The Creation of *CD209* Gene Knockout Sheep as a Model for Bovine Leukemia Virus Resistance

By

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Abstract

In this study, we report the generation of *CD209* gene knockout sheep, utilizing electroporation-mediated CRISPR-Cas9 genome editing, as a model to test whether this might make cattle resistance against Bovine Leukemia Virus (BLV). This approach exploits the *CD209* gene's role as a receptor for BLV, hypothesizing that its knockout would confer resistance to infection. Our methodology involves specific guide RNAs targeting the sheep *CD209* gene, followed by electroporation into ovine zygotes to induce targeted gene disruptions. It was hypothesized that a gene knockout of *CD209* would result in the inability of the virus to bind and enter the cell; therefore, creating disease resistance. The resultant lambs exhibited varied mosaicism and phenotypic outcomes associated with the gene edit, indicative of the CRISPR-Cas9 system's effectiveness and efficiency. This study not only demonstrates a novel application of gene editing in livestock but also underlines the potential of sheep as surrogate models in BLV research, due to their analogous immunological responses and shorter gestational periods compared to cattle. The successful application of this technology paves the way for future research in genetic engineering for livestock disease resistance, with significant implications for animal health management and agricultural productivity.

Table of Contents

Figures and Tables

Chapter 1: Literature Review

Bovine Leukemia Virus

Bovine Leukemia Virus (BLV), is an enzootic oncogenic retrovirus with a global spread among domesticated cattle that causes bovine leukemia or enzootic bovine leukosis disease (Kettmann et al., 1976). The first reported case of BLV was described in 1871 in Klaipeda, Lithuania where the infected cow was described as having superficial lymph node hypertrophy and splenomegaly (Schwartz & Lévy, 1994). In the early 1970s, U.S. researchers noticed a rise in cow herd lymphosarcoma (Kettmann et al., 1976). BLV is now endemic in Europe, Asia, the Middle East, and the Americas.

Characterization of the BLV

Research into the potential cause underlying of the lymphosarcoma was undertaken and determined to be the retrovirus now known as BLV. Further investigations by Kettmann et al. (1976) showed that BLV particles are RNA-exogenous viruses with an RNA reverse transcriptase enzyme complex, a discovery that categorized BLV among the oncogenic retroviruses. Investigations by Buehring et al. (2019) defined BLV as a deltaretrovirus closely related to the human T-cell leukemia virus types I and II (HTLV-I and HTLV-II). BLV belongs to the genus Deltaretrovirus within the family Retroviridae (Gillet et al., 2007; Marawan et al., 2021; Polat, Takeshima, & Aida, 2017; Straub & Lévy, 1999). BLV is an enveloped virus with a single-stranded RNA genome.

Pathogenesis of BLV

Understanding the pathophysiology of BLV infection and how it affects host organisms requires understanding the precise targets of BLV infection at the cellular and species level. The B lymphocytes, white blood cells involved in the immune response, are the cells most often infected by BLV at the cellular level (Kettmann et al., 1976; Krasikov et al., 2020; Petropavlovskiy, Donnik, & Bezborodova, 2019).

Upon entering the organism, the virus infects the lymphocytes and converts its RNA genome into DNA using the enzyme reverse transcriptase, a characteristic feature of retroviruses (Gillet et al., 2007). The viral DNA is then integrated into the host cell's genome, becoming a provirus. Once integrated, the provirus directs the production of viral proteins and allows the virus to replicate within the host cell (Buehring et al., 2019; Gillet et al., 2007; Kettmann et al., 1976; Lucas & Roberts, 1982; Mammerickx et al., 1987).

BLV most commonly affects water buffalo (*Bubalus bubalis*) and domestic cattle (*Bos taurus*) (Gillet et al., 2007). Water buffalo are common carriers but do not appear to be significantly impacted by the disease and frequently act as infectious carriers of the disease. Cattle show negative health effects upon proliferation of the virus; which can be varied between individuals, usually taking years for infected phenotypes to be visible (Djilali, Parodi, & Levy, 1987). Infection may cause weight loss, lymph node enlargement, reduced milk production, and increased illness susceptibility in domestic animals (Rhodes, Pelzer, & Johnson, 2003; Zhang et al., 2022). BLV infection may produce immunosuppression and lymphoproliferative disorders in alternative hosts, albeit the symptoms differ from those reported in cattle. Cattle appear to have a lesser of a B cell proliferation response to the virus than small ruminants, namely sheep and goats. This increased

B cell proliferation in the smaller ruminants in-turn causes a more widespread and rapid infection. (Djilali, Parodi, & Levy, 1987; Gatei et al., 1989; Gillet et al., 2007; Mammerickx et al., 1987).

Methods of Transmission, Infection, and Genetic Integration

Iatrogenic transmission, defined as an illness caused by medical treatment, horizontal, and vertical transmission are all potential routes of BLV infection (Gatei et al., 1989). The study on the mechanism of leukemogenesis by Gillet et al. (2007) established that iatrogenic transmission of BLV to an animal occurs primarily through the transfer of infected lymphocytes, particularly during medical treatment involving the use of a needle, such as vaccination. Horizontal transmission occurs when an animal comes into contact with another animal that already has the disease. Vertical infection refers to a parent giving the disease to their offspring. An infected dam may transmit the BLV virus to her fetus vertically, during pregnancy or indirectly, via milk and colostrum (Ruiz et al., 2018). Iatrogenic transmission is demonstrated to be likely the largest source of BLV infection in the U.S (Rodríguez et al., 2011). It is common practice in the United States for the same vaccination needle to be used in multiple animals, spreading blood and therefore blood-borne disease (such as BLV) (Rodríguez et al., 2011).

Prevalence and Distribution of BLV in the United States

The first study in the U.S. to provide a baseline for the prevalence of BLV and inform on the control measures was conducted by Baumgartener et al. (1975). The study involved 100 operations and 4394 dairy cattle which were tested using an Agar Gel Immuno-diffusion method (AGID). The findings revealed a 66% prevalence where at least one cow on the operation tested positive. A recent study by LaDronka et al. (2018) that aimed to understand BLV's antibody

prevalence among U.S. dairy cattle also produced a higher modern rate of infection. The LaDronka study involved 4120 dairy cows from 103 operations across eleven states. Using ELISA, or Enzyme-Linked Immunosorbent Assay, milk samples were tested for BLV antibodies. The findings revealed that 94.2% of the herds had at least one positive BLV ELISA Antibody test. The average within-herd standardized AP, Antibody Prevalence, was 46.5%, lactation-specific AP increased from 29.7% to 58.9% from first to fourth and greater lactation cows. The findings were consistent with no significant differences between states, regions, breeds, or herd size. LaDronka et al. (2018) concluded that there is an increasing trend of BLV among dairy cattle. These findings are consistent with the latest statements from the United States Department of Agriculture (USDA, 2021). The USDA data reveal that in 2021, 89% of dairy and 38% of beef cattle tested seropositive for BLV, indicating an increasing trend in prevalence.

Eradication Efforts in the US

Eradication efforts for BLV in the United States have been underway since the discovery of the virus (Kuczewski et al., 2021). After a comprehensive evaluation of available published articles on BLV prevalence and control, Marawan et al. (2021) concluded that the lack of a vaccine has been the major factor that has made control infeasible. The USDA (2021) also states that currently, the evidence available shows that the United States lacks defined Federal regulations for curbing the spread of the virus among the cattle population. Marawan et al. (2021) and USDA (2021) conclude that early detection and subsequent diagnosis are essential in noting the affected animals and eliminating them to diminish the spread of the virus. Rodríguez et al. (2011) has found three control approaches that are currently in use by farmers in affected regions. These approaches include: test and eliminate, test and segregate, and test and manage. Test and eliminate involves

identifying the infected cattle and slaughtering them (Rodríguez et al., 2011). Test and segregate requires detecting the affected cattle and isolating them for separate management from the non-infected. Test and management, on the other hand, involves testing for infection, and then taking biosafety management measures to minimize animal exposure to infectious agents.

Eradication Efforts in Other Countries

Governments in several countries have BLV management and elimination policies (Marin et al., 1978). These initiatives promote testing and monitoring, biosecurity, and disease identification and elimination. BLV has been reduced or eliminated in numerous countries, such as England, Australia, and the European Union. Individual infection rates of between 34% and 50% have been reported in South American nations including Colombia, Venezuela, Chile, and Uruguay (Alfonso, Almansa, & Barrera, 1998; Islas, 1990; Marin et al., 1978). In Argentina individual and herd-prevalence levels reached as high as 32.8% and 84% respectively (Trono et al., 2001). In Brazil, the individual prevalence levels reached 50% (D'angelino, Garcia, & Birgel, 1998). Seroprevalence rates are 86.8% in Korea and 68.1% in Japan but are significantly low at only 5% in Cambodia and Taiwan (Murakami et al., 2011; Suh et al., 2005; Wang, 1991). Middle Eastern countries are the regions with lower prevalence averaging 20%, except Turkey (48.3%) and Iran (64.7%) (Burgu et al., 2005; Hafez et al., 1990).

