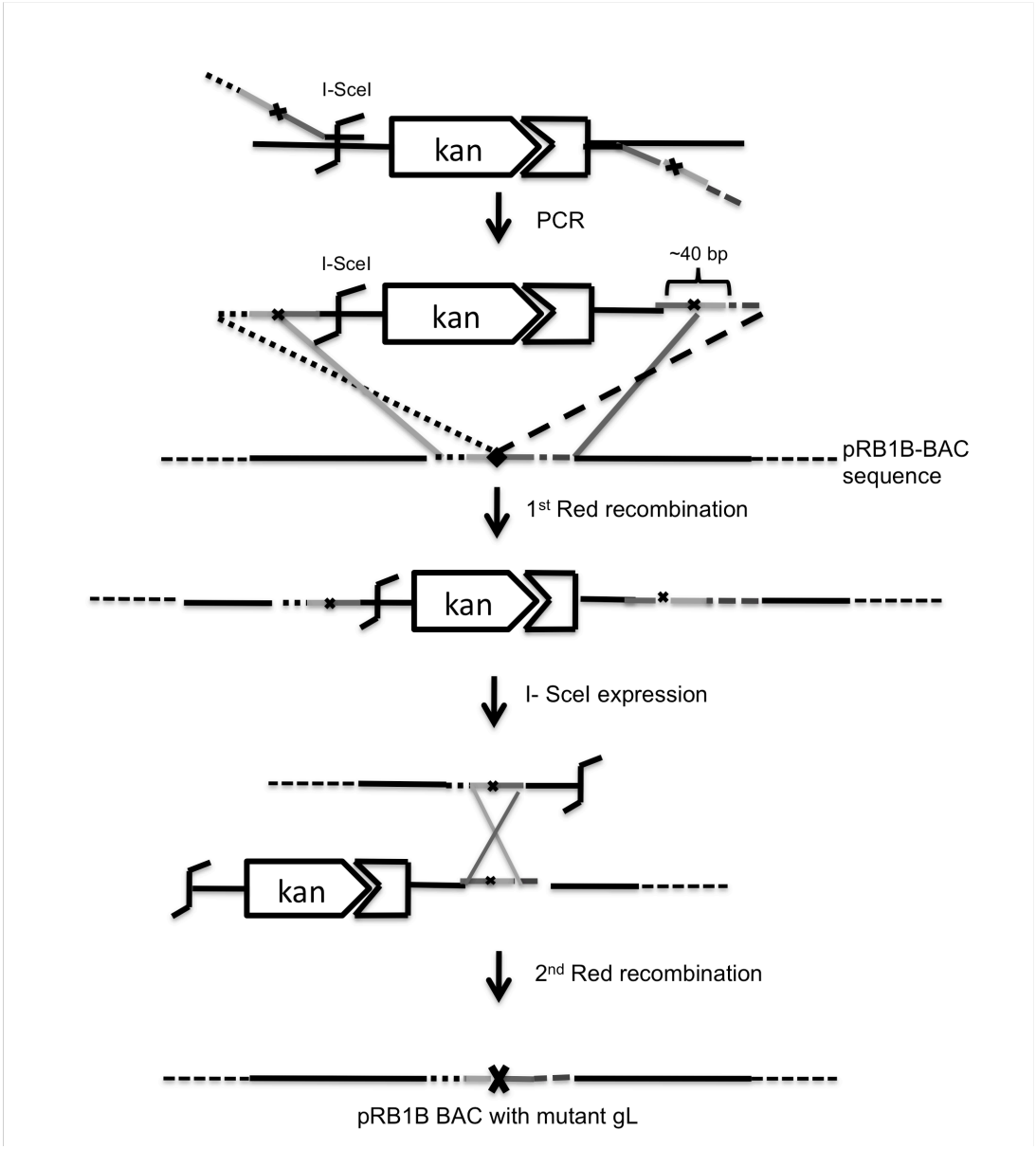


Figure 1 Diagram of two-step Red mediated mutagenesis.
The diagram above shows the strategy of the two-step recombination method employed to obtain the desired mutation at target locus without retention of foreign sequences.

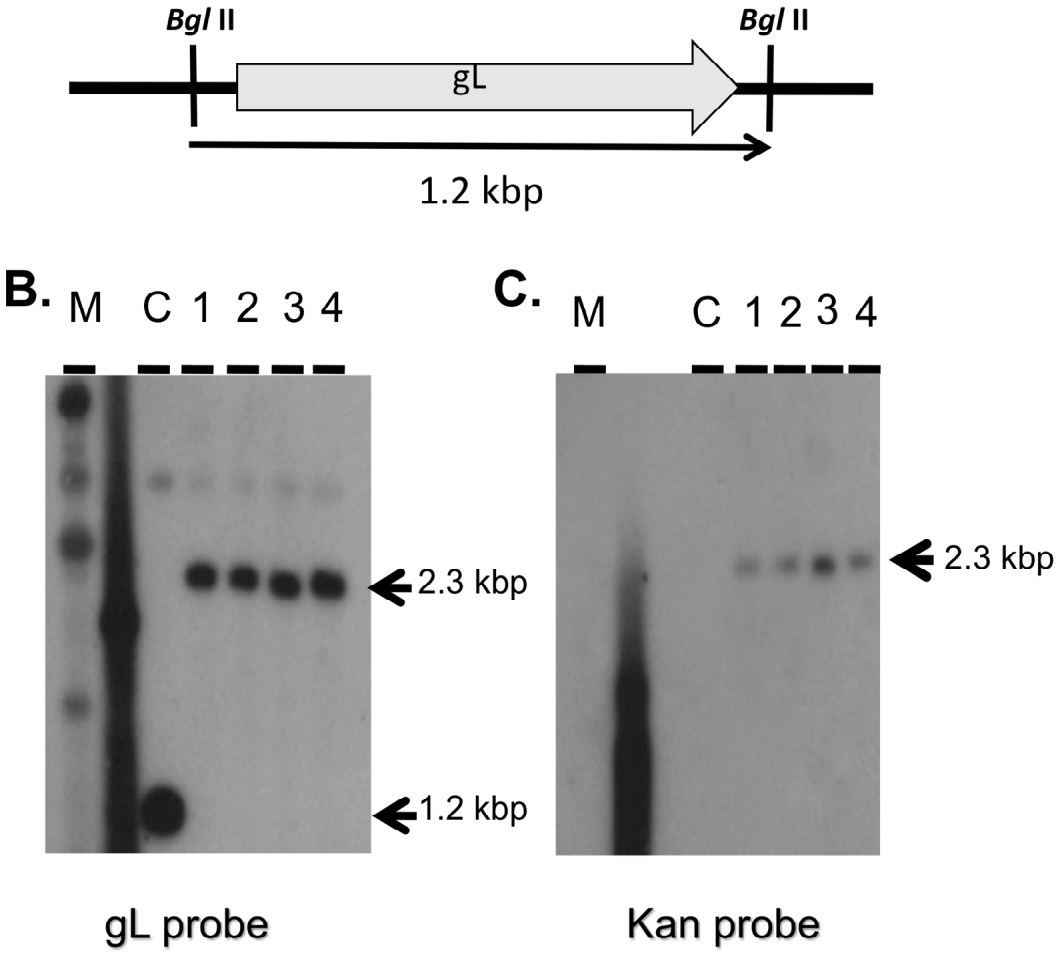


Figure 2 Southern blot analysis of kanamycin cassette insertion.
Panel A shows a map of the wild type gL locus with flanking *Bgl* II sites. Panel B shows a Southern blot of pRB1B- and gL mutant 1, 2, 3 and 4 BAC DNAs digested with *Bgl* II and probed with a gL probe. Note: increase in the band size of gL genes of mutants. Panel C shows a Southern blot of the same DNAs probed with a Kanamycin cassette probe. Note: band of increased size hybridized with increased with Kan probe, indicating that the increase in size (from 1.2 kbp to 2.3 kbp) is due to kanamycin cassette insertion.

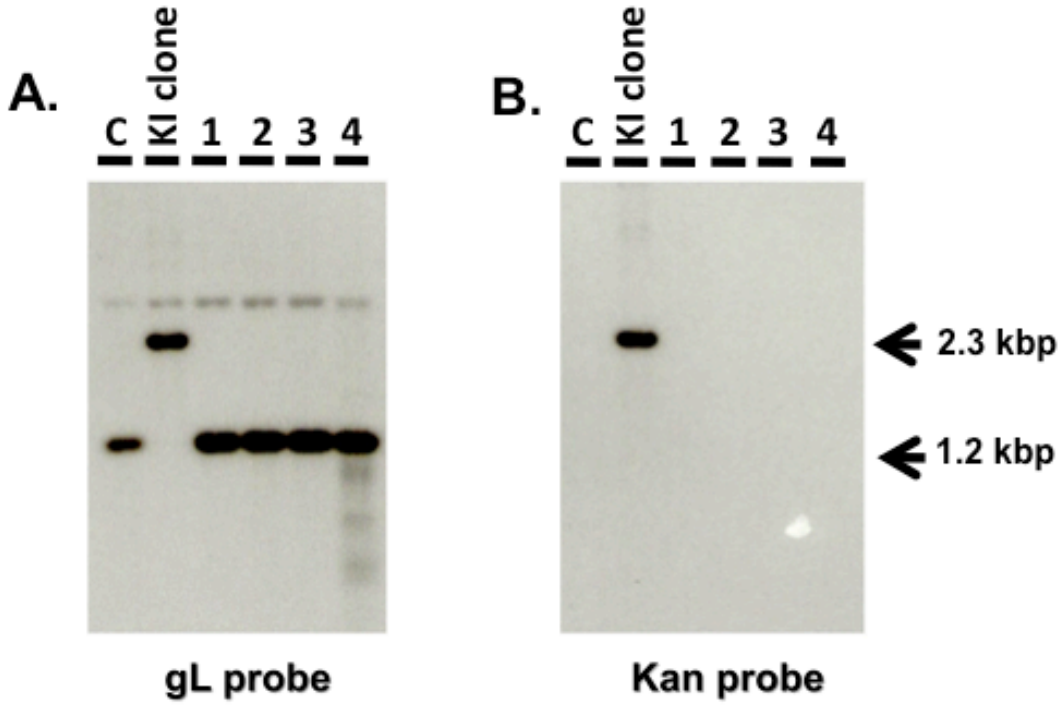


Figure 3 Southern blot analysis of final recombinant clones after knock out of kanamycin cassette.