Economic and Health Implications of BLV infection in Cattle

BLV infection has serious health ramifications for cattle with negative economic impacts (Hsieh et al., 2019; Jaworski et al., 2019). Animals with infections often have suboptimal

productivity and health (Takeshima, Ohno, & Aida, 2019). The direct and indirect costs incurred by farmers with seropositive animals include high veterinary costs, death of animals, poor quality semen and embryos, and reduced or lost production (Da et al., 1993; Juarez, 2019; Kuczewski et al., 2021; Nakada et al., 2022; Nieto Farias et al., 2018; Ott et al., 2003; Watanabe et al., 2019; Yang et al., 2016). For example, a study by Ott et al. (2003) on the relationship between BLV seroprevalence and herd-level productivity in U.S. dairy farms found that sub-clinically infected animals produced 218 kilograms less milk per year as compared to those without a seropositive outcome. Additionally, BLV-associated lymphoproliferative diseases such as lymphosarcoma may impair fertility, cause weight loss, and necessitate the early euthanasia of affected animals. The whole cattle sector is affected financially, not just individual farms. Losses caused by BLV in cow herds include lower milk production, decreased reproductive effectiveness, higher replacement costs, and trade restrictions (Juarez, 2019; Kuczewski et al., 2021; Nakada et al., 2022; Ott, Johnson, & Wells, 2003; Watanabe et al., 2019; Yang et al., 2016). These financial repercussions highlight the need to implement efficient control strategies to lessen the financial burden brought on by BLV infection.

Economic and Health Implications for Humans

Concerns have been raised about the safety of dairy products due to the potential presence of BLV in the food chain (Marawan et al., 2021). Nakada et al. (2022) found that among BLV-infected cattle, only 5% developed lymphoma requiring exclusion of their products from consumption, while the remaining 95%, though infected, showed no clinical signs and their products were considered safe for consumption. Additionally, a meta-analysis by Marawan et al. (2021) found no evidence of BLV transmission to humans, despite studies exploring this

possibility. Buehring et al. (2014) reported finding BLV DNA and proteins in commercial bovine products and in human breast cancer patients, raising concerns about potential health implications. Although the study by Buehring found no causation, only a correlation between BLV DNA presence in breast cancerous tissue, fear around the zoonosis of the disease and potentially negative effects of the virus on human health persist (Marawan et al., 2021).

Historical Advances in Gene Technology - Mice

Traditional breeding methods, such as selective mating, were used in early genetic research on mice to examine inheritance patterns and the function of genes in phenotypic characteristics. Researchers were able to start directly modifying genes in the lab thanks to the development of molecular biology tools in the 1970s and 1980s, such as recombinant DNA technology and the capacity to isolate and clone genes (Thomas & Capecchi, 1987).

The development of knockout mice, achieved through the inactivation or "knockout" of specific genes, revolutionized the field. The knock-out mice were created through disruption of genes in Embryonic Stem Cells and then subsequent introduction into mouse embryos; creating chimeric KO mice (Smithies et al., 1985). This technology was not possible in other species until Embryonic Stem Cells could successfully be cultivated. Knockout mouse models have played a crucial role in elucidating gene function in development, physiology, and disease (Capecchi, 2005). Pioneering studies using knockout mice have yielded significant insights into various biological processes. For instance, research on the tumor suppressor gene p53, using knockout mice, demonstrated its pivotal role in preventing cancer development (Donehower et al., 1992). In addition to knockout mice, other genetic technologies in mice emerged, including conditional gene knockout, which allowed researchers to selectively delete genes at specific developmental stages or in specific

tissues (Gu et al., 1994). Conditional knockout technology further enhanced the understanding of gene function in a context-dependent manner.

The development of programmable nucleases in the 1980s, like Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems was a significant milestone in gene technology. Utilizing a nuclease to precisely alter the genome is what is commonly known as gene editing (Wang et al., 2022). ZFNs, for example, are nucleases capable of precisely binding to a DNA domain, enabling targeted DNA cleavage (Klug, 2010). Similarly, TALENs utilize TAL effector domains to recognize specific DNA sequences and introduce double-strand breaks (Bogdanove & Voytas, 2011).

The discovery of Clustered Regularly Interspaced Short Palindromic Repeat-associated protein 9 (CRISPR-Cas9) introduced simplicity, versatility, and efficiency in gene editing (Gostimskaya, 2022). CRISPR-Cas9 is a revolutionary gene-editing innovation from the bacterial immune system (Ishino et al., 1987). CRISPR uses RNA to precisely target and create a double strand breaks in DNA (Jinek et al., 2013). The RNA molecule, termed a Guide RNA, contains the specific DNA binding site for precise nuclease action. Upon binding at the guide RNA directed site the CRISPR protein creates a double strand break in the DNA.

Applications in Livestock - Cattle and Sheep

Modern researchers have realized the promise of gene editing methods in livestock animals after successful applications in mice (Wang et al., 2022). Numerous experiments have been conducted on cattle and sheep, two commercially significant species, to enhance production traits

and disease resistance (Wang et al., 2022). Due to its potential to improve disease resistance, meat quality, and milk output in cattle, gene editing has drawn a lot of interest. In previous experiments intended genetic alterations were successfully introduced into bovine embryos using CRISPR-Cas9, improving calves' disease resistance, muscular growth, and decreasing susceptibility to disease infections (Wang et al., 2022).

Electroporation as a Tool for Gene Editing

An integral element of gene editing is the addition of the editing reagents (namely nuclease, template DNA, and guide RNAs in the case of CRISP editing system) to the DNA of the cell (Tavakoli et al., 2021). Historically the editing reagents were introduced through microinjection of early stage embryos; the injection of reagents directly into the embryo through the zona pellucida using a micro-manipulator and glass needle (Lin & Van Eenennaam, 2021). Electroporation, a technique characterized by the application of brief electrical pulses to cells, causing pores to form in the cellular membrane ((Tanihara et al., 2016)). Electroporation and the resultant cellular pores have emerged as a potential tool for the introduction of editing reagents into cells. Electroporation requires fewer steps and equipment, making it easier to perform. It further involves the use of a stereomicroscope, an electroporator, and an electroporation cuvette, whereas microinjection requires specialized micromanipulation equipment and skills (Lin & Van Eenennaam, 2021).

Electroporation as a Tool for Gene Editing in Livestock

Gene editing via electroporation has found further application in livestock (Namula et al., 2022). Electroporation-mediated genome editing was hypothesized to have promising results in livestock

species after successful trials in mice (Wang et al., 2022). Specifically, gene knockouts using the non-homologous end joining (NHEJ) pathway have been particularly successful, with several experiments documenting high rates of the bi-allelic mutation using electroporation. Limited studies have been published demonstrating the technology in livestock species but the technology is rapidly advancing (Mahdi, Medrano, & Ross, 2022; Namula et al., 2022; Tanihara et al., 2016). Additionally, it was observed that there are already targets in the sheep and goat genomes that have been edited using microinjection of CRISPR Cas9 genome-editing reagents (Wang et al., 2022).

Viral Resistance through the use of Specific Gene Knockouts

In 2016, Whitworth et al. produced the first PRRS (porcine reproductive and respiratory syndrome virus) resistant piglets through a successful knockout of the *cd163* gene using CRISPR/Cas9. The *cd163* gene is an antibody binding protein responsible for viral cell entry. These resistant knockout piglets showed full resistance to the virus; showing no signs of infection for over 35 days post exposure. *cd163* knockout piglets appeared to be grossly phenotypically normal (Whitworth et al., 2016).

In 2019, Whitworth et al. successfully used CRISPR/Cas9 to create ANPEP (Amino Peptidate N Gene) knockout pigs to investigate the role of the ANPEP in susceptibility to TGEV (transmissible gastroenteritis virus) and PEDV (porcine epidemic diarrhea virus). ANPEP is a membrane bound enzyme shown to be involved in the entry of viruses into the cell. The resulting knockout piglets were resistant to TGEV but still susceptible to PEDV. The researchers hypothesized that PEDV must have a different or redundant avenue of viral entry. In this study all knockout animals appeared phenotypically healthy, though no in depth phenotypic study into their

potential immune cell morphology and expression differences was conducted (Whitworth et al., 2019).