Panel A shows a Southern blot of pRB1B- and gL mutant (1, 2, 3 and 4) BAC DNAs digested with *Bgl* II and probed with a gL probe. Note the decrease in the band size of gL genes of mutants due to knock-out of the kanamycin cassette. Panel B shows a Southern blot of the same DNAs probed with a kanamycin cassette probe. Note the knock out of kanamycin cassette in all 4 clones.

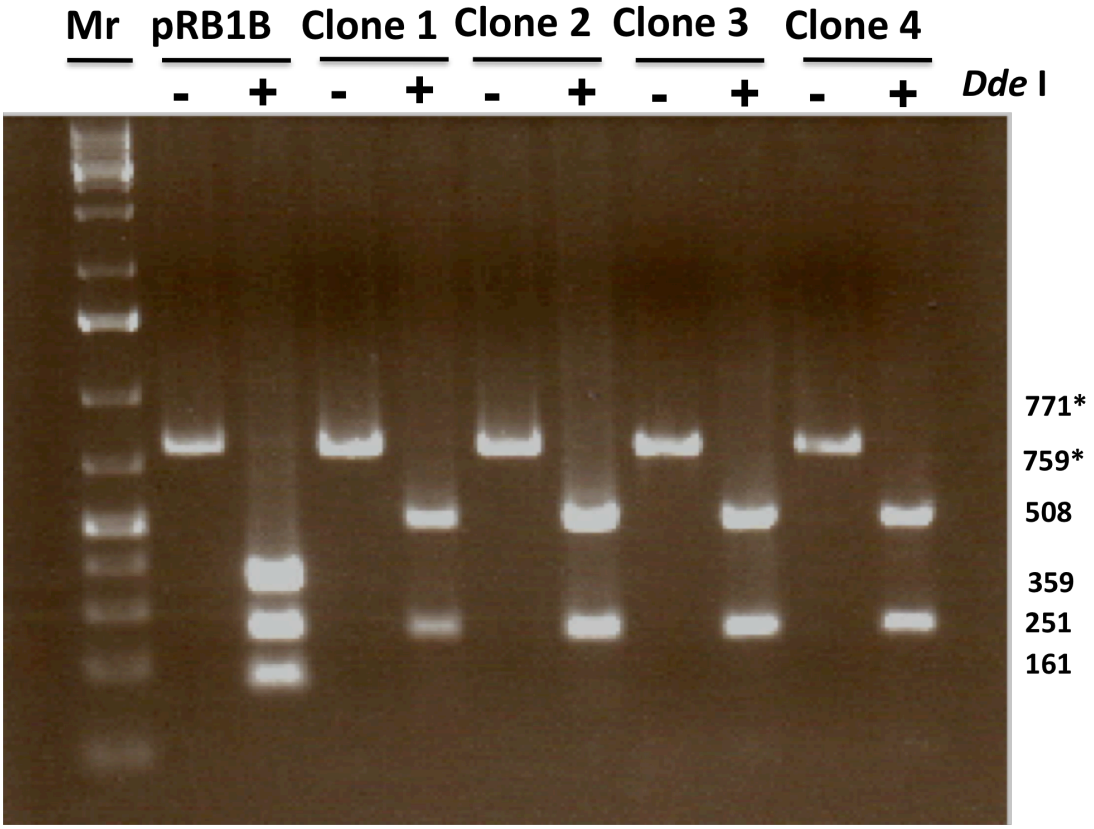


Figure 4 PCR/RFLP analysis of gL loci of parental and recombinant viruses.

The agarose gel above shows the PCR/RFLP analysis of parental RB1B and recombinant RB1BgLΔ clones. PCR amplification of the gL gene of parental RB1B yielded 771-bp; similarly gL genes were PCR amplified from recombinant clones to yield 759-bp. Amplicons are shown with and without *Dde I*-digestion. For RB1B, the bands are 771*(undigested amplicon), 359,251 and 161 bp, and for recombinants amplicons with the gL deletion, bands are 759*(undigested amplicon), 508, and 251 bp.

A.

MUT ATCTTTCCTTCGTGCTATGTT----------TACGGTT
WT ATCTTTCCTTCGTGCTATGTT**TACTAAGACCAA**TACGGTT

B.

MUT MKIYRVLVHLSFVLGMF—-----TVLAWSKYD
WT MKIYRVLVHLSFVLGMF**TKTN**TVLAWSKYD

Figure 5 Sequence alignment of glycoprotein L genes of RB1B and RB1BgLΔ
Panel A shows sequence alignment of glycoprotein L genes of mutant and wildtype RB1B showing 12 nt deletion. Panel B shows the amino acid sequence alignment of mutant and parental RB1B showing deletion of 4 amino acids within the gL coding sequence.

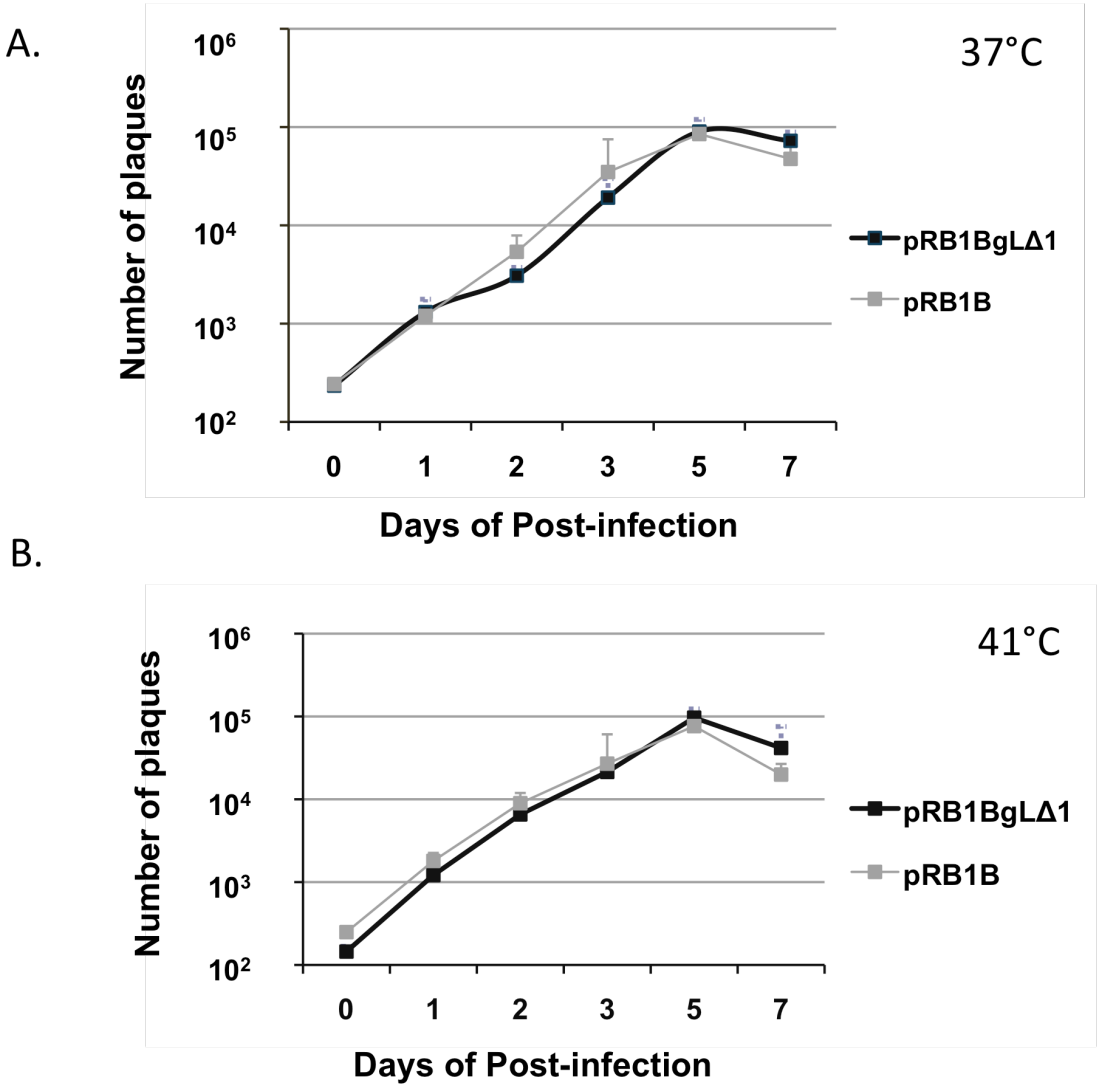


Figure 6 Single step growth curves.
Growth curves are shown of the parental RB1B and recombinant RB1BgL□1 infected CEFs grown at 37°C (A) and 41°C (B). Each point represents the mean number of plaques counted on total of six titer dishes (duplicate dishes at three dilutions of 1:10,1:100, and 1:1000 for days 1 and 2, and 1:100, 1:1000, and 1:10000, for days 3, 5 and 7) counted at 5-6 days post-infection. The standard deviation and error bars are shown.

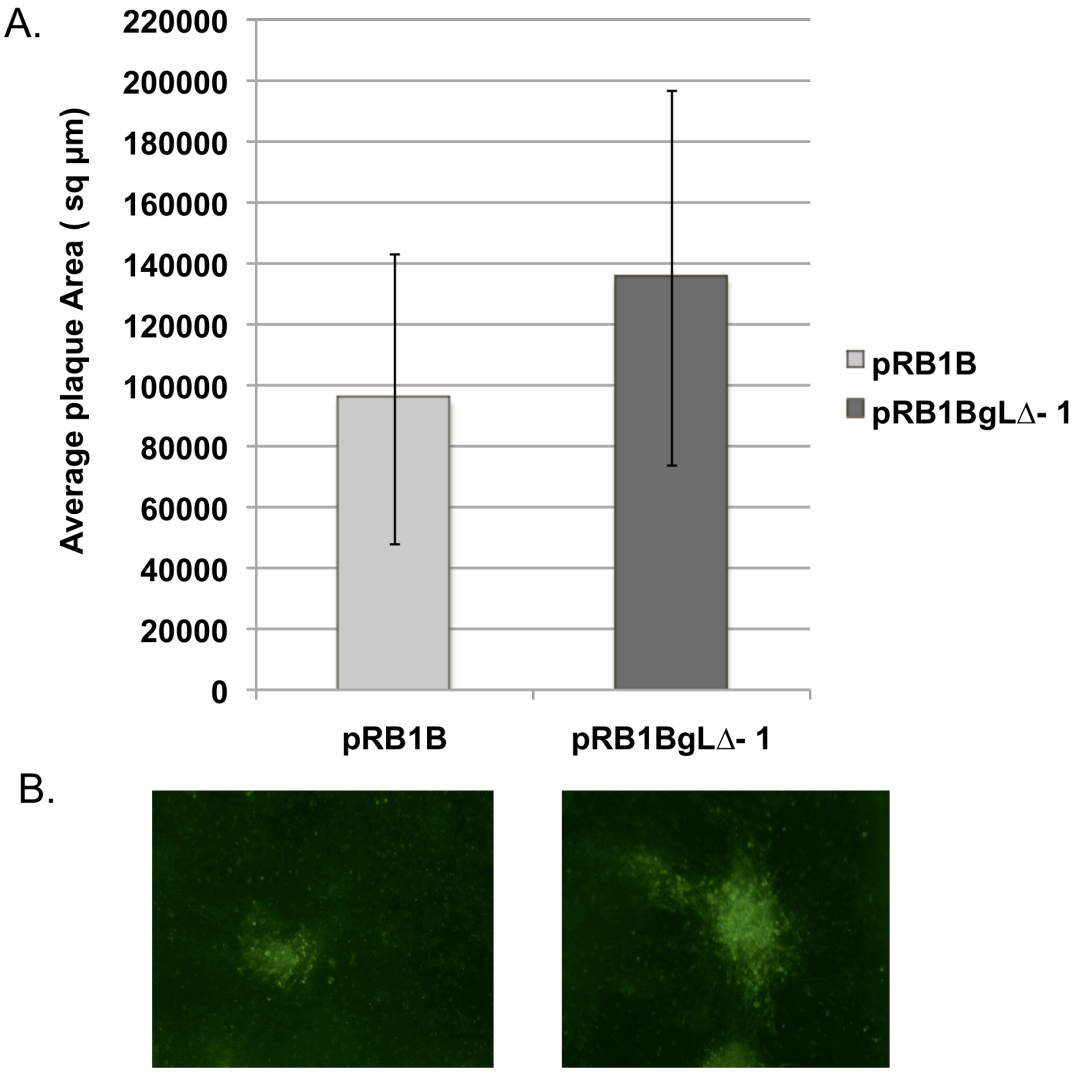


Figure 7 Plaque area analysis.
(A) The relative plaque size induced by parental RB1B and recombinant RB1BgLΔ1 is shown. CEFs were infected with 200 PFU of both parental RB1B and recombinant RB1BgLΔ1 and plaque areas were measured 6 days post-infection. (B) Representative plaques of parent (left) and mutant (right) are shown.

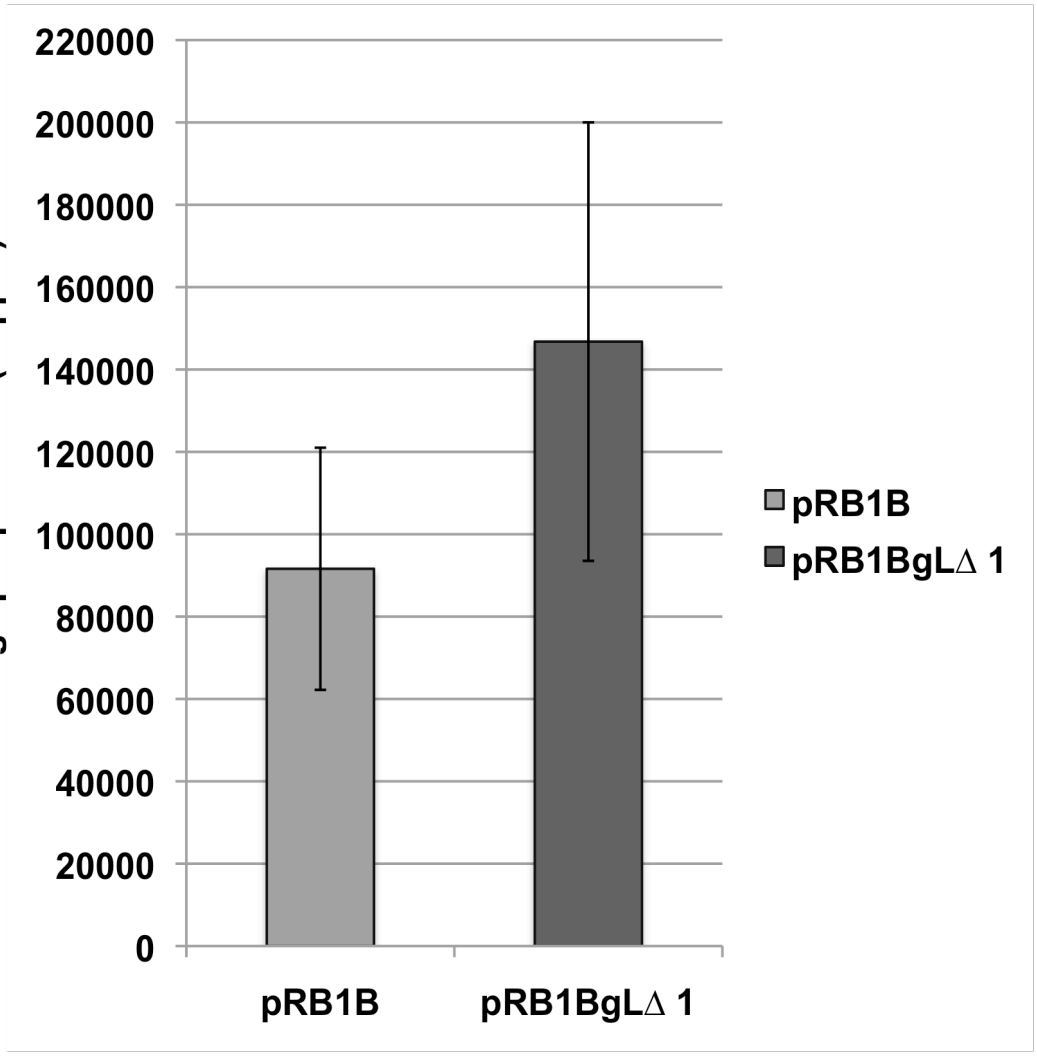


Figure 8 Plaque area analysis.
The relative plaque areas induced by parental RB1B and recombinant RB1BgLΔ1 are shown. CEF were infected with 200 PFU of both parental RB1B and recombinant RB1BgLΔ1 and plaque areas were measured 6 days post-infection The mean plaque area of RB1B is 91612 **sq µm** ± 29401 and mean plaque area for RB1BgLΔ is 146773 **sq µm** ± 53235.