Xu et al (2020) attempted to create PRRSV (porcine reproductive and respiratory syndrome virus), TGEV (transmissible gastroenteritis virus), and PDCoV (Porcine deltacoronavirus) through the knockout of the *cd163* and *papn* genes (both hypothesized viral entry genes). The researchers used CRISPR/Cas9 to precisely knock out each gene. The researchers were successful in creating double knockout piglets which were then shown to be resistant to PRRSV and TGEV with a reduced susceptibility to PDCoV. These piglets did not show any clear phenotypic adverse effects of the double knockout (Xu et al., 2020).

Koslová et al. (2021) created ALV (avian leukosis virus)-resistant chickens through a CRISPR/Cas9 mediated knockout of the TVA gene. The TVA gene is a cell surface binding protein responsible for entry by the ALV. Resulting homozygous knockout chickens were fully resistant to ALV A and K both *in vitro* and *in vivo*. Heterozygous knock-out animals were still susceptible to the virus. Homozygous knockout chickens did present a disorder in their B12 metabolism, believed to be caused by the function of the Tva gene as a cobalamin receptor (Koslová et al., 2021).

Qi et al (2022) created *pcbp1* gene knockout pigs using CRISPR/Cas9 for the goal of creating CSFV (Classical Swine Fever Virus) resistance pigs. *pcbp1* is an RNA and DNA binding protein known to play a role in virus replication and resistance. The study found that knockout cells showed a reduced CSFV infection rate due to upregulation of virus interfering genes in knockout cells. Compared to wildtype littermates, the knockout piglets did not show any distinct phenotype differences (Qi et al., 2022).

CD209 Knockout for Resistance in Cattle

The knockout of the *CD209* gene in cattle to increase resistance to transmission of the BLV has been proposed (Deykin et al., 2020). Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), also known as *CD209*, is a protein present on the surface of cells and functions as a receptor for BLV entrance into host cells (Klimov, Shevtsova, & Kovalchuk, 2018). DC-SIGN also functions as a pivotal pattern recognition receptor in dendritic cells, orchestrating antigen capture and presentation, and modulating T-cell responses to maintain immune homeostasis. Furthermore, it plays a critical role in establishing immune tolerance and regulating dendritic cell survival and trafficking, essential for effective immune surveillance (Rahimi, 2021). It has been hypothesized that removing the *CD209* membrane proteins through a targeted gene knockout could create resistance to the BLV. *CD209* facilitates the binding and internalization of pathogens, including viruses like BLV, prompting antigen response, and initiating an immune response. In mice, there are multiple homologs of *CD209* (Kalantari et al., 2018). A study investigating a *CD209* (*CD209a*) homolog knockout mice have shown that these mice exhibit ordinary susceptibility to bacterial infection (Tanne et al., 2009). They show diminished T and B cell proliferation and extramedullary hematopoiesis in the spleen, indicating an essential role for *CD209*a in immune regulation and hematopoiesis.

Sheep as a model for BLV

Sheep are a valuable model organisms in the study of BLV due to their ability to become easily infected with BLV but show research advantageous infection dynamics, disease

progression, and immunological responses (Gatei et al., 1989; Mammerickx et al., 1987; Porta et al., 2019). One key advantage of using sheep as a BLV model is their susceptibility to the virus. When experimentally infected with BLV, sheep develop persistent infections that closely mimic the course of infection seen in cattle but in a much more rapid and intense fashion. Sheep routinely exhibit signs of the disease (antibody presence, lymphocyte proliferation, etc) in as early as a few weeks where cattle sometimes take years to develop these symptoms (Porta et al., 2019). Sheep also have a shorter gestation period, easier handling, and less cost, making them an efficient model organism in research studies. Mammerickx et al. (1987) experimentally infected cattle, sheep, and goats with BLV through injection of BLV+ blood. They found that the required infectious dose of BLV was lower in sheep and goats than in cattle. In addition, the incubation period for BLV infection was also longer in cattle than that observed in sheep or goats. Sheep experimentally infected with BLV were more susceptible than cattle to the oncogenic effects of viral infection A recent study by Porta et al. (2019) confirmed that sheep continue to be experimentally advantageous for BLV research. Porta et al. (2019) investigated in depth the exact requirements of BLV infection in sheep. Cell-Free BLV was extracted from a Fetal Lamb Kidney (FLK) cell line and experimentally injected into sheep. In addition, FLK cells infected with BLV were directly injected into the sheep. Porta et al. (2019) found the minimum dose of BLV-FLK cells required to infect sheep was 500 cells. The cell-free FLK-BLV supernatant was infective down to a 1:1000 dilution.

Immune Response in Sheep

Current studies indicate that sheep are better BLV models than cattle because they have a more rapid and intense immune response; showing antibody production within a matter of days and clinical symptoms within weeks (Djilali, Parodi, & Levy, 1987; Gatei et al., 1989;

Mammerickx et al., 1987; Porta et al., 2019). Sheep infected with BLV exhibit higher proviral loads, viral replication, and more rapid disease onset than cattle. However, innate and adaptive immunological components may play a role in the processes driving this strengthened immune response in sheep (Gatei et al., 1989). Sheep generate larger quantities of BLV-neutralizing antibodies, which aid in diagnosing the disease. (Gatei et al., 1989). Upon infection sheep produce a larger number of lymphocyte cells when compared to cattle; this both aids in detection of the disease and proliferation of the virus.

Sheep are useful for BLV studies because they have a shorter gestational period as compared to cattle. One of the major barriers in livestock gene editing is the long generation time interval (Wang et al., 2022). When discussing the potential of gene editing in livestock for disease resistance, specifically in sheep, a reduced generation interval has favorable implications for research time and discovery.

Sheep are also prolific breeders, often conceiving 2-3 young during a single gestation. The creation of larger cohorts of BLV-infected sheep made possible by this high reproductive rate offers an efficient aid in animal research numbers. Additionally, these offspring offer a chance to look at the vertical transmission of BLV and analyze how maternal antibodies affect the offspring's vulnerability to illness (Ruiz et al., 2018). Compared to cattle, sheep are smaller, more docile, and manageable, making experimental techniques like blood sampling, immunization, and other treatments easier. Their more compact size and cooperative behavior make frequent monitoring of disease development, immunological responses, and therapeutic results more feasible.

Overall sheep have many benefits over cattle as a model organism in the study of BLV infection. Their increased immune response allows for rapid detection and monitoring of the disease (Mammerickx et al., 1987). Their gestation period allows for shorter generation periods in

producing F1 animals. The common birth of multiple offspring allows for more samples in F1 generations and allows for the potential for replicates in vertical transmission studies. Sheep size and management ease allows for efficient and inexpensive disease monitoring, blood collection, and storage.

Presented Research

Enhancing livestock genetics is paramount for optimizing animal production and welfare. Traditional breeding methods often include limitations such as lengthy timelines and, in some cases as in disease resistance, face genetic impasses, such as the ability to naturally breed in genetic resistance when no resistance is currently found in the species. To circumvent these constraints, gene-editing technologies have emerged as a potential alternative, enabling the immediate and precise integration of desirable traits into animal genomes (Hallerman et al., 2022). In this context, specific genetic knockouts are a powerful strategy for creating viral resistance in livestock. Viral resistance can be achieved through gene editing precise knockouts of specific viral entry ports (Koslová et al., 2021; Qi et al., 2022; Whitworth et al., 2016; Whitworth et al., 2019; Xu et al., 2020).

While genome editing in mammalian livestock traditionally leverages somatic cell nuclear transfer or direct zygote microinjection, these methods encounter challenges such as suboptimal efficiency and the incidence of undesired birth effects (Liu et al., 2013; Namula et al., 2022). Moreover, microinjection demands substantial technical expertise and time constrained workflows. Electroporation offers a high-throughput method of generating gene-edited animals via the treatment of large numbers of early-stage embryos. Operating through controlled electrical pulses, electroporation disrupts the lipid bilayer of the plasma membrane, allowing the entry of editing reagents (Tanihara et al., 2016).

Gene editing has been shown to be a successful strategy in the creation of viral resistant livestock (Whitworth et al., 2016). The potential of creating a *CD209* knockout bovine for resistant to BLV has been hypothesized (Deykin et al., 2020). Sheep have been proven as a preferred and successful model organism to cattle for BLV studies due to their rapid onset of the disease, shorter gestation periods, and ease of handling sheep are the preferred model organism (Porta et al., 2019). Demonstrated here is the use of electroporation to create gene edited *CD209* knockout sheep hypothesized to be resistant to BLV as a model for the disease in bovids.