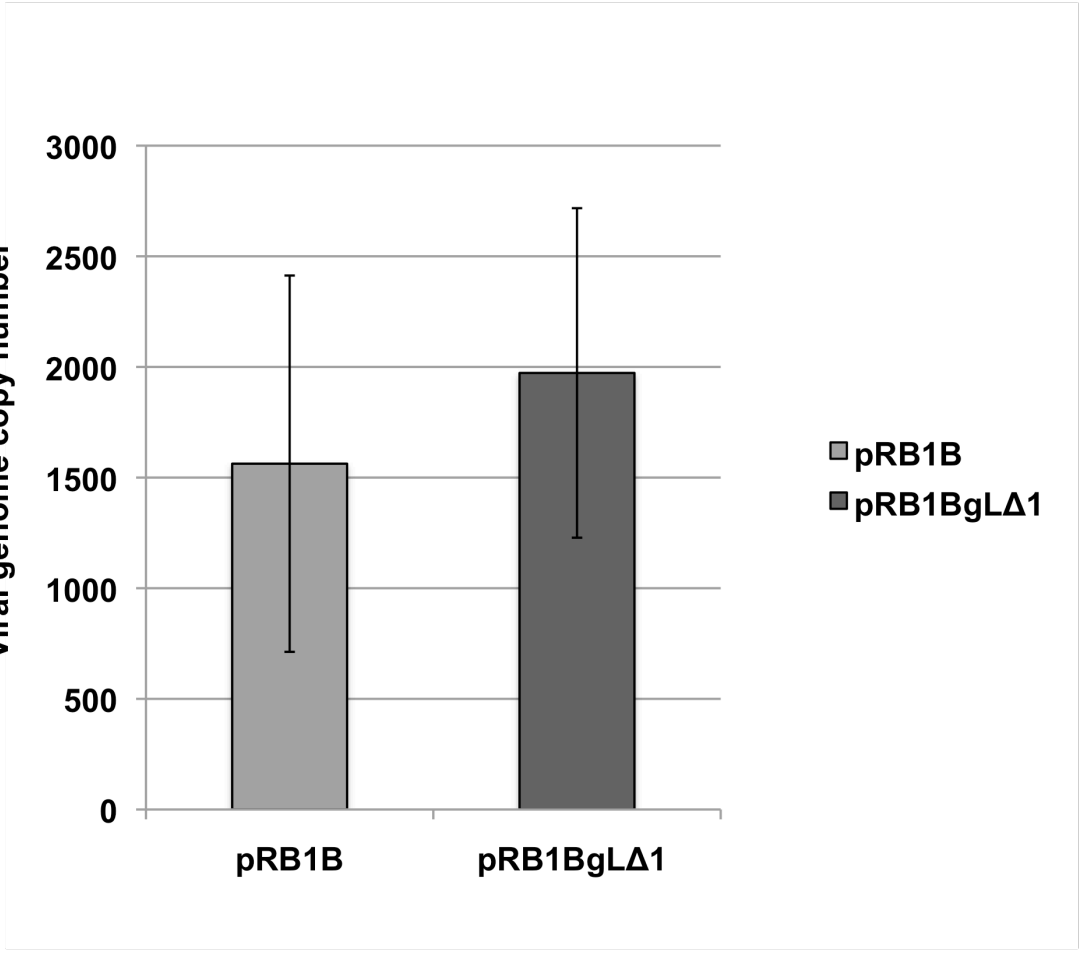


Figure 9 Viral genome copy number analysis.
qPCR analysis of the viral gB gene and a chicken ovotransferrin genes in total DNA isolated from cells infected with parental RB1B and recombinant RB1BgLΔ1 is shown. The mean viral genome copy number per plaque for RB1B is 1563 ± 850 and for RB1BgLΔ is 1973 ± 745. The difference in viral genome copy number per plaque was not considered significant (p value = 0.43).

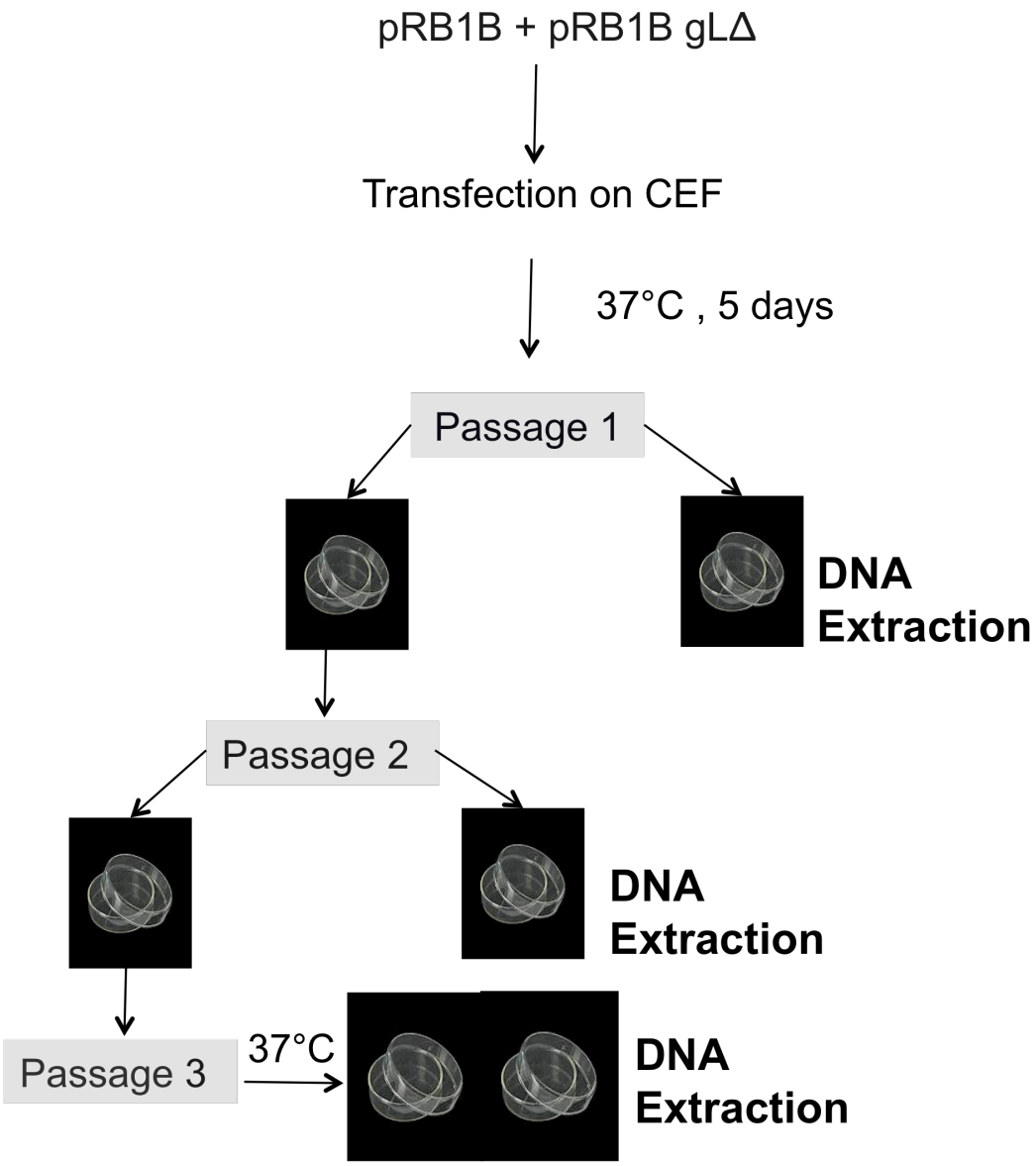


Figure 10 Schematic of competition assay of RB1B and RB1BgLΔ1 viruses in cell culture.

DNAs of parental and mutant viruses were mixed at various ratios and passaged 3 times in cell culture. The DNA was extracted at each passage for PCR/RFLP analysis for the gL deletion.

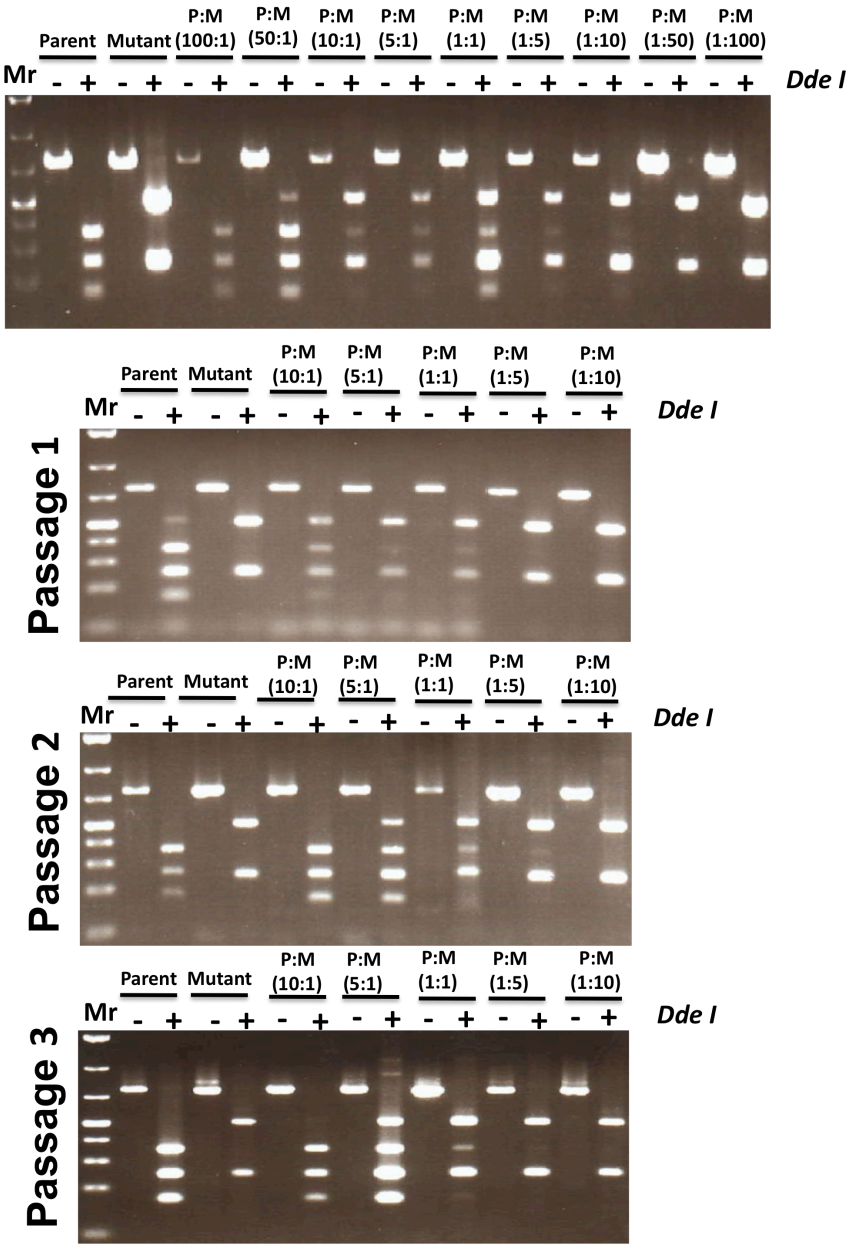


Figure 11 Competition assays of RB1B and RB1BgLΔ1 viruses in cell culture.

Panel A shows the control group where DNAs of parental and mutant viruses were mixed at various ratios for PCR/RFLP analysis for gL mutation. Panel B shows the gel picture for gL mutation assay of DNA extracted from passage 1 in cell culture, Panel C and D is show the PCR/RFLP analysis of DNAs extracted fron passage 2 and 3 respectively.

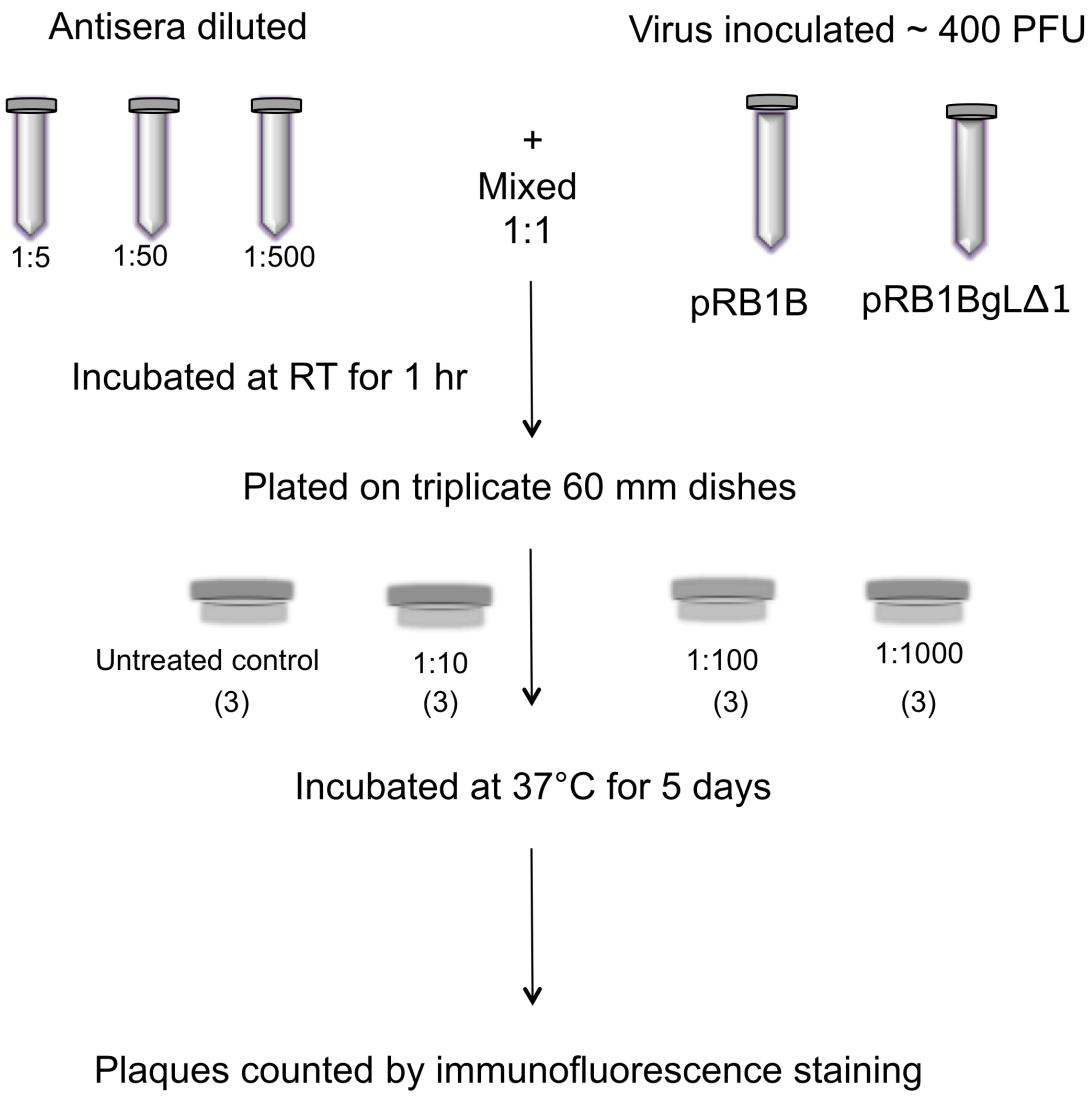


Figure 12 Virus neutralization assay of parental RB1B and mutant RB1BgLΔ1 viruses.

The diagram shows that the antisera from unvaccinated, commercially obtained broiler chicks was diluted at 1:5, 1:50 and 1:500 dilution and were mixed 1:1 with virus to obtain 200 PFU per treatment. Following 1-hour incubation at RT, each treatment was inoculated on freshly plated CEF in triplicate. Plaques were enumerated at 5 days of post-inoculation.

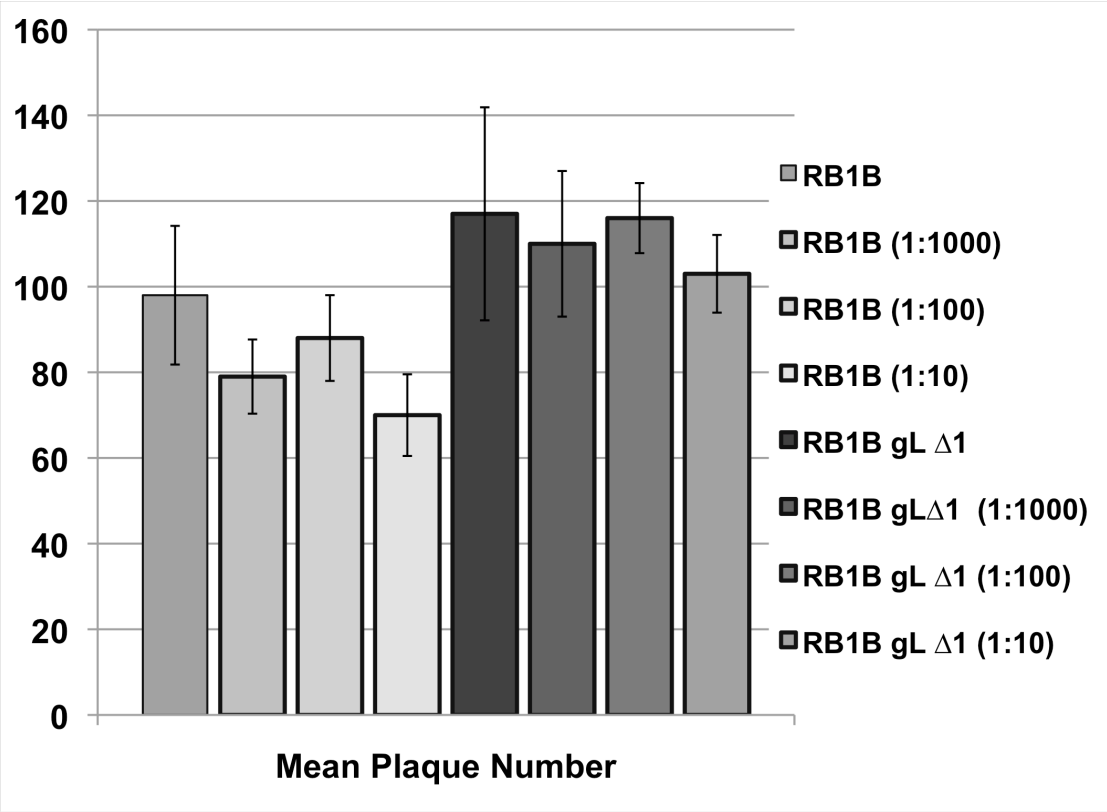


Figure 13 Virus neutralization assay in cell culture.
A Bar graph of virus neutralization data is shown, neutralization of both parent and mutant virus infected CEF is insignificant at 1:1000, 1:100 and 1:10 dilution of antisera in cell culture.

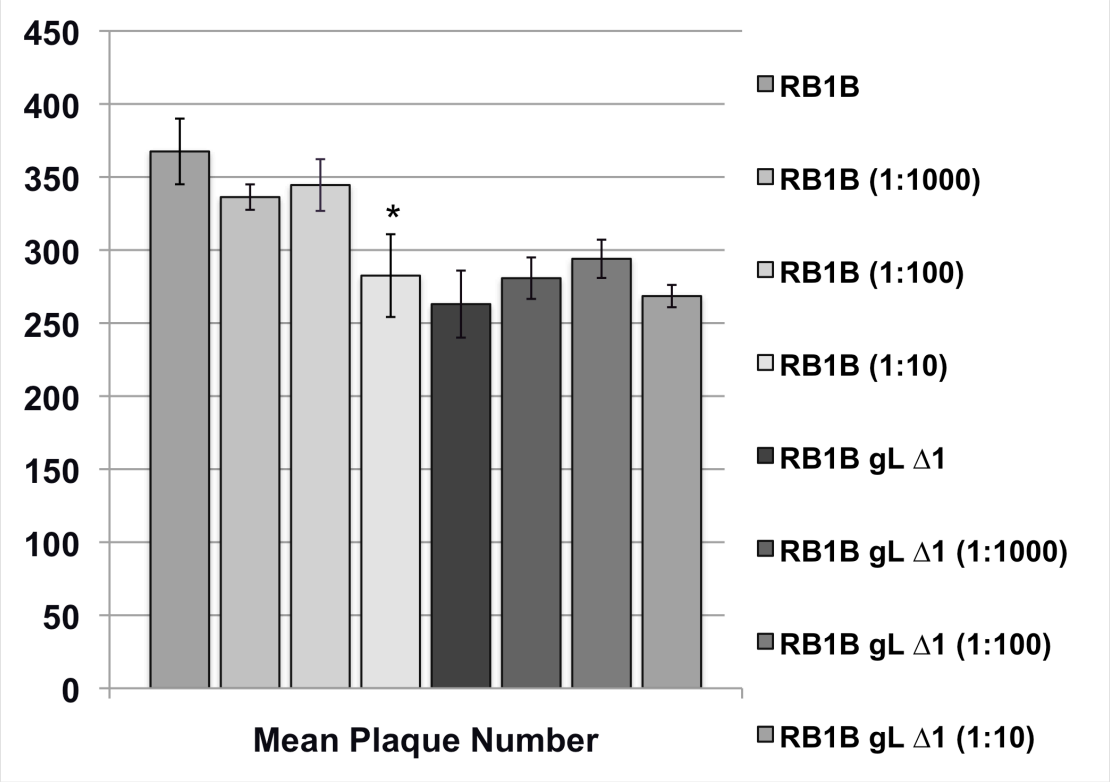


Figure 14 Virus neutralization using RB1B- and RB1BgLΔ1- infected SPC from *in vivo* experiment.