Chapter 2: Creation of Genome Edited *CD209* Knockout Sheep

Introduction

Animal health and disease resistance has been a common breeding goal in domestic livestock. With the development of modern genetic technologies there becomes potential for gene editing to create these desirable traits. There have been many successful gene edited knock-out virus resistant animals (Koslová et al., 2021; Qi et al., 2022; Whitworth et al., 2016; Whitworth et al., 2019; Xu et al., 2020). BLV is an oncogenic retrovirus causing bovine leukemia (Kettmann et al., 1976). This is an extremely prevalent viral disease with no current vaccine or cure and as such, stands as a strong potential target for creating genetic viral resistance (Marawan et al., 2021).

BLV was first identified in 1871 (Kettmann et al., 1976). BLV has since been recognized as a global cattle disease agent, impacting both the health and productivity of livestock (Rhodes, Pelzer, & Johnson, 2003). The virus primarily targets B lymphocytes, leading to lymphocytosis and lymphosarcoma. Interestingly, BLV affects different species variably. While cattle show a delayed immune response, sheep and goats exhibit a more rapid and intense reaction (Djilali, Parodi, & Levy, 1987).

Transmission of BLV occurs primarily through the transfer of infected lymphocytes and is prevalent in several countries, posing significant economic and health challenges (Alfonso, Almansa, & Barrera, 1998; Marin et al., 1978; Ruiz et al., 2018). It has been hypothesized that the *CD209* antibody protein is the viral entry point on the cell membrane (Deykin et al., 2020).

This study hypotheses that creating a *CD209* gene knockout in sheep will create resistance to BLV and function as a model for bovine resilience to the disease. By targeting this gene, we aimed to remove the virus's ability to enter cells, thereby resulting in viral resistance. This approach aligns with previous successful applications of gene editing in livestock, where targeted gene knockouts have shown promise in conferring resistance to various diseases (Whitworth et al., 2016; Xu et al., 2020).

The choice of sheep as a model organism is strategic for their benefits to the study. Their susceptibility to BLV, rapid disease progression, and analogous immunological responses to cattle make them ideal candidates for this study (Gatei et al., 1989; Mammerickx et al., 1987). Additionally, sheep offer practical advantages, such as shorter gestational periods and ease of handling, which facilitate the research process.

Our editing method is electroporation-mediated CRISPR-Cas9 genome editing. Electroporation-mediated gene editing offers a high throughput, efficiency, and low cost alternative to traditional micro-injection (Lin & Van Eenennaam, 2021). A single guide approach, where one RNA-Guide is inserted with the CRISPR/Cas9 protein into the embryo, is selected here with the intent to create an INDEL (insertion or deletion) directed knockout at the target site in the *CD209* gene.

Materials and Methods

Animals

All experiments involving adult sheep and their lambs were approved and performed in accordance with the University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol #23019). All ovaries and oocytes were collected from Superior Farms in Dixon Ca.

Guide Design

Single gRNAs (sgRNAs) were designed targeting the sheep *CD209* gene using Chop-Chop guide design software (Montague et al., 2014) for a targeted single guide directed knockout of the *CD209* gene (Table 1). The top 3 guides were chosen based on the Chop-Chop off-target rating and tested *in vitro* through electroporation and subsequent genetic analysis through PCR and Sanger Sequencing. These guides are titled A-C and target the Exon 5' splice site or internal to Exon 5. DNA was extracted from a control animal through a QIAGEN DNeasy tissue collection kit from blood (QIAGEN, Hilden, Germany). PCR was performed with the DNA as follows, 10 µL of Promega (Promega, WI, USA) GoTaq Green Master Mix (2x), 1 µL forward primer (5µM), 1 µL reverse primer (5µM) (Table 2), 100ng of genomic DNA, and molecular grade nuclease-free water to a final volume of 20 µL. Cas9 and guide were thawed at room temperature (RT) and 5 µl of Cas9 at a concentration of 500ng/ µl was incubated for 10 minutes at RT with 5µl of guide, also at a concentration of 500 ng/µl to form a ribonucleoprotein (RNP). The PCR product was diluted to 100 ng/µl and 5 µl of PCR product was mixed with 1 µl of the Cas9/Guide RNP solution in a PCR tube. The reaction tube was incubated for 4 hours at 37°C in

a thermocycler. The subsequent product was then run on a 2% agarose gel for 30 minutes at 100V and visualized for the presence of two distinct bands.

Guide Name	*Sequence 5'->3'*
A	GAACGAAGCACTCCCGTACCTGG
B	AGATCCTACAGCGCCTGACCCGG
C	GCCCAACAACCACGGAGATGAGG

Table 1: Guide RNA names and coordinating sequences.

Primer Name	*Sequence 5'->3'*
O*CD209*FA1	GCTTTCAAGGTCTCCCCACG
O*CD209*RA1	GCTGGGAAGAGGAACAGAAAGG
O*CD209*FA2	GCTTTCAAGGTCTCCCCACG
O*CD209*RA2	GCTGGGAAGAGGAACAGAAAGG
O*CD209*2k_2F	GCCGATCCAGGTGGGTTTAT
O*CD209*2k_2R	CTGGGTGAAGACTGGGAGAC
T3	GCGCGAAATTAACCCTCACTAAAG
M13F	GTAAAACGACGGCCAG

Table 2: DNA Primer names and coordinating sequences.

Production of Ovine Embryos

Ovine ovaries were collected from a local abattoir and transported in 38.5°C sterile saline to the lab. In the lab, cumulus-oocyte complexes (COCs) were aspirated using a WTA vacuum pump (WatanabeTecnologia Aplicada, College Station, Texas, USA) connected to a sterile butterfly needle. COCs were washed and placed into 50 µl of Small Ruminant *In vitro* Culture Media (SR-IVC) (Stroebechmedia, Hovedstaden Denmark). COCs were incubated in SR-IVC for 18 hours at 38.5°C in a 5% CO2 humidified incubator. Ovine sperm was collected through electro-ejaculation and cryopreserved using standard protocols (Álvarez et al., 2012). One straw of semen (0.5 mL) was prepared through a 5-minute incubation in a 38.5C water incubator, after which it was transferred to a 1.5 mL tube and centrifuged at 200*x*g for 5 minutes. One hundred microliters of the pellet were removed and resuspended with 100 µl of Small Ruminant *In vitro* Fertilization Media (SR-IVF) (Stroebechmedia, Hovedstaden Denmark) and centrifuged again at 200g for 5 minutes. One hundred microliters of the pellet was again collected and resuspended with 100µl of SR-IVF. Groups of 25 COCs were then placed into 50µl drops of SR-IVF and incubated with 2×10^6 sperm per mL and incubated at 38.5C in a 5% CO2 humidified incubator for 6 hours.

Electroporation of Ovine Embryos

Post-fertilization experimental zygotes were vortexed for 3 minutes to remove cumulus cells. Per 15 zygotes, 5µl of CRISPR-Cas9 at a concentration of 500 ng/µl was incubated at RT

with 5µl of guide RNA A at a concentration of 500ng/µl at RT in a 1.5 ml tube. Up to 500 embryos were then washed through five 50µl drops of SOF-Hepes medium (Stroebechmedia, Hovedstaden Denmark). Embryos were then washed through 3 drops of 35µl of Optimum Medium (Stroebechmedia, Hovedstaden Denmark). A 10µl drop of the Cas-9 and Guide solution was created and 15 zygotes placed in this solution and mixed by pipetting.

An aliquot of 20µl of the zygote, Cas-9, and Guide RNA mix were then placed in an electroporation cuvette. Electroporation was performed at a poring pulse of 300V, 3.5 msec pulse length, 50.0 msec pulse interval, 2 pulses, 0% decay rate, +/- polarity, and Transfer Pulse of 20.0V, 50.0 msec pulse length, 50.0 msec pulse interval, 0 pulses, 40% decay rate, and +/- polarity. Zygotes were then washed from the cuvette using 5 repeat washings of 50µl SR-IVC media. Zygotes were collected from washing media and placed in groups of 25 embryos into 50µl SR-IVC media under ovoil (Stroebechmedia, Hovedstaden Denmark). Zygotes were incubated at 38.5C in a WTA EVE embryo incubator (WatanabeTecnologia Aplicada, College Station Texas) for 7 days and then either collected for genetic analysis post-blastocyst or transferred to synced ovine recipients.