A bar graph for virus neutralization data is shown (above), depicting the neutralization of RB1B- and RB1BgLΔ-infected spleen cells. Data shown are the mean of quadruplicate neutralization reactions (± SD). The asterisk indicates that the number of plaque is significantly different from the medium only control (p = 0.0066).

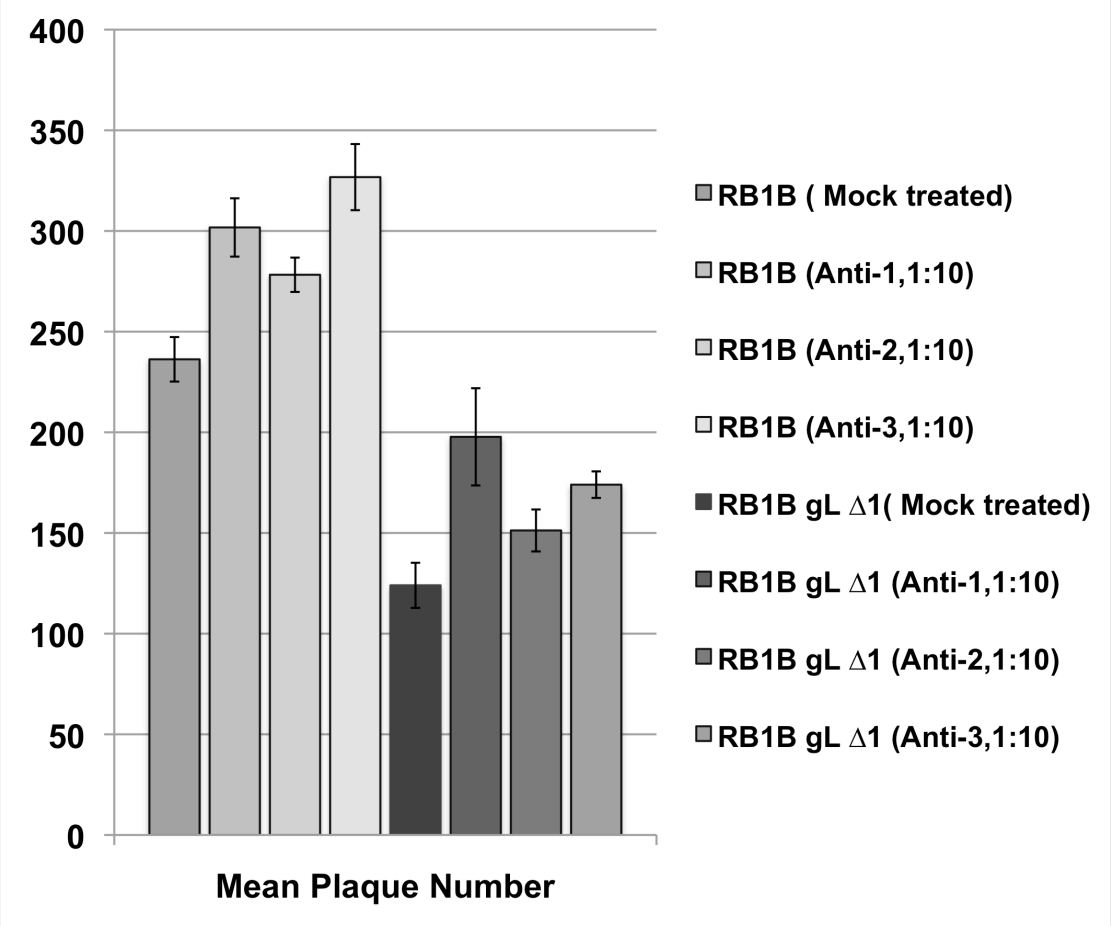


Figure 15 Virus neutralization using RB1B and RB1BgLΔ1 infected SPC from the *in vivo* experiment.

A bar graph for virus neutralization data is shown (above), depicting the neutralization of RB1B- and RB1BgLΔ-infected spleen cells. Data shown are the mean of quadruplicate neutralization reactions (± SD). The differences were not less than the mock treated SPF chicken serum.

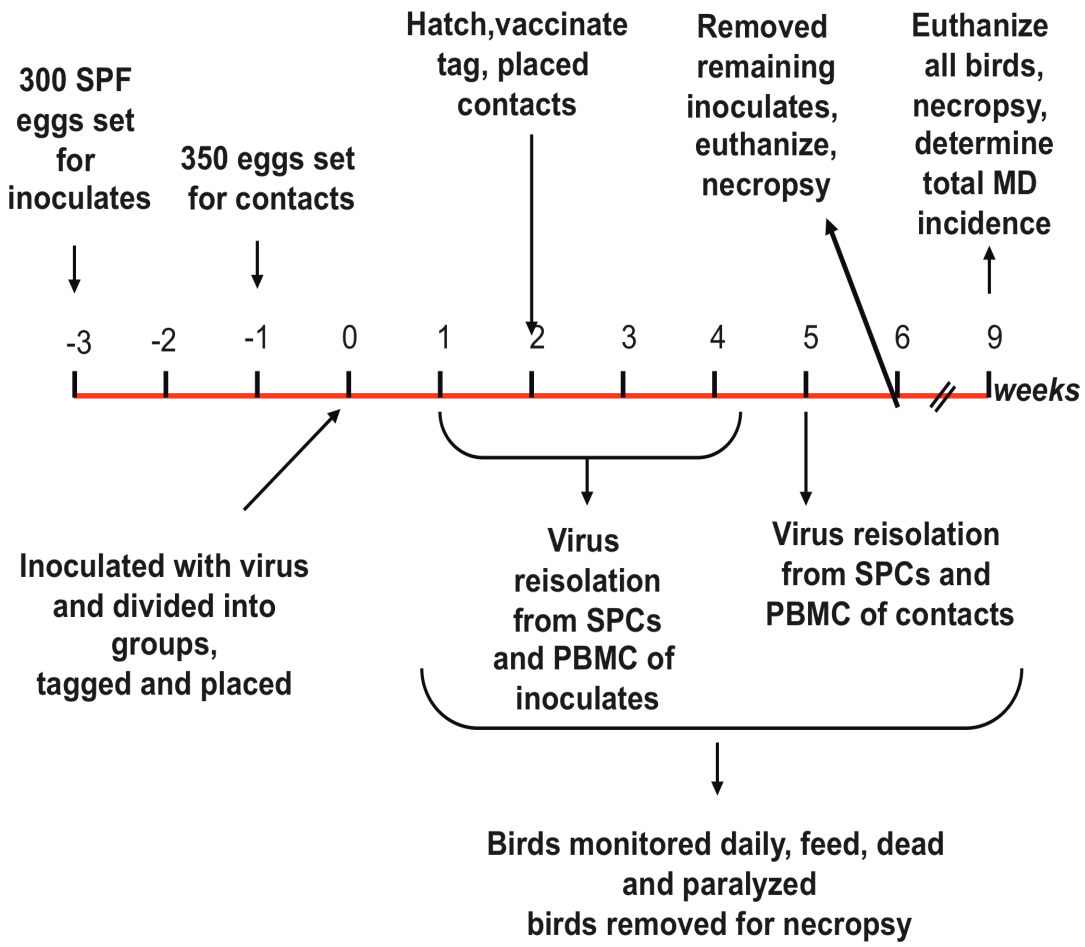


Figure 16 Experimental design of the *in vivo* study for characterization of parental RB1B and mutant RB1BgLΔ1 viruses.

Treatment groups are described in text. The schematic shows the placement of inoculates, vaccinates, and uninfected contacts in respective isolators.