Zygotes that developed to the blastocyst stage were collected for genetic analysis using polymerase chain reaction (PCR) and subsequent Sanger Sequencing through GeneWiz (Azenta, South Plainfield, New Jersey). DNA primers were designed targeting 350 bp upstream and downstream of the target site. Individual blastocyst DNA was extracted through incubation with 10µl of QuickExtract™ DNA extraction solution (Lucigen, Toronto, CA) at 37C for 10 minutes. PCR was performed on the extracted blastocyst DNA in two rounds. The first round of PCR reagents as follows, 3µl H2O, 10µl Promega Go-Taq Green (Promega, WI, USA), 1µl Primer O*CD209*FA1 at a concentration of 5 µM, 1µl Primer O*CD209*RA1 at a concentration of 5 µM,

and 5µl extracted blastocyst DNA. These reagents were mixed in a PCR tube and vortexed before PCR, 3 minutes at 95C, and for 40 rounds at 30 second minutes 95 degrees C, 3 minutes 55 degrees C, 1.5 minutes at 72C, and a final 72C incubation for 10 minutes (Table 3). The second round of PCR reagents was 8µl H2O, 10µl Go-Taq, 1µl Primer OCD209FA2 at a concentration of 5 µM, 1µl Primer OCD209RA2 at a concentration of 5 µM, and 5µl extracted blastocyst DNA. The second round of PCR was run for PCR, 3 minutes at 95C, and for 35 rounds at 30 second minutes 95 degrees C, 3 minutes 55 degrees C, 1.5 minute(s) at 72C, and a final 72C incubation for 10 minutes (Table 3). PCR products were visualized on a 1% agarose gel (0.5g of agarose dissolved in 50ml of TE-Buffer, microwaved at 70 seconds, and 3µl Sybr Safe™ (.6X) added post-microwave). Five µl of Round 2 PCR product was added and run at 100V for 30 minutes. Bands were then excised using a scalpel and DNA extracted through a QIAGEN commercial kit (QIAGEN, Hilden, Germany). Gel extracted DNA was diluted to 20ng/µl and 5µl of DNA, 5µl of H2O, and 5µl of the forward primer (5 µM) were submitted for sanger sequencing through GeneWiz (Azenta, South Plainfield, New Jersey). Results were analyzed on SnapGene Software (version 7.0) (Figure 1).

Embryo Transfer

Embryos were graded using standard the International Embryo Technology Society standards (Barfield & Demetrio, 2023) and those determined to be of the highest quality through visual inspection (grade 1) were transferred laparoscopically to synchronized recipient ewes. All drugs were sourced from Merck Animal Health, Rathway, New Jersey, USA unless otherwise specified. Recipient ewes were synchronized through a protocol consisting of CIDR insertion (progesterone 0.3g. (Zoetis, New Jersey, USA)) on day 0, injection of 0.5mL (125mg) Estrumate (Cloprostenol, Merck, New Jersey, USA) intramuscularly on day 9, removal of CIDRs and

subsequent injection of 5ml PG-600 intramuscular on day 10, and finally an injection of .5mL Factrel (Gonadotropin releasing hormone, Zoetis, New Jersey, USA) on day 12 intramuscularly. Heat was detected using a live ram wearing a no-mate harness (Rurtec, Hamilton, NZ) starting on day 10 and ending on day 12. 20 ewes were estrus synced with 13 showing estrus between day 10 and 12. On day 18 embryos were transferred to the 13 estrus showing recipients. Presence of corpora lutea on the ovaries of the recipients was confirmed prior to transfer of the embryos. Ewes received either 3 or 4 embryos with two control ewes receiving 3 control embryos each. Blastocysts that were not transferred were submitted for Sanger sequencing.

Pregnancy Diagnosis

On day 34 of gestation pregnancy and fetus counts were determined through transabdominal ultrasound. Pregnancy was confirmed at day 72 of gestation.

DNA Extraction for Genotyping

Hair was collected from lambs at birth from between the distal limb. DNA was then extracted using the QIAGEN DNeasy Blood and Tissue® kit (QIAGEN, Hilden, Germany). DNA extraction from blood was not successful as the lambs had insufficient buffy coats, indicating leukopenia.

Short Read PCR and Sanger Sequencing

PCR reactions were prepared with 10 µL of Promega (Promega, WI, USA) GoTaq Green Master Mix (2x), 1 µL forward primer (5µM), 1 µL reverse primer (5µM), 100ng of genomic DNA, and molecular grade nuclease-free water to a final volume of 20 µL. PCR products were run on 2% agarose gels prepared with 60 mL of 1% tris base acetic acid and EDTA (1% TBE), and 2µl SYBR™ Safe gel stain. Visualization of bands was performed under blue and UV light. DNA fragments were cut out of gels individually using a scalpel

and purified PCR products were gel-extracted using a standard gel extraction kit. Gel-purified PCR products were outsourced to GeneWiz (Azenta, South Plainfield, New Jersey) for purified PCR product Sanger sequencing. Visualization of Sanger sequencing results was performed in SnapGene alignment analysis. SnapGene aligns the sequence to the reference genome and reports any gaps (deletions or insertions) or mismatches (nucleotide base differences).

	CD209 short	*CD209* long	TOPO TA Plasmid	*Embryo* Round 1	*Embryo* Round 2
Forward Primer	O*CD209*FA1	O*CD209*2k_2F	T3	O*CD209*FA1	O*CD209*FA2
Reverse Primer	O*CD209*RA1	O*CD209*2k_2R	M13F	O*CD209*RA1	O*CD209*RA2
DNA Amount	100ng	100ng	100ng	5µl Blast DNA	5µl PCR Product
Wild Type Amplicon Size	718	3826	784	718	718
Initial Denaturation Time	3'	3'	3'	3'	3'
Denaturation Temp (°C)	95	95	95	95	95
Denaturation Time	30"	30”	30"	30"	30"
Annealing Time	30"	30”	30"	30"	30"
Annealing Temperature (°C)	55	55	55	55	55
Extension Time	1'30"	3'30”	1'30"	1'30"	1'30"
Extension Temperature (°C)	72	72	72	72	72
Final Extension	10'	10’	10'	10'	10'

Time					
Volume (µL)	20	20	20	20	20
Cycle #	35	40	35	40	35

Table 3: Primer sets and conditions for PCR amplification.

Long Read PCR and Sanger Sequencing

To determine the potential presence of a large knockout near the guide region a longer PCR product was designed. This PCR aimed to amplify 3826 of the region (~1.9kb upstream and down) of the guide suite. PCR reactions were prepared with 10 µL of Promega GoTaq Green Master Mix (2x) (Promega, WI, USA), 1 µLforward primer (5µM), 1 µL reverse primer (5µM), 100ng of genomic DNA, and molecular grade nuclease-free water to a final volume of 20 µL. PCR products were run on 2% agarose gels prepared with 60 mL of 1% tris base acetic acid and EDTA (1% TBE), and 2µl SYBR™ Safe gel stain. Visualization of bands were performed under blue and UV light.

TIDE Analysis

TIDE, *tracking of indels by decomposition*, is a software program with the purpose of identifying the presence and rate of indels (Brinkman et al., 2014). The algorithm accurately reconstructs the spectrum of indels from Sanger sequence traces. Sanger Sequencing results from the edited lambs was analyzed for INDEL rates using TIDE software (Figure 5).

TOPO-TA Mosaicism Analysis

Mosaicism is common in animals produced through gene editing due to variable editing in the embryo. To analyze the details of the mosaicism in the edited lambs TOPO-TA cloning

was performed. TOPO-TA cloning is a process where a DNA fragment (PCR product surrounding the guide site) is inserted into an E.Coli vector and grown to create single genotype colonies capable of submitting for Sanger Sequencing and mutation analysis (Ata et al., 2018).

To create the donor DNA fragment a PCR was performed on extracted DNA from the edited lambs. The PCR was performed using a reaction of 10 µL of Promega GoTaq Green Master Mix (2x) (Promega, WI, USA), 1 µL forward primer O*CD209*FA1 (5µM), 1 µL reverse primer O*CD209*RA1 (5µM), 100ng of genomic DNA, and molecular grade nuclease-free water to a final volume of 20 µL. PCR products were loaded (5µl) on a prepared with 60 mL of 1% tris base acetic acid and EDTA (1% TBE), and 2µl SYBR™ Safe gel stain. The resulting gel band was extracted using a standard gel extraction kit from QIAGEN (QIAGEN, Hilden, Germany). Topo TA cloning was performed using a standard Invitrogen kit (Thermo Fisher, MA, USA). Once cloning success was confirmed by the presence of colonies on agar plates colonies were grown individually in 15ml sterile containers with 5ml LB (Luria-Bertani) broth containing ampicillin at a concentration of 100 micrograms per milliliter. A 20µl pipette tip was used to collect a single colony and place in the LB broth. Broth was grown and shaken overnight at 37C. Minipreps were performed on this TOPO-TA broth following standard protocols (Thermo Fisher, MA, USA). Mini-prep DNA was then subsequently submitted for Sanger Sequencing analysis through GeneWiz (Azenta, South Plainfield, New Jersey) for analysis.

ECOR1 Restriction Digest

The TOPO-TA cloning plasmid vector contains ECOR1 restriction enzyme digestion sites flanking the insertion site. A restriction enzyme digest of the TOPO-TA miniprep DNA was conducted to investigate the insertion success. Restriction enzyme digest was conducted through a incubation at 37C for 20 minutes of a reagent mix consisting of 1µg of DNA, 5µl

10X NEBuffer (New England Biolabs, MS), 1µl EcoR1 Enzyme, Water to 20µl. Product was mixed with 3µl 6x purple loading dye (New England Biolabs, MS) and run on a 1.5% agarose gel for 30 minutes at 100V.

Results

In vitro Guide Results

All three guides (A, B, and C) cut the target region successfully *in vitro*. Guide A was chosen for subsequent *in vivo* testing, analysis, and blastocyst creation.

Blastocyst Results

All Blastocysts not transferred were submitted for Sanger sequencing (n=46). Of these, 36 of the blastocysts showed a clear INDEL at the guide site (Figure 1). Ten blastocysts appeared to be wildtype based on Sanger Sequencing results viewed on SnapGene alignment analysis. Blastocysts identified with no gaps or mismatches are assumed to be wildtype. Editing efficiency was therefore calculated at 36/46 or a 78.2% efficiency (Figure 2).

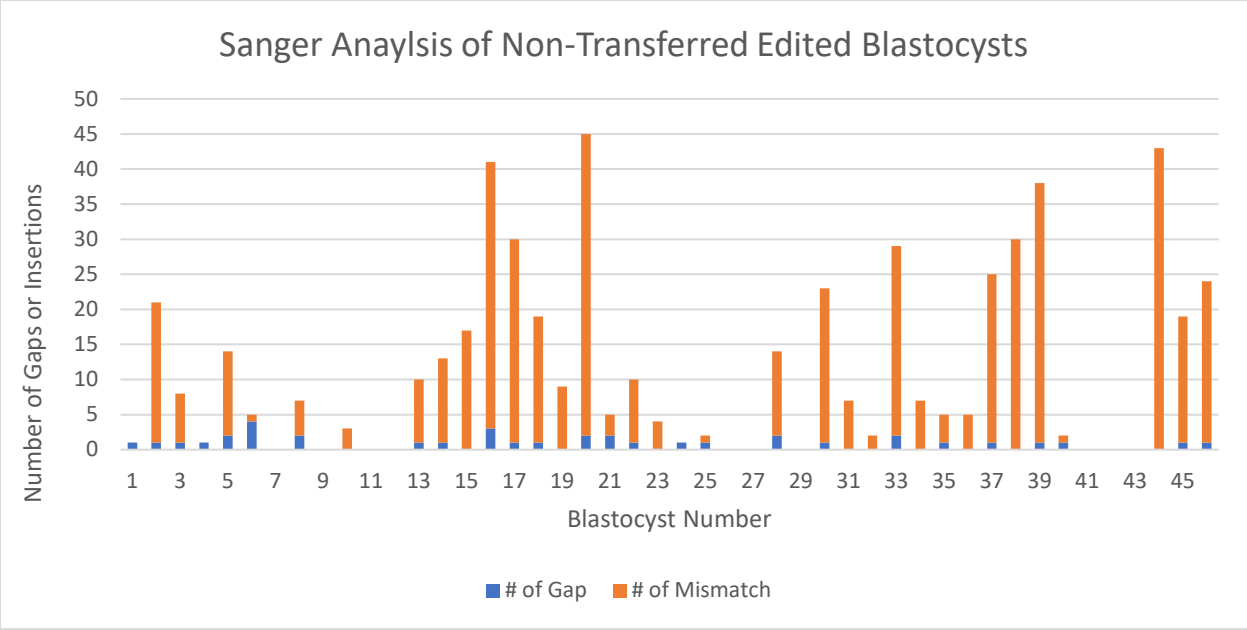

Figure 1: Blastocysts identified with their number of gaps (insertions or deletions) and/or base pair mismatches when aligned to an un-edited reference genome.

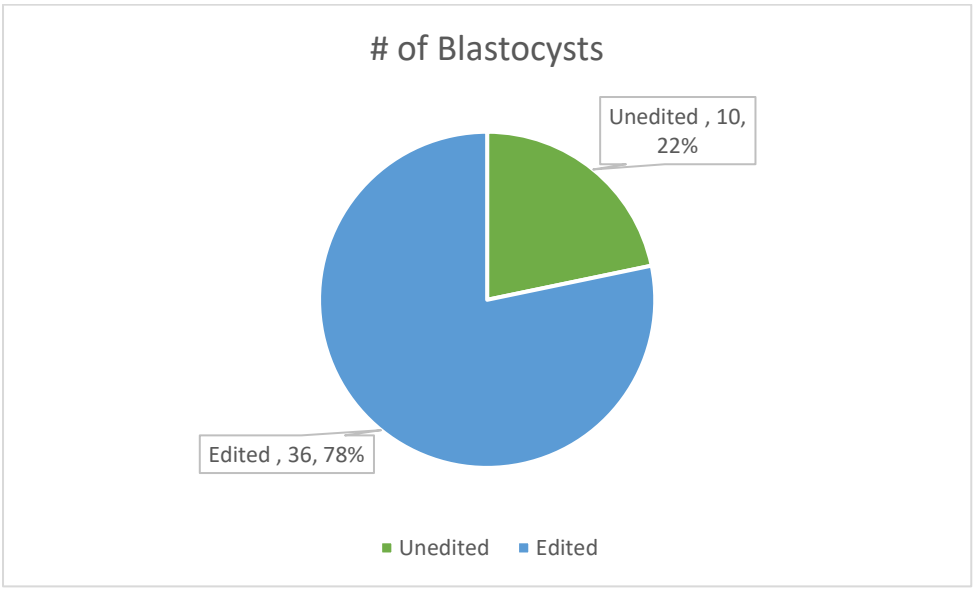

Figure 2. Pie chart demonstrating the percentage of blastocysts not transferred that contained edits compared to unedited blastocysts.

Pregnancy Results

Pregnancy rate was determined through transabdominal ultrasound at day 34 and day 72 of pregnancy. On day 34 of pregnancy, it was determined that 6 ewes were pregnant. All ewes pregnant at day 34 were carrying electroporated embryos. No control pregnancies remained. On day 72 pregnancy was confirmed in 5 ewes; ewe #7238 appeared by ultrasound to no longer be pregnant.

Lamb Birth

Seven lambs were born from the 5 recipients carrying pregnancies. Six lambs were born alive. The subsequent birth circumstance, condition, and weight are demonstrated below in Table 4. All lambs were terminated, either dying or being euthanized, by 1 month of age. The associated cause of death, dates, and ages are demonstrated below in Table 5.

Recipient	Lamb ID	Lamb ID	SEX	DOB	Birth	Birth Condition	Birth Weight
7013	N/A	1	F	8/9/2023	Delivered unassisted	Stillborn	1.8kg
8048	7171	2	F	8/10/2023	Unassisted	Live	7 kg
8048	7170	3	M	8/10/2023	Unassisted	Live	not recorded
CA037-9138	7186	4	M	8/11/2023	C-Section	Live, severely contracted forelimbs	7.5kg
CA037-9138	7187	5	M	8/11/2023	C-Section	Live	6.4kg
CA037-9138	N/A		F	8/11/2023	C-Section	Mummified fetus	N/A
1717	no ID	6	M	8/12/2023	Dystocia (one leg back)	Live, mild contracture of forelimbs	7.7kg
20171	7188	7	F	8/14/2023	C-Section	Live, contracted forelimbs	8.4kg

Table 4: Representing lambs born and their recipient along with birth data.

Recipient	Lamb ID	Lamb ID	SEX	Outcome	Reason	Date of Death	Age of Death
7013	N/A	1	F	N/A	N/A	N/A	N/A
8048	7171	2	F	Died	Pneumonia & Colitis	8/25/2023	15 Days
8048	7170	3	M	Died	Pneumonia	8/12/2023	2 Days
CA037-9138	7186	4	M	Euthanized	Respiratory Failure	8/18/2023	7 Days
CA037-9138	7187	5	M	Euthanized	Typhlocolitis	8/19/2023	8 Days
CA037-9138	N/A		F	N/A	N/A	N/A	N/A
1717	no ID	6	M	Euthanized	Respiratory Failure	8/13/2023	1 Days
20171	7188	7	F	Euthanized	Broncho-pneumonia	9/5/2023	22 Days

Table 5: Representing lambs born and recipients and their outcome.

Short Read PCR and Sanger Sequencing

PCR was performed on extracted DNA from each lamb born (Table 4). All lambs showed an expected 718 base pair product (Figure 3). The gel product was then extracted and submitted for Sanger Sequencing. The resulting sanger product showed a large level of mosaicism with clear edits and INDELs (insertions and/or deletions) surrounding the guide cut site in 6/7 sheep (Figure 4).

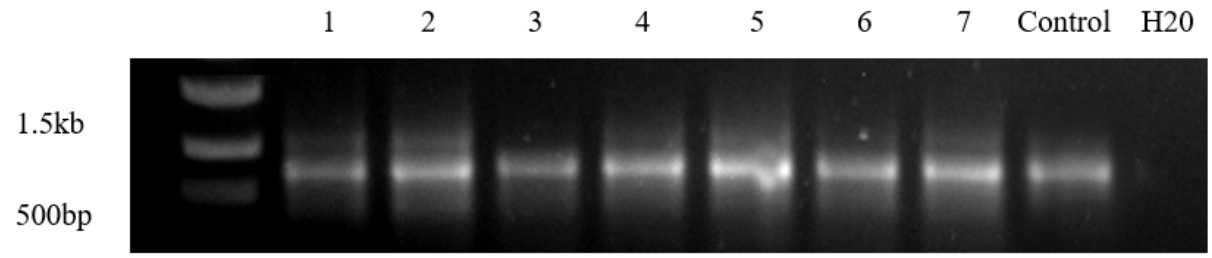

Figure 3: Gel product demonstrating all 7 sheep DNA band alignments to the control band of the expected size.

Figure 4: Snapgene visualization of the 7 edited lambs aligned to a reference genome demonstrating associated gaps and mismatches to the reference genome as well as Sanger Sequencing peaks of the amplified region.

Long Read PCR

A long PCR (3825bp) was performed on extracted DNA from each lamb born to analyze the potential presence of a large deletion. All lambs showed an expected 3825 base pair product with a single band.

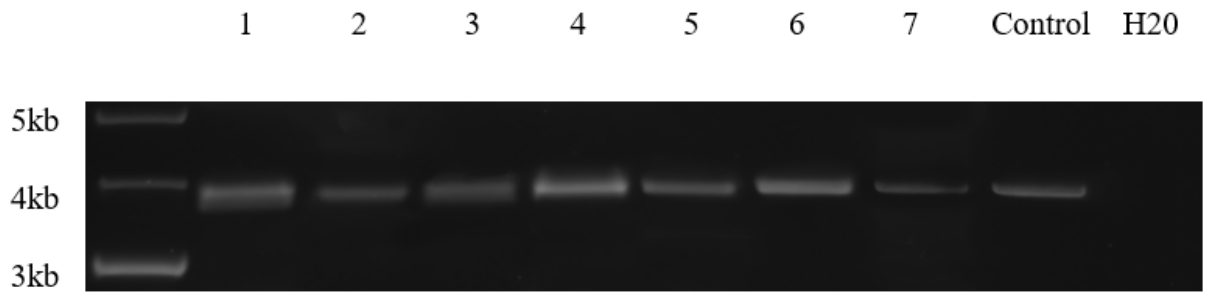

Figure 5: Edited sheep and control bands from the long PCR (3825bp) showing similar band length to control.

Blastocyst TIDE

Tide Analysis was performed on all edited blastocysts not transferred. Results are split into embryos tested pre-transfer day (verification) (Figure 7) as well as embryos produced for the embryo transfer but not transferred (Figure 6). Average editing efficiency determined by TIDE analysis pre-transfer was 61.28%. Average editing efficiency in blastocysts not transferred on the day of transfer (non-transferred) was 44.78%.

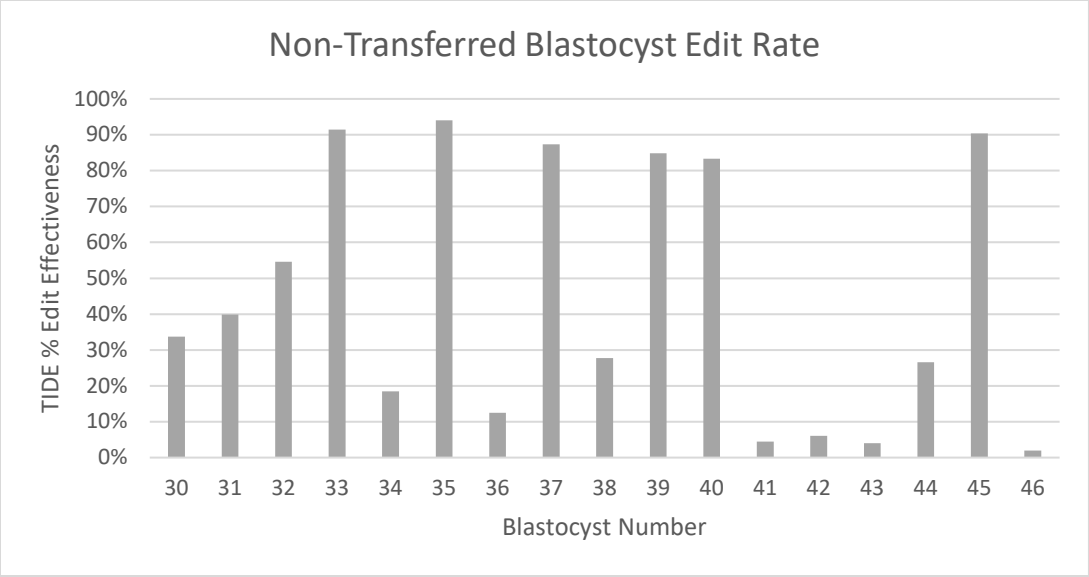

Figure 6: Bar chart showing the TIDE analysis estimated percent edited based off Sanger sequencing data of blastocysts created for transfer that were not transferred.

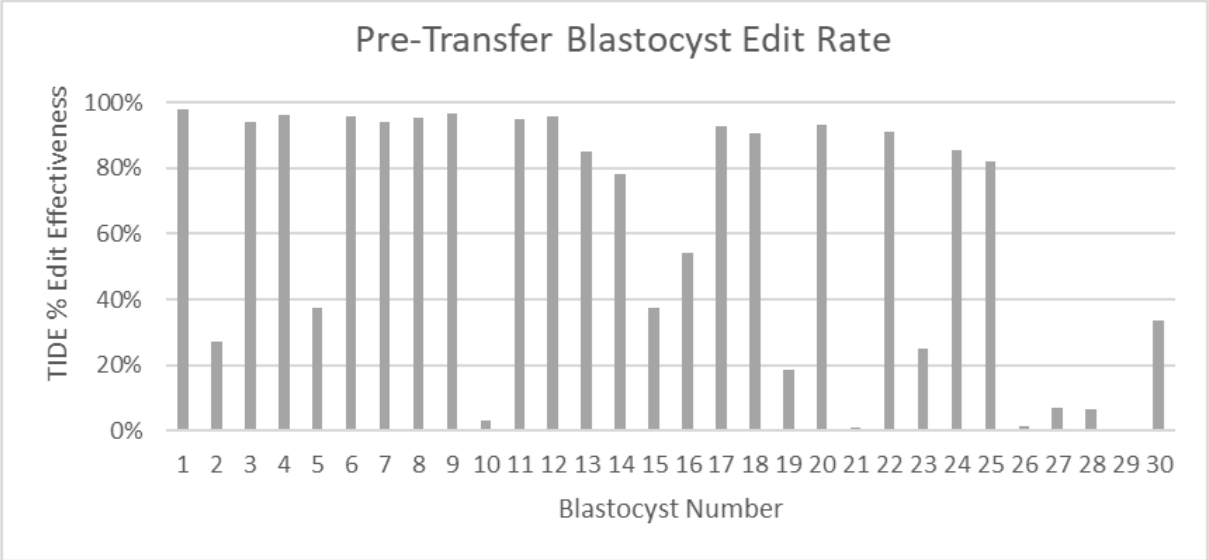

Figure 7: Bar chart demonstrating TIDE analysis estimated percent edited based off Sanger sequencing data of blastocysts created prior to transfer embryo creation date with guide A and embryo round 1 and 2 primers.

Lamb TIDE Analysis

TIDE INDEL Analysis software (Brinkman et al., 2014) found INDELS in 6 of the 7 edited lambs based on Sanger Sequencing (Figure 4). TIDE edit efficiency rate varied between 15.1% (lamb 1) and 62.0% (lamb 7) (Table 6). Average TIDE edit efficiency (mosaicism) was 34.19%.