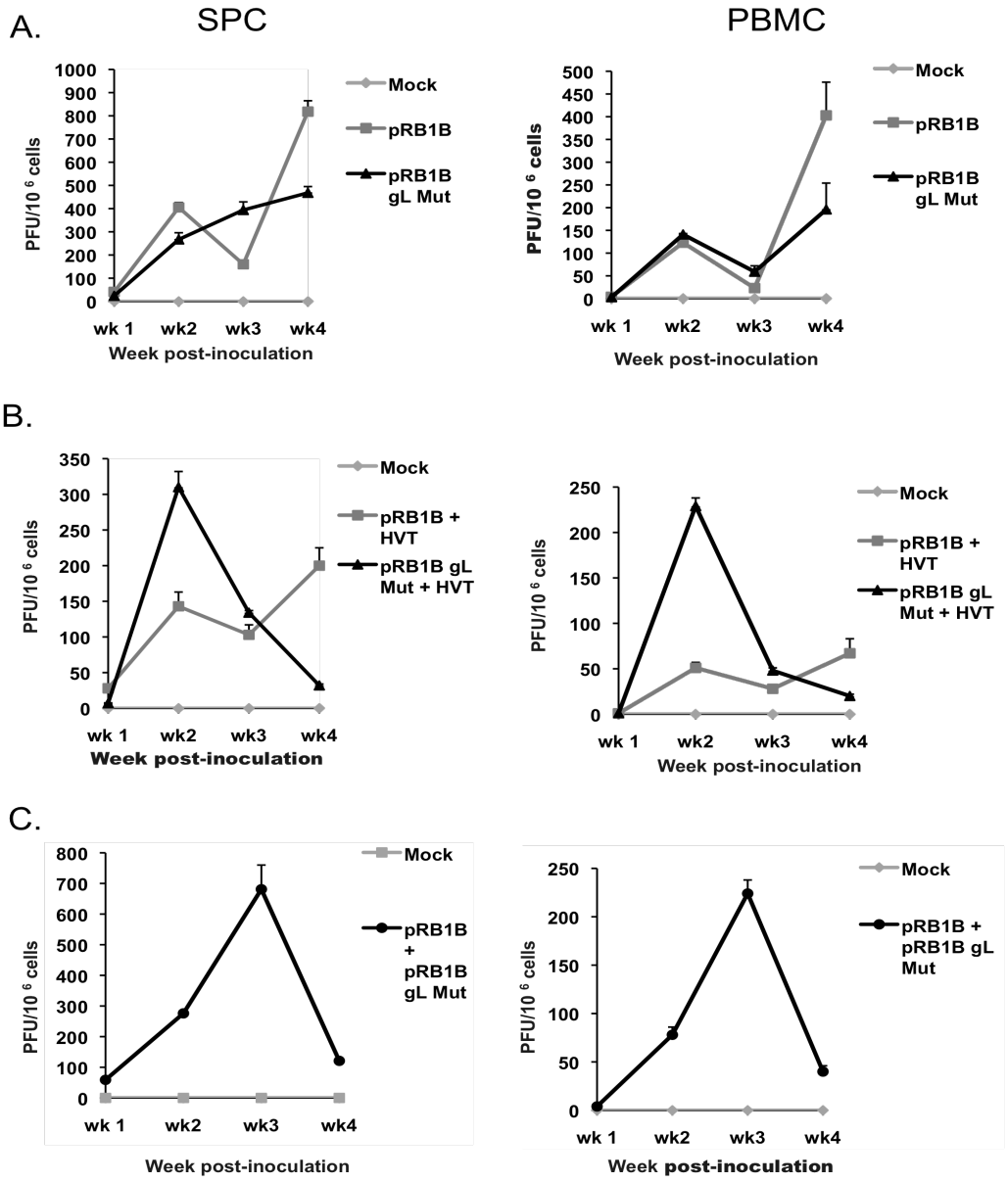
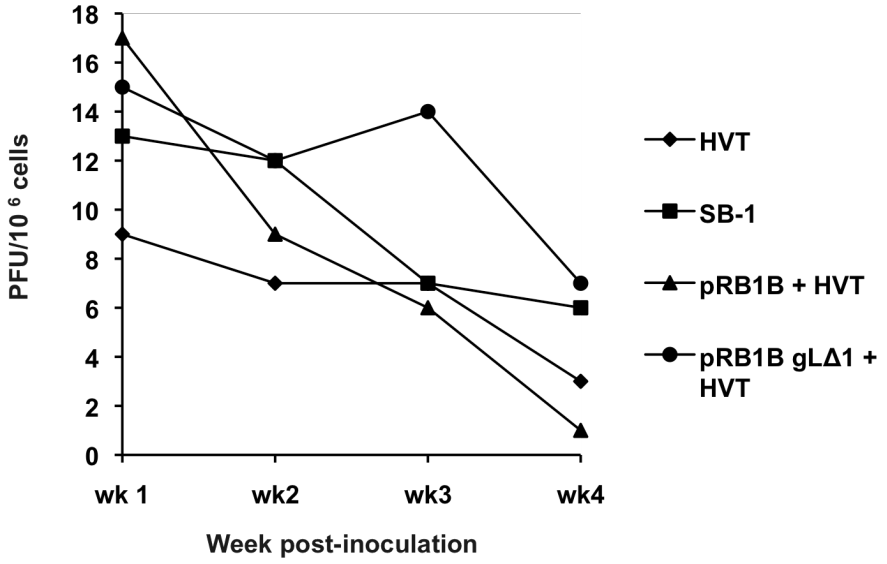


Figure 17 Viremia data of inoculates from spleen cells and PBMC. Three designated birds were taken per treatment group. Curves were generated from virus plaques formed on CEF monolayers infected with spleen cells (left) and from PBMCs (right). (A) Virus reisolation from pRB1B and pRB1B gL mutant. (B) Virus reisolation from pRB1B + HVT and pRB1B gL mutant + HVT infected spleen cells (left) and PBMCs (right). (C) Viremia curves obtained from pRB1B + pRB1B gL mutant infected spleen cells (left) and PBMCs (right). Data are shown as mean PFU/106 plated as (± SD).

A. SPCs



B. PBMC

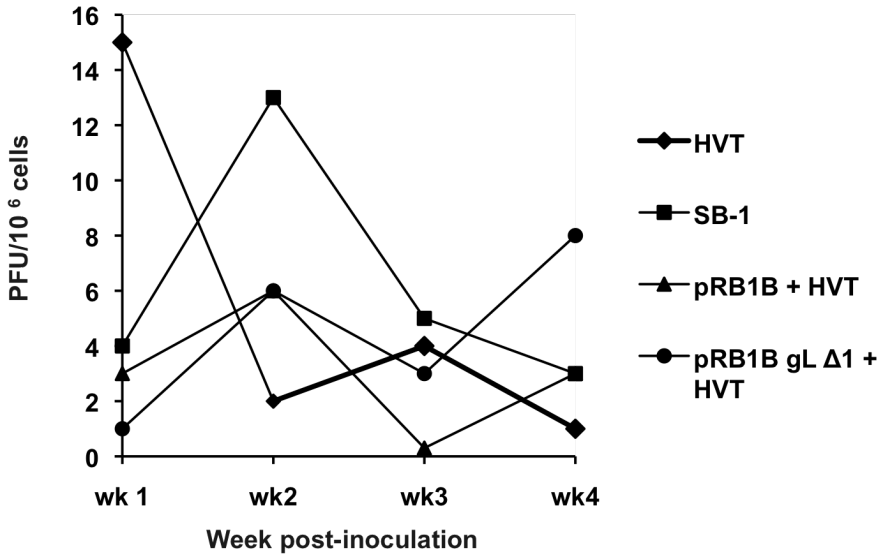


Figure 18 Viremia data of HVT from spleen cells and PBMC of inoculates. HVT virus reisolation from HVT/SB1, pRB1B + HVT and pRB1B gLΔ1 + HVT infected spleen cells (A) and PBMCs (B). Viremia curves obtained from plaques formed on CEFs from infected spleen cells (left) and PBMCs (right). Data are shown as mean PFU/106 plated as (± SD).

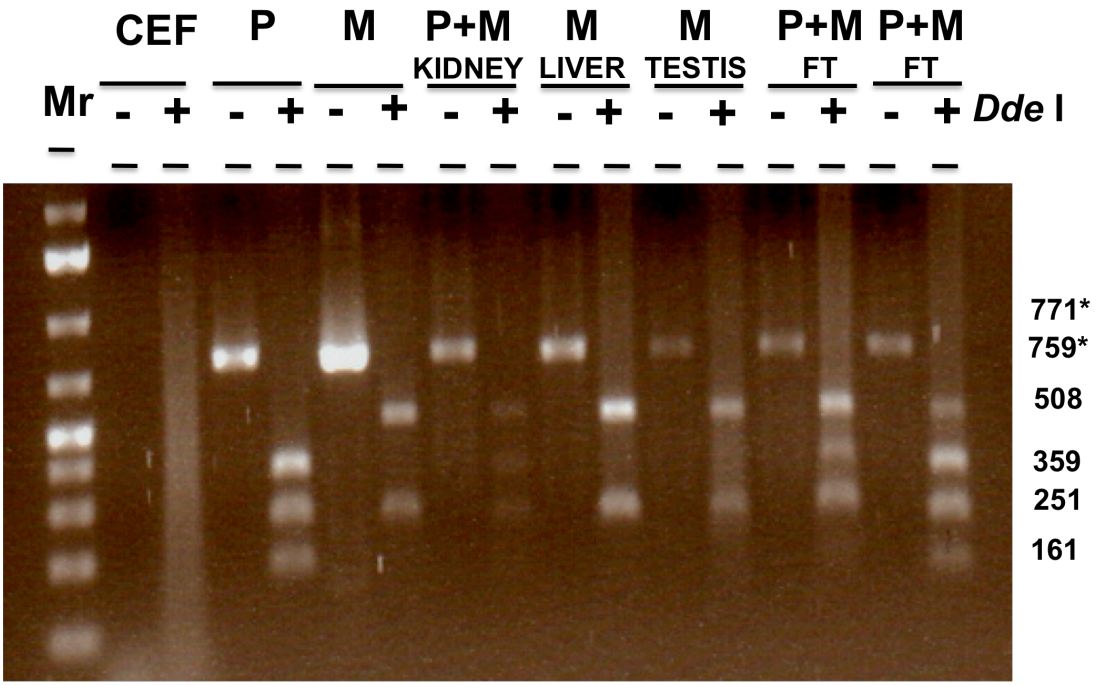


Figure 19 . PCR/RFLP analysis of the gL loci of tumor samples from mutant and mix groups.