Lamb Number	Percent Edited (TIDE Analysis)
Control	0.0%
1	15.1%
2	27.7%
3	52.5%
4	45.6%
5	22.6%
6	62.0%
7	48.0%

Table 6: Table demonstrating edited lamb DNA TIDE analysis and resulting predicted edit rate (mosaicism) based on Sanger Sequencing of the edit region.

TOPO-TA Cloning Analysis

TOPO-TA showed preliminary promise. After transformation many hundred colonies grew. Minipreps were performed on 11 colonies from each edited sheep transformation. Miniprep DNA concentration measured on a ThermoScientific Nanodrop showed a DNA

concentration in the range of 100ng/µl. Mini-prep plasmids submitted for Sanger Sequencing consistently showed no priming using internal ovis primers (O*CD209*FA1 & O*CD209*RA1) nor the kit provided T3 and M13F universal primer. One sequence did return primed and showed a random insertion (671bp) in the plasmid backbone along with a short sequence (21bp) of the sheep amplicon (Figure 8). The 671bp random insertion does not align to any reference genome when run through NCBI Basic Local Alignment Search Tool (BLAST).

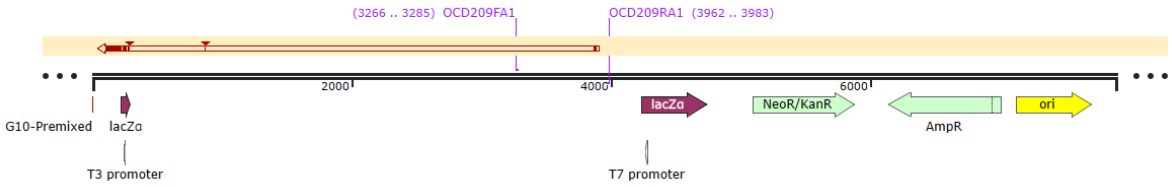

Figure 8: SnapGene alignment demonstrating the aligned sequence with a large unaligned insertion.

ECOR1 Restriction Enzyme Digestion Analysis

155 (154 edited and one control) TOPO-TA plasmids were restriction enzyme digested using ECOR1 enzyme for analysis of the insert region. Results were extremely variable with bands of varied length being present (Figure 9).

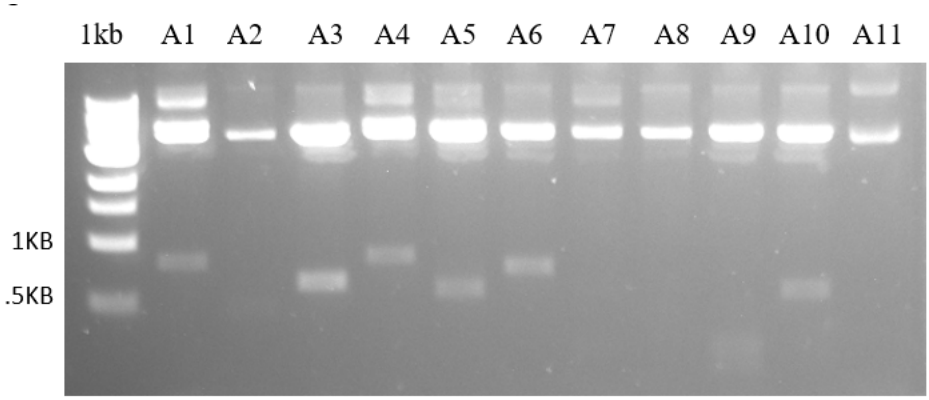

Figure 9: Gel image demonstrating the Topo TA bands.

Discussion

BLV is an endemic virus in the United States with a clear economic and health impact (Rhodes, Pelzer, & Johnson, 2003). Traditional eradication control methods have proven to be unsuccessful (Marawan et al., 2021). Gene editing has recently been hypothesized to be a potentially widespread successful eradication and management method (Deykin et al., 2020). BLV has been shown to enter through a single cellular entry port and it has been hypothesized that the gene knockout of this entry point will provide protection to edited organisms from infection of the virus. Gene editing has been proven to be an effective method for disease control in other livestock species and diseases (Whitworth et al., 2016). A major limitation of gene editing in livestock historically has been the reliant on somatic cell nuclear transfer or microinjection (Lin & Van Eenennaam, 2021). Somatic cell nuclear transfer is a very hard procedure requiring a large amount of training and resources while also being associated with many undesired procedural effects (large offspring syndrome, etc) (Liu et al., 2013). Microinjection is not usually associated with the same undesired effects of somatic cell nuclear

transfer but requires the use of expensive equipment and a trained individual (Namula et al., 2022). Microinjection is also an extremely slow process and limits the commercial application of the technology.

Electroporation has emerged as an efficient means of gene editing livestock (Tanihara et al., 2016). Electroporation is capable of editing hundreds of embryos in a short amount of time using a relatively inexpensive machine and a much lower level of training. Electroporation has also been shown to achieve similar if not higher edit rates in livestock when compared to microinjection. Sheep have been proven to be a desirable model organism for BLV research in the past (Djilali, Parodi, & Levy, 1987). Cattle show a varying response to infection and can take years to show detectable symptoms and response to viral infection. Sheep have a much shorter disease period when experimentally infected with BLV; showing infection within a few weeks of injection with blood containing viral infected cells. Sheep also have a shorter gestation period, allowing for a quicker experimental timeline. Combining the technologies of gene editing for viral resistance, electroporation, and sheep as a model allows for a relatively simple and low-cost technique of creating model livestock resistance to BLV. *In vitro* production of blastocysts showed a high edit efficiency rate based on Sanger Sequence analysis. Seven lambs were successfully created and birthed following the experimental electroporation. The edit state of these lambs is still under investigation. Important to note is that no control embryo implantations were successful and as such as all lamb births were hypothesized and expected to be edited. One lamb was stillborn and therefore a phenotype could not be truly characterized. The birth of lambs was not without issue; 4/6 live lambs required assistance with birth and all birthing ewes showed signs of dystocia. All six lambs did subsequently die or were humanely euthanized post-parturition. The lifespan of these lambs was varied with some dying almost immediately after

birth and others living for weeks, but all showed struggle with life. All lambs which lived past birth eventually developed respiratory issues and/or gastrointestinal disease which ultimately led to their death: either through euthanasia or natural death. The genetic analysis of these lambs faced intense struggle. The lambs had an extremely low white blood cell counts and therefore blood DNA extraction was difficult. DNA extraction was therefore performed on hair follicles due to the failure of the blood extraction. The Sanger results shows possible mosaicism and irregularities. This mosaicism is expected in edited organisms derived from electroporation (Mahdi, Medrano, & Ross, 2022). The TIDE analysis of the edited lambs showed a relatively low efficiency rate compared to the analyzed un-transferred blastocysts. Blastocysts produced day of transfer showed an average edit efficiency lower than previous embryos but still higher than the edited lambs presented. Contamination of DNA samples from the Dam or other outside sources cannot be ruled out as a potential source of background wild-type contamination. This background wild type contamination would in effect lower the edit efficiency seen in the TIDE analysis. All lambs showed severe phenotypes hypothesized to be the result of their edits and therefore the low edit efficiency seen in analysis is unexpected. The issues seen in the TOPO-TA cloning may be the results of potentially contaminated DNA as well as the random DNA insertion seen in the restriction enzyme analysis. This study demonstrated the potential success of creating knockout blastocysts through the electroporation of a single guide and Cas9. The creation of live edited organisms demonstrated much more struggle and shows some of the issues surrounding compounding points and the negative effects of the lack of control organisms in editing experiments. The subsequent phenotype of these lambs was not as expected and showed large detriment to health resulting in their early death.

In conclusion it has been demonstrated that creating *CD209* knockout blastocysts is possible through electroporation of a single guide RNA with CRISPR-Cas9, and subsequent lambs may be born. The exact genotypes of the lambs cannot be determined with data yet collected. The phenotypes of the lambs were incredibly unusual with all lambs dying less than a month after birth. The resulting birth weight of the lambs was also well above average and might relate to the resulting issues present post-parturition. It cannot be clearly stated whether the irregular and unhealthy phenotypes were due to their edits but is none the less drastic. The resulting phenotypes of the lambs might be from *in vitro* embryo production effects, edit effects, or alternative effects. Gene editing work is not without risk, demonstrated here is the birth of 7 unhealthy lambs. It is unknown the disease resistance level of these lambs due to their untimely and unhealthy deaths. Future work will be completed to attempt to fully analyze the genotypes of the effected lambs and to determine the exact genetic or environmental causes of the phenotypes demonstrated here.

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