The agarose gel above shows the PCR/RFLP analysis of tumor samples collected from mutant and mixed infection groups during the *in vivo* study. PCR amplification of gL gene of parental RB1B yielded a 771bp amplicon, similarly the gL genes were PCR amplified from mutant control to yield 759 bp amplicon. Amplicons are shown with and without *Dde I* digestion. Fragment sizes are given at right. Parent pattern: 359, 251 and 161 bp . Mutant pattern: 508 and 251 bp. Abbreviations are: P = RB1B, M = RB1BgLΔ, FT = feather tip purified DNA.

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Appendix

Pathogenic comparison of BAC-based recombinant Marek's Disease Viruses (strains RB1B, MD5, CVI988 and derivatives)

Registration #: 10-018

University of Delaware
Department of Occupational Health & Safety
Recombinant DNA Registration

Directions: Please complete this form to register recombinant DNA research with the University Biosafety Committee (UBC) as required by the most current "Guidelines for Research Involving Recombinant DNA Molecules" (NIH *Guidelines*) and University Policy 7-19.

Submit a separate form for each project. **A copy of the current *Guidelines* is available at the DOHS web site: http://www.udel.edu/OHS/. For questions, please contact the Biosafety Officer at 831-8475.**

Section I- to be completed for all projects

Principal Investigator: Mark S. Parcells, Ph.D.
Department: Animal and Food Sciences
Address: 052 Townsend Hall
Phone Number: 831-0114 Fax: 831-2822
Email: Parcells@UDel.edu
Labs to be used: L313 Worrilow Hall, also locations off-site (see narrative)
For exempt work: General Work Description:
For non-exempt (covered) work: Project Title: Pathogenic Comparison of BAC-based Recombinant Marek's Disease Viruses (strains RB1B, MD5, CVI988 and derivatives)
Proposed start date for research: 5/1/10
Your signature below indicates that you acknowledge all requirements and restrictions of the most current NIH *Guidelines* for the biosafety level you have indicated, unless modified by the UBC, that you accept responsibility for the safe conduct of the experiments conducted at this biosafety level and that you have informed all associated personnel of the conditions required for this work. It is the Principal Investigator's responsibility to follow the NIH *Guidelines* and notify the Biosafety Officer and the UBC of any adverse events, including research-related accidents and illnesses. The Principal Investigator certifies that the work description is accurate. Any work performed which is not approved under this permit may be subject to the loss of grant funds. This registration must be updated annually. Signature of Investigator: Mark S. Parcells Date: 07/26/10

Section II- to be completed for all projects

Check the appropriate registration category for experiments covered by the NIH Guidelines:
All categories are defined in the NIH Guidelines

A. Experiments which are exempt and do not require registration.

☐ Examples include rDNA that is: not in organisms and viruses; entirely DNA segments from a single nonchromosomal or viral DNA source; entirely from a prokaryotic host including its indigenous plasmids or viruses when propagated only in that host or when transferred to another host by well established physiological means; entirely from a eukaryotic host when propagated only in that host or a closely related strain of the same species; entirely segments from different species that exchange DNA by known physiological processes; or not a significant risk to health or the environment.
If work is exempt, attach a description of the recombinant DNA procedures to be performed.

B. Experiments that Require IBC Approval, Recombinant DNA Advisory Committee Review, and NIH Director Approval Before Initiation.

☐ Deliberate transfer of a drug resistance trait to a microorganism that is not known to acquire the trait naturally, if such acquisition could compromise the use of the drug to control disease agents in humans, veterinary medicine, or agriculture

C. Experiments that Require NIH/ORDA and IBC Approval Before Initiation.

☐ Cloning of toxin molecules with LD_{50} of less than 100 nanograms per kilogram body weight

D. Experiments that Require IBC Approval, Human Subjects Approval, and NIH/ORDA Registration Before Initiation. Submit completed Appendix M, I-V from the NIH *Guidelines* along with this document.

☐ Deliberate transfer of recombinant DNA or DNA or RNA derived from recombinant DNA into one or more human subjects (human gene transfer)

E. Experiments that Require IBC Approval Before Initiation

☐ 1. Experiments using Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents as Host-Vector Systems

☐ 2. Experiments in which DNA from Risk Group 2, Risk Group 3, Risk Group 4, or Restricted Agents is cloned into nonpathogenic prokaryotic or lower eukaryotic Host-Vector Systems

☐ 3. Use of infectious DNA or RNA viruses or defective DNA or RNA viruses in the presence of helper virus in tissue culture systems

X 4. Experiments involving recombinant DNA in animals or transgenic whole animals

☐ 5. Experiments involving whole plants, to include exotic infectious agents that may impact ecosystems, transmissible exotic infectious agents in the presence of their specific arthropod vectors, sequences encoding vertebrate toxins introduced into plants or associated organisms, or microbial pathogens of insects/animals associated with plants if microorganism may impact ecosystem

☐ 6. Experiments involving more than 10 liters of culture

F. Experiments that Require IBC Notice Simultaneous with Initiation

☐ 1. Formation of recombinant DNA molecules containing no more than two-thirds of the genome of any eukaryotic virus in tissue culture with no helper virus

☐ 2. Recombinant DNA modified plants that are noxious weeds or can interbreed with noxious weeds. Plants associated with recombinant DNA modified non-exotic microorganisms which have the potential for serious impact on ecosystems. Recombinant DNA modified arthropods or small animals associated with plants if these materials have no serious impact on ecosystems

☐ 3. Experiments involving recombinant DNA modified whole plants or organisms (if not included in Category E5 above)

☐ 4. Generation of transgenic rodents where genome is altered by stable introduction of rDNA into germ line, if it requires only BSL1 containment

Section III- to be completed for covered (non-exempt) projects only

1. Names of individuals participating in project, with job title: Mark S. Parcells, Project Leader; Phaedra Tavlarides-Hontz, technician; Upendra Katneni, Huimin Dong, Shireen Shaikh, graduate students;
2. Source(s) of DNA/RNA sequences (include genus, species, gene name and abbreviation): gallid herpesvirus 2, Mardivirus 1, Marek's disease virus, RB1B, MD5 and CVI988 strains
3. Is a vector required? **Yes** ☐ **No X** If yes, identify specific phage, plasmid, or virus: Virus vector: Adenovirus ☐ Retrovirus ☐ Other ☐ Defective: **Yes** ☐ **No** ☐ Replication competent: **Yes** ☐ **No** ☐ If viral vector, what percent of the viral genome remains?
4. If the recombinant contains viral DNA, does the insert represent more than 2/3 of the viral genome? **Yes X No** ☐
5. Is a helper virus required? **Yes** ☐ **No X** If yes, specify:
6. What is the biological activity of the gene product or sequence inserted? BAC sequences within genome contain chloramphenicol resistance gene and guanine phosphoribosyl transferase gene, mutants made using Kanamycin or Ampicillin resistance cassettes
7. Will a deliberate attempt be made to obtain expression of the foreign gene encoded in the recombinant DNA? **Yes X No** ☐ **(for purpose of mutant construction in bacteria)**
8. Host strain for propagation of the recombinant (give genus, species, and parent strain): E. coli, K-12 derivatives, DH10B, GS1783, etc.
9. Target recipient of recombinant DNA (indicate species or cell lines used): Animals: *Gallus gallus* Tissue Culture: chicken embryo fibroblasts (CEF) Plant cells: Plants: Gene therapy: Specify target host(s) - human, animal species:
10. Proposed biosafety level for project (check one): **1** ☐ 2 **X** 3 ☐
11. Have all personnel involved in this project been trained to the appropriate biosafety level? **Yes X No** ☐
12. Dual Use Research- Check any categories below that apply to your project: ☐ Renders a useful vaccine ineffective ☐ Adds antibiotic resistance affecting response to a clinically useful drug ☐ Enhances pathogen virulence ☐ Increases pathogen transmissibility ☐ Widens a pathogen's host range ☐ Enables a pathogen to evade diagnostic or detection modalities ☐ Weaponization (e.g. environmental stabilization of pathogens) **X None of the above**
13. **Be sure to attach a description of the recombinant DNA procedures to this form.** Include the following items: nature and purpose of the project; outline the procedures and techniques; risk to

personnel; practices/equipment/facilities to protect the personnel; methods to inactivate and dispose of the agents. Sufficient detail must be provided to understand the project and review the rDNA procedures.

Section IV- For UBC Use Only

☐ Project/work exempt from recombinant DNA NIH *Guidelines*.
(**Make sure Work Description is attached**).

☒ Project/work requires registration according to NIH *Guidelines*. The PI and staff can safely perform this work with the training, work practices, and lab facilities listed.

The following signatures indicate provisional approval by the University Biosafety Committee for this project involving recombinant DNA technology. The work is to be performed according to NIH requirements. Final approval for projects that are NOT exempt from the NIH *Guidelines* will not be granted until after review by the entire UBC at the next meeting. Non-exempt work covered under this approval cannot begin until final approval is received.

UBC Member Conducting Review

Print Name: NICOLE M. DONOFRIO

Signature: [signature]

Date: 8/24/10

Biosafety Officer

Print Name: Krista Murray

Signature: Krista Murray

Date: 8/26/10

Expiration date: N/A

Final UBC approval date: 9/9/10

UBC Representative Signature: [signature] Krista Murray

UBC Comments: