Development and Characterization of Ectromelia Virus-Moscow in the BALB/c Mouse Model for Smallpox Therapeutic and Prophylaxis Drug Efficacy Testing Under the FDA Animal Rule

THESIS

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Abstract

Variola virus (VARV) is the causative agent of smallpox and has been globally eradicated with no naturally occurring infections reported since 1977. Because Variola virus (VARV) has been eradicated from the environment, alternative animal models are used to evaluate potential treatments for smallpox. One model of smallpox utilizes intranasal (IN) inoculation of BALB/c mice with ECTV, causative agent of mousepox disease in mice, due to the genetic and disease presentation similarities between ECTV and VARV. The ECTV-mouse model reproduces many features similar to that of human smallpox including severe, acute systemic disease caused by a low virus infectious dose; lack of pulmonary involvement early in the disease progression; and a presentation of a characteristic pustular rash in mice that survive the acute phase of disease. Thus, the BALB/c mouse model was developed to characterize and study ECTV infection and pathogenesis. First, the propagation of ECTV resulted in a certified working stock of virus and the LD<sub>90</sub> and LD<sub>50</sub> were determined to be 32.10 PFU and < 2.44 PFU, respectively. The natural history study resulted in the characterization of the disease progression resulting from intranasal infection in BALB/c mice. The clinical parameters that provided the earliest indication of disease onset following challenge were defined, in addition to the clinical parameters that correlated with mortality and time-to-death. The final study aimed to evaluate the combination of various clinical signs of disease as
reliable markers to define the onset of ECTV disease within the context clinical disease progression in the BALB/c mouse model using a combination of clinical and laboratory parameters. In all, the ECTV BALB/c mouse program resulted in a well-characterized small animal model that is suitable for use under the FDA “Animal Rule” in order to test the efficacy of therapeutics for smallpox.
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Chapter 1: Introduction

1.1 Bioterrorism and Current Public Health Concerns

Terrorism, defined by the Federal Bureau of Investigation (FBI) as ‘‘the unlawful use of force and violence against persons or property to intimidate or coerce a government, the civilian population, or any segment thereof, in furtherance of political or social objectives’’, is a harsh reality of the world in which we live (Anderson & Bokor, 2012). At first thought, many envision terrorism as physical acts of violence, such as explosives or nuclear attacks, or possibly chemical releases, but these methods are only a small portion of what could potentially be utilized. The deliberate use of biological agents, such as viruses, bacteria, or other agents to cause illness or death in people, animals, or plants is a form of terrorism called bioterrorism, which can be far more detrimental and deadly than physical harm and destruction (Centers for Disease Control and Prevention, 2013). What makes biological agents stand out from other methods of terrorism is the fact that they are not easily detected, and do not typically cause disease until hours or even several days after their release. Also, unlike chemical or physical forms of attack, biological agents not only affect their immediate victims, they can also affect multiple other people that may not have been present at the original site of attack. In some cases, once an individual becomes infected with certain agents, he or she may transmit the bacteria or virus naturally to multiple new hosts. Many methods of agent dispersal can be utilized in a bioterrorist attack, including, but not limited to, the
contamination of food and water sources and agent aerosolization, depending on the ease of administration and the agent’s most harmful route of transmission (Abraham, Walls et al., 2012). Two classifications of infectious agents exist, replicating and non-replicating agents. Agents classified as replicating include live organisms or spores, where non-replicating agents are toxins, or poisonous substances produced by living organisms (Anderson & Bokor, 2012). Bioterrorism agents have been categorized into classes (A, B, and C) by the Centers for Disease Control and Prevention (CDC) based on their ability to spread, or disseminate, and the severity of the illness or death in which they cause (Centers for Disease Control and Prevention, 2013). Category A bioterrorism agents are the highest-priority agents, posing the greatest risk to public and national security due to the fact that they a) can be easily transmitted from person to person; b) can result in high death rates and have the potential for a major public health impact; c) might cause a state of public panic; and d) require special action for public health preparedness (Centers for Disease Control and Prevention, 2013). Category B and C agents are more readily available, not as easily spread, and generally have lower morbidity and mortality rates.

Bioterrorism is not a new concept, and has in fact been utilized for centuries (Anderson & Bokor, 2012; Chomel & Sun, 2010; Clarke & Rinderknecht, 2011; Wallin, Luksiene et al., 2007; Christian, 2013). The earliest reports of bioterrorism activities involve employing bees, wasps, ants, and other stinging insects as weapons in attacks against enemies (Christian, 2013). During the middle ages, corpses of animals and humans who died of various diseases were catapulted into enemy territories to weaken the forces before physical attack. In the nineteenth century time period, the blankets from
patients suffering from smallpox were purposely given to unknowing South and North American indigenous peoples during the French and Indian war. It is reported that Germany shipped livestock infected with glanders and anthrax to the Allied countries before World War I to disrupt the food supply. More recently, letters contaminated with anthrax spores circulated in the United States after the 2001 September 11th terrorist attacks, resulting in twenty-two cases of disease and five deaths (Christian, 2013). Many more instances of bioterrorism have occurred throughout world history, and many more are likely to happen. Since the terrorist attacks and subsequent anthrax letters in the early 2000s, The United States has been on heightened alert for potential bioterrorist attacks. Studies show that the US is inadequately prepared to face further biological attacks (Danzig, 2012). This can be partially attributed to the lack of government organization, but can also be due to lack of the appropriate vaccines, prophylaxis, therapeutics, and inefficient detection and diagnostic tools. Critical care physicians would be one of the first groups of people to identify that a bioterrorism outbreak has occurred, and studies show a large percentage of clinicians would not be able to recognize outbreaks caused by rare or non-endemic disease causing agents (Switala, Coren et al., 2011). In particular, one of the most frightening, yet realistic agents that could be utilized in an attack is variola major (VARV), the causative agent of smallpox disease. After the global eradication of smallpox, declared by the World Health Organization (WHO) in 1980, routine vaccination of the public ceased completely in 1984, with several countries, including North America, Europe, and the Western Pacific, discontinuing vaccination programs even before the last confirmed case of smallpox in 1977 (Wittek, 1982; Jezek, Khodakevich et al., 1987). Because of this fact, the majority of the current public
population has never been vaccinated, and is potentially susceptible to smallpox infection. It is speculated that the protective effect of the smallpox vaccine may largely wear off after about twenty years, leaving individuals who were vaccinated in the 1980s and before vulnerable to a potential outbreak as well (Thomas H. Maugh II, 2001). Therefore, virtually the entire US population is susceptible to smallpox infection after removing the few individuals vaccinated for occupational purposes. Although there are only two known locations where viable VARV samples are kept, the CDC in Atlanta, Georgia, USA, and the Laboratory for Applied Microbiology at Koltsovo in Novosibirsk region (Russian Federation), it is speculated that several other countries may hold secret stocks to be potentially utilized in a bioterrorist attack (Wallin, Luksiene et al., 2007). Along with having a vulnerable population, another motivator of employing VARV in a bioterrorism attack is the fact that it is relatively easy to produce and disperse if in the hands of the wrong individual (Anderson & Bokor, 2012). For these reasons, most bioterrorism experts believe that smallpox would be one of the most likely agents to be utilized by terrorists and therefore the development of safe and effective biomedical countermeasures is a current public health priority. (Wallin, Luksiene et al., 2007; Henderson, 1998)

1.2 Orthopoxviruses

VARV belongs to the family Poxviridae, genus Orthopoxvirus, the most well-known and heavily studied genus of the poxviruses that is also home to eight other species, including Cowpox virus (CPXV), Monkeypox virus (MPXV), Vaccinia virus (VACV), and Ectromelia virus (ECTV) (Damon, 2007). The genus Orthopoxvirus is
comprised of a group of single linear, double stranded DNA viruses with a genome of 170-250 kb in length (Wittek, 1982; Shchelkunov, 2011; Viral Zone, 2013). Viruses belonging to the *Orthopoxvirus* genus are enveloped, brick-shaped, approximately 250 nm long by 200 nm wide, and large enough to be seen using a light microscope (Fenner, 2000). The family name *Poxviridae* is derived from the Latin word for “spotted”, or ‘pox’, and refers to the raised bumps that occur on the body and face of an infected person or animal (Centers for Disease Control and Prevention, 2004). Poxviruses are unique in the fact that DNA replication occurs completely in the cytoplasm of an infected cell. Replication in the cytoplasm as opposed to the nucleus of the host cell separates the viral and host DNA, thus allowing for easier biochemical analysis of host-pathogen interactions (Wittek, 1982). There are two infectious forms of poxviruses, the first being the intracellular mature virion (MV), and the extracellular enveloped virus (EEV). The MV consists of a nucleic acid core enveloped by a single membrane bilayer. The MV particles mostly remain in the cytosol and are released due to cell lysis. While most of the resulting enveloped virus remains attached to the plasma-membrane as a cell-associated virus (CEV), important for cell-to-cell spread, some enveloped virus is released as EEV important for long-range virus spread in tissue culture. The EEV occurs when the MV is released from the host cell via budding after acquiring a second double membrane from the trans-Golgi, as opposed to being released after the host cell is lysed (Viral Zone, 2013; Ma, Xu et al., 2013). In general, replication sites of *Orthopoxviruses* are dependent on the route of initial virus exposure, and are mostly limited to the skin and respiratory tract. When virus enters the body via the skin, replication first occurs in the Malphigian layer of the epidermis and subsequently is carried to the lymphatic system to initiate
systemic illness (Damon, 2007). After exposure to virus via the respiratory tract, a combinations of upper and lower respiratory tract infection can occur. The virus then replicates in the alveolar macrophages and small bronchioles, after which virus migrates to the regional pulmonary lymph nodes (Damon, 2007). After replication in the regional lymphatics, virus disseminates through the blood to the spleen, liver, bone marrow, and other reticuloendothelial organs for further replication. A period of secondary viremia occurs next, and results in the generation of the characteristic generalized rash (Damon, 2007).

1.3 Smallpox

Smallpox is believed to have first appeared around 10,000 BC in northeastern Africa as the first agricultural settlements began to arise (Riedel, 2005). Around 4,000-3,000 BC, descriptions similar to smallpox infection appeared in ancient Indian, Egyptian, and Chinese writings. The earliest concrete evidence of smallpox disease was uncovered in the remains of Egyptian mummies who died approximately 3,000 years ago with smallpox-like rashes apparent on the skin. The most notable mummy discovered with signs of disease was that of the Egyptian Pharaoh Ramses V, who reportedly died in 1157 BC. Smallpox quickly spread worldwide causing frequent epidemics during the Middle Ages, with historical recordings of disease incidence occurring in mostly larger cities of Greece, China, Japan, France, and India, killing as many as 30% of the infected population (Geddes, 2006;Riedel, 2005). By the end of the 16th century, it was a significant cause of human disease and death in Europe, India, China, and southwest Asia. The disease was carried along with European explorers and African slaves from
endemic areas to the Americas circa 1520, and by the mid-18th century, it became a major endemic disease across the globe with the exception of Australia (Riedel, 2005; Geddes, 2006). Smallpox was unintentionally spread world-wide via the movement of infected people, including explorers, armed forces fighting in various wars, and slaves. It was also spread intentionally, most notably in what are believed to be some of the first acts of bioterrorism via contaminated blankets given as gifts to Native Americans by the British during the French-Indian war (1754-1767) (Riedel, 2005). Smallpox infection did not discriminate, occurring in all socioeconomic classes with a case-fatality rate varying from 20-60% in adults and up to 80% in infants in the late 1800s (Riedel, 2005).

VARV is a species-specific virus only able to produce smallpox disease in humans and select nonhuman primates (NHPs). Disease caused by VARV is highly contagious and sometimes fatal (Eyler, 2003). Transmission of disease occurs by direct face-to-face contact with an infected individual through respiratory secretions, along with direct contact with body fluids, such as puss from smallpox lesions, or direct contact with contaminated fomites such as clothing or bed linens (Centers for Disease Control and Prevention, 2004). Two genetically different variants exist within the VARV species, those being variola major and variola minor (Damon, 2007). Disease caused by the variola major variant of VARV is characterized by four disease presentations: ordinary smallpox, modified smallpox, flat smallpox, and hemorrhagic smallpox, with the ordinary type accounting for more than 90% of all cases with a case fatality rate of approximately 30% (Centers for Disease Control and Prevention, 2004). Variola minor causes a less common form of smallpox disease-causing VARV and is much less severe, causing approximately 1% case fatality (Eyler, 2003). Ordinary smallpox disease is characterized
by seven disease stages: incubation (7-17 days), prodrome (2-4 days), early rash (4 days), pustular rash (5 days), pustules and scabs (6 days), resolving scabs (6 days) and scabs resolved periods, ensuing in a disease course of five weeks on average after initial exposure (Centers for Disease Control and Prevention, 2013). The incubation period lasts approximately 7 to 17 days and at this time, the infected individual is not contagious. The prodromal period, where the first signs of disease occur, follows incubation and lasts about 2 to 4 days. During this period, individuals experience flu-like illness including fever, body aches, and malaise, and may be contagious. Early rash, or the enanthem phase of rash, appears first on the tongue and mouth where the spots develop into sores that lyse, spreading large amounts of virus to the throat, causing the person to become highly contagious via respiratory secretions. A body rash starts to form around the same time that the mouth sores rupture, starting at the head and face, spreading to the arms and legs, and continues to the outer extremities in approximately 24 hours. By the third day of cutaneous presentation, or the exanthema period, the rash begins to form small raised bumps, which progress to pustules with a depression in the center by day five. By the end of the second week of rash presentation, mostly all of the pustules crust and form scabs. The infected individual is contagious until the last scab has fallen off, leaving disfiguring scars where the scabs once were. Reports suggest that survivors were not only left with physical scars, but also blindness was a common sequela, occurring in one third of survivors (Riedel, 2005). Because the last case of smallpox occurred in 1977 before the invention of many scientific advances that allow for a detailed description of disease pathogenesis, limited data into the molecular aspects of the systemic pathology of human smallpox exists. Smallpox disease was primarily
characterized by lesion presentation, along with other clinical symptoms of disease such as fever, malaise, head and body aches, and occasional vomiting (Cann, Jahrling et al., 2013; Centers for Disease Control and Prevention, 2004). Clinical pathological evaluations were rarely performed, with available data largely being limited to complete blood counts and coagulation studies. Some of the few reported changes in individuals with ordinary smallpox infections included mild anemia, general leukocytosis, thrombocytopenia, and low platelet counts during the vesicular stage of disease. The same studies reported these parameters as returning to normal levels during the pustular stage of disease (Koplan, Monsur et al., 1975; Roberts, Coffee et al., 1965; Ikeda, 1925).

Reported pathological findings associated with ordinary smallpox include, but are not limited to, congestion and small hemorrhages in the spleen, bronchopneumonia, edema, and atelectasis in the lungs, hepatomegaly, congestion, and lipidosis in the liver (BRAS, 1952; Councilman, Magrath et al., 1904). Bronchopneumonia is generally believed to be the most common and most serious complication of ordinary smallpox, ultimately thought to be the main cause of death in people who succumbed to disease (Cann, Jahrling et al., 2013).

Starting in 1967, large eradication efforts were conducted world-wide lead by the WHO under the WHO Strategic Action Plan for the Intensified Smallpox Eradication Program, which was built on five critical concepts, including mass vaccination. Routine vaccination was ended in 1972 after sufficient herd immunity was established. Today, only military personnel and laboratory workers in contact with Orthopoxviruses are normally vaccinated. Smallpox was declared eradicated in 1980 and a naturally occurring or laboratory acquired case has not since been reported.
With the threat of smallpox being used as a bioterrorism weapon, it is important that safe and effective vaccines, prophylactics, and therapeutics are available if needed. The current smallpox vaccine, a live, attenuated vaccinia virus (VACV), has many limitations and is not adequate for the current population due to many vaccine-associated side-effects. The CDC has listed multiple adverse effects of the VACV vaccine, and there are many people with contraindications in which vaccination is not recommended, including individuals affected by chronic and immunosuppressive diseases, such as HIV/AIDS diabetes, and many forms of cancer, along with individuals suffering from certain skin disorders such as eczema or atopic dermatitis, and women who are pregnant (CDC, 2003). Because it is a live virus vaccine, there is potential for replication and dissemination in the vaccinated individual causing disease. Additionally, inadvertent spread to other unvaccinated individuals via direct contact with secretions from the site of inoculation is possible (CDC, 2003; CDC, 2013). Also, there are currently no FDA approved pharmaceutical treatments for smallpox. And the only treatment is supportive care. Along with health concerns of the current vaccine, it is not ethical to infect humans with deadly diseases in order to test medical countermeasures (MCMs). Thus, surrogate animal models of smallpox disease are utilized in scientific studies to elucidate disease mechanisms, as well as efficacy studies to test novel vaccines, prophylactics, or therapeutics (U.S. Food and Drug Administration, 2009; Xiao, Aldaz-Carroll et al., 2007).
1.4 Animal Modeling and the FDA Animal Rule

Developing new vaccines and therapeutics MCMs against VARV is challenging. Due to its eradication, there are no infected human subjects that are able to be employed in clinical trials for drug efficacy testing. Developing medical countermeasures for other diseases that have not been eradicated but have a low incidence rate proves equally as difficult. It is highly unethical to knowingly infect humans with disease causing agents, especially those that are highly debilitating and virulent, such as VARV. Because of this, the Food and Drug Administration (FDA) released new regulations in 2002 concerning the approval of new drugs or biological products when human efficacy studies are neither ethical nor feasible that are known as “the Animal Rule” (U.S.Food and Drug Administration, 2009; Xiao, Aldaz-Carroll et al., 2007). The Animal Rule states that ‘the evaluation of new drugs or biological products that target diseases that are unethical or unfeasible to test in the human population can gain FDA marketing approval based on adequate and well-controlled animal studies and phase I clinical trials when the results of those studies establish that the drug of biological product is reasonably likely to produce clinical benefit in humans’. In order to do so, surrogate animal models for the disease at hand must be developed. Gaining FDA approval for a biological countermeasure using only animal models to prove efficacy is difficult and requires extensive background knowledge into the natural history of disease and potential animal model at hand. Therefore, certain preliminary criteria must first be established before any product is considered for marketing approval. Those criteria include: (a) a reasonably well-understood pathophysiological mechanism of the toxicity of the substance and its prevention or substantial reduction by the product must be provided; (b) the effect of the
product must be demonstrated in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated in a single animal species that represents a sufficiently well-characterized animal model for predicting response in humans; (c) the animal study endpoint is clearly related to the desired benefit in humans; and (d) the data on the pharmacokinetics and pharmacodynamics of the product in animals and humans should allow for the selection of an effective dose in humans (U.S. Food and Drug Administration, 2009; Xiao, Aldaz-Carroll et al., 2007). To satisfy all four criteria, surrogate animal models must first be developed. The process of developing an animal model for the efficacy evaluation of drug or biomedical products is reliant on multiple factors. First, the infectious agent that will be used in the efficacy testing needs to be characterized. The challenge agent that is used in the animal model studies is ideally the identical pathogen that causes disease in humans. If this is not feasible, surrogate agents can be used as long as justification that the agent used in the animal model is suitable for producing evidence of the effectiveness of the intervention in humans. The agent should demonstrate pathogenic determinants of disease in the animal model that are similar to those that are understood in the human. The route of exposure of the challenge agent to the animal model should be similar to the expected route of exposure in humans. Various routes of exposure include, but are not limited to aerosol, intranasal (IN), subcutaneous (SC), intraperitoneal (IP), intradermal (ID), and intravenous (IV) exposures, with aerosol exposure being the most likely employed route of exposure in a bioterrorism attack due to the massive area and numbers of people that can be infected from a single release. The quantification of the challenge dose in the animal model should be reproducible and reliable across multiple studies and a scalar
relationship of the animal dose to the dose anticipated in human disease should be established. After the characterization of the etiologic agent is completed, the susceptibility and host response of the animal model to the agent is to be determined. The animal response to the agent should be as similar to the disease in humans as possible, including morbidity and mortality rates that are representative to that of human disease. After susceptibility in the animal model has been shown, the natural history of the disease in the animal needs to be characterized and certain factors should be closely examined, those being time-to-onset, time course of disease progression, and the clinical signs and symptoms of disease. A trigger for intervention, whether it is a clinical symptom or disease specific biomarker, should be identified in the animal model to better define the timing of the delivery for the intervention in human patients for a therapeutic product. When characterizing an animal model for drug efficacy testing, it is critical that all of the above considerations are closely examined and the resulting studies carried out. Animal models for smallpox infection have been characterized, including the New Zealand white rabbit, NHP models, and multiple murine models, but most models currently have very limited pathology descriptions and need further refinement (Chapman J.L., Nichols D.K. et al., 2010). In line with the FDA Animal Rule, the preferred animal models for smallpox drug efficacy testing are the rabbit, mouse, and NHP models of infection. The NHP model is to be one of the two required animal models for vaccine testing. Proof of efficacy of any therapeutic or prophylactic drug requires the rabbit and/or mouse models (U.S.Food and Drug Administration, 2009; U.S.Food and Drug Administration, 2011).
1.4.1 Rabbitpox virus in the New Zealand White Rabbit Model

The New Zealand white rabbit has been utilized in multiple studies using Rabbitpox virus (RPXV), strain Utrecht, an Orthopoxvirus highly virulent in rabbits which causes up to 100% mortality (Li, Chen et al., 2005; Adams, Rice et al., 2007). Most studies utilized the ID method for inoculation, resulting in up to 100% mortality in nine-week-old rabbits with a median time to death of nine days post-infection of 1,000 PFU/animal of RPXV-Utrecht (500 PFU bilaterally). Gross lesions at the inoculation site, secondary skin lesions on the outer extremities and areas away from inoculation site, and respiratory involvement have been reported, along with an increase in body temperature and decreases in body weight beginning between 3 and 4 days post-infection (Adams, Rice et al., 2007). Studies utilizing aerosol exposure of RPXV to rabbits have been conducted, resulting in 100% mortality by five to seven days post-challenge (Nalca & Nichols, 2011; Nalca, Hatkin et al., 2008; Garza, Hatkin et al., 2009)). Results showed gross necropsy congestion and edema of the mandibular and mediastinal lymph nodes and edema of the lungs and mediastinum. Most lesions present were internal and located in the respiratory tract, liver, gonads, and/or adrenal cortices. Skin lesions were present in longer surviving animals and consisted of 1- to 2-mm light red/tan macules and papules on the lips, eyelids, and occasionally ears, where larger lesions occasionally appeared on areas shaved prior to viral challenge. Aerosol transmission of RPXV from ID infected animals to sentinels has been reported, with clinical findings suggesting the route of infection in the sentinel rabbits is inhalational (Adams, Rice et al., 2007; Westwood, Boulter et al., 1966). Animals that develop disease after natural aerosol transmission have a reported survival rate of 21.3%, a survival rate similar to that of
human smallpox. The difference in survival rates between ID infections and natural aerosol infections can be attributed to the viral dose, where virus administered via the IN route requires an infectious dose 10-100 times more than ID infectious to cause high lethality (Rice, Adams et al., 2011).

1.4.2 MPXV in the NHP Model

NHP models have been utilized in multiple MPXV studies, producing disease results similar to that of human VARV infection (Meyer, Perrichot et al., 2002; Nalca, Livingston et al., 2010; Rimoin, Kisalu et al., 2007). Current MPXV models include both rhesus (Macaca mulatta) and cynomolgus macaques (Macaca fascicularis), although the latter have been shown to be more sensitive to infection and display more severe clinical signs (PRIER & SAUER, 1960; SAUER, PRIER et al., 1960). Current models utilize IV, aerosol, ID, or intratracheal (IT) routes of inoculation, with aerosol and IT routes preferred for drug efficacy testing, as they create respiratory models of infection thought to be similar to the human pathophysiology after an aerosol bioterrorism attack and the natural route of secondary infection thereafter (Zaucha, Jahrling et al., 2001; Chapman J.L., Nichols D.K. et al., 2010). Extensive characterization of the pathology in cynomolgus macaques after aerosolized MPXV challenge has been described. These studies have indicated systemic dissemination of the virus via monocytic cell-associated viremia and the development of a generalized vesiculopustular rash in a centrifugal distribution similar to that of VARV infection in humans (Huggins, Goff et al., 2009; Zaucha, Jahrling et al., 2001). Death of the animals is attributed to respiratory
complications and necrotizing lesions in lymphoid organs, skin, and mucosal surfaces, and reproductive organs. Although the NHP disease models are mostly considered to be ideal overall in terms of the anatomical and immunological similarity to humans, there are many disadvantages to using NHP models in drug efficacy testing. In general, NHPs are expensive to obtain, difficult to handle, can harbor life-threatening zoonotic diseases, and acquiring the numbers needed to properly produce statistically significant results can be challenging (Chapman J.L., Nichols D.K. et al., 2010). Along with NHP models being used for applications pertaining to smallpox pharmaceutical research, most anti-orthopoxvirus drugs can potentially be applied to MPXV infections in humans (a current public health concern in parts of Africa) due to viral genetic similarity (Reynolds, Carroll et al., 2012; Reynolds & Damon, 2012).

1.5 Ectromelia Virus in the Mouse Model

ECTV (210 kbp) is the causative agent of mousepox and was first described in 1930 as a fatal disease in laboratory mice (Marchal J., 1930). It is a species-specific virus with a very narrow host range, only known to cause disease in specific mice, with voles being a speculated natural reservoir (Esteban & Buller, 2005). The virus is transmitted naturally among wild and laboratory mice populations via direct contact or contact with virus contaminated fomites (Chapman J.L., Nichols D.K. et al., 2010; Esteban & Buller, 2005). The Hampstead strain of ECTV was the first to be indentified and described by Marchal in 1930. The virus is infectious at low doses, with reported lethal dose required to kill half of the study population (LD$_{50}$) numbers below 10 plaque forming units (PFUs) in specific mouse strains (Esteban & Buller, 2005). Primary viremia occurs in the mouse
after localized replication in regional lymph nodes and consequently results in infection of the liver and spleen. Secondary viremia occurs shortly after infection of the spleen and liver. Generalized maculopapular rash similar to the classic pustular rash seen in human smallpox cases has previously been reported in mice that survive the acute phase of disease. Skin ulcerations and necrosis of the tail and feet has been reported in mice that develop chronic disease. Ultimately, death in acute cases results from necrosis of the liver and spleen (Chapman J.L., Nichols D.K. et al., 2010). After being studied in the laboratory setting, it was determined that the genetic and clinical disease course similarities between VARV and ECTV rendered ECTV a good model of smallpox and exanthematous disease pathogenesis, and subsequently anti-orthopoxvirus drug efficacy testing (Fenner, 1948; Parker, Handley et al., 2008). These similarities include a required low virus dose to cause disease in the upper and lower respiratory tract, no obvious lung involvement during early disease after IN inoculation, the detection of virus in respiratory gases during the pre-exanthem period, and presentation of characteristic exanthematous rash (Buller & Palumbo, 1991; Roberts, Coffee et al., 1965; Fenner, Henderson et al., 1988). Differences between ECTV infection in mice and VARV in humans do exist, including a shorter overall disease course, where death in infected mice usually occurs seven to fourteen days post infection. In contrast, death in humans infected with smallpox occurs approximately 18 to 22 days post-infection (Roberts, Coffee et al., 1965). Unlike smallpox infection in humans, there is major liver and spleen involvement in ECTV infected mice, as major lesions can be observed in these organs (Roberts, Coffee et al., 1965; Buller, 2004).
The disease course and susceptibility is largely dependent on the mouse species, the virus strain, and route of infection. There are several species of mice that are highly susceptible to ECTV infection – including A/J, BALB/c, DBA/2 and C3H strains of mice – where C57BL6, SKH1 and AKR strains of mice are resistant to severe disease (Esteban & Buller, 2005; Schriewer, Buller et al., 2004; Jacoby RO, Fox JG et al., 2002; Roberts, Coffee et al., 1965). ECTV strain Moscow is the most thoroughly studied, infectious, and virulent ECTV strain in mice (Esteban & Buller, 2005; Buller, 2004). Various routes of infection have been utilized in the mouse model, including ID, SC, IN, IT, IV, IP, intracerebral (IC) and aerosol (Schriewer, Buller et al., 2004; Fenner & Buller, 1997). Because the natural route of infection is believed to be through abrasions in the skin, footpad scarification is the most commonly used method of infection, which produces localized replication in regional lymph nodes, primary viremia resulting in infection of the liver and spleen, followed by secondary viremia. A generalized maculopapular rash can be seen in animals surviving the acute phase of disease, along with bilateral conjunctivitis (Fenner & Buller, 1997; Chapman J.L., Nichols D.K. et al., 2010; Esteban & Buller, 2005). Aerosol and IN routes of infection are most commonly used when developing a model for VARV drug efficacy testing to better mimic the most likely exposure of humans to VARV, an aerosol release of the virus during a bioterrorism attack (Parker, Schriewer et al., 2008). In these models, virus is taken up by alveolar macrophages and/or epithelium of the upper and lower respiratory tract and transported to the regional lymph nodes and spleen (Roberts, Coffee et al., 1965; Fenner & Buller, 1997). The IN route of infection more closely imitates the natural route of exposure of
smallpox, while also capturing the respiratory route of infection most likely utilized in a bioterrorism event (Parker, Touchette et al., 2008).

Although multiple studies have been conducted looking at disease pathogenesis in the mouse model after ECTV infection, there are remaining gaps in the data pertaining to potential ECTV models used for the evaluation of smallpox drug and vaccine efficacy. Capturing a route of exposure that mimics the predictive mechanism of agent release along with the subsequent natural transmission of the virus and identifying an animal model that will exhibit similar susceptibility and disease progression to VARV-infected humans are two important factors that need to be addressed. Because predictions of a potential bioterrorism attack utilize aerosolization of VARV for infection, a mouse model with respiratory disease involvement is critical for the evaluation of smallpox biological countermeasures and vaccines. It was determined that a susceptible mouse model utilizing the IN route of exposure to the virulent ECTV-Mos needed further development and refinement for therapeutic and prophylaxis drug efficacy studies.

BALB/c mice are extremely susceptible to low infectious doses of ECTV, with reported LD$_{50}$ of about 5 PFU after IN infection with ECTV-Mos in 12-14 week old BALB/c mice, and presenting clinical symptoms similar to human VARV infection (Xiao, Aldaz-Carroll et al., 2007). The natural history of IN infection of ECTV-Mos in the BALB/c mouse model is not well characterized. This was the basis for the development of a smallpox pathogenesis surrogate model using IN infection of BALB/c mice with ECTV-Mos. In order to do so, the virus was propagated and characterized; the potency and the LD$_{50}$ and LD$_{90}$ of the virus in the mouse model was assessed; and the
natural history of infection was characterized. Determining the clinical parameters that provide the earliest indication of disease onset following challenge along with highlighting certain parameters that correlate with time-to-death, survival were the main objectives that were assessed. A description of this model may allow for innovative in vivo studies designed to elucidate the molecular mechanisms of ECTV pathogenesis, as well as to aid in the efficacy assessment of novel anti-orthopoxvirus vaccine, therapeutic, and prophylaxis candidates.
2.1 In-vitro Study: The Propagation, Harvest, and Characterization of ECTV-Mos Working Stock

2.1.1 Viral Propagation

ECTV-Mos (ATCC, Manassas, VA) was stored in liquid nitrogen until time of use. African Green Monkey kidney epithelial cells (Vero E6 cells) were used for ECTV propagation. The cells were expanded to forty-eight T-162 flasks and were infected with a target multiplicity of infection (MOI) of 0.01. The flasks were examined microscopically daily until an adequate level of cytopathic effect (CPE) had occurred (90-100% CPE). All work was performed in a biosafety level 2 (BSL-2) laboratory under aseptic conditions.

2.1.2 Viral Harvesting

After approximately 144 hours, ≥ 90% CPE had occurred and the infected cells were scraped from the flasks and collected into centrifuge bottles. The infected cells were centrifuged for one hour at 10,000 x g at a temperature of 4°C. After centrifugation, the supernatant was removed and the remaining cell pellet was resuspended in 1mM Tris-buffer and rinsed. Three freeze-thaw cycles were performed to lyse any remaining intact cells to release intracellular virus by placing the cell slurry in a ≤-70 °C freezer for 30 minutes and then immediately incubate in a 37 °C water bath until completely thawed.
The slurry was centrifuged at 300 x g for five minutes at 4°C to remove cellular debris. The supernatant was aliquoted and placed on ice. The remaining pellet was resuspended in 10 mL of 10 mM Tris buffer and subject to centrifugation, and the supernatant was removed and combined with the initial supernatant from the previous centrifugation. The combined supernatant, containing the virus, was kept on ice and sonicated. The virus slurry was purified by centrifugation at 48,000 x g for one hour at 4°C using a 36% sucrose solution cushion. The supernatant was then removed and the pellets were resuspended in 3 mL of sterile phosphate buffered saline (PBS) (four pellets in 3 mL PBS each).

2.1.3 Plaque Assay to Determine Viral Titer

The resulting viral working stock was titrated via plaque assay. Vero E6 cells were seeded onto 12-well cell culture flat bottom plates and were grown to ≥90% confluent monolayers overnight. Serial dilutions of the ECTV working stock were prepared, and 0.1 mL aliquots were inoculated in triplicate into the monolayers. After a one hour incubation period at 37°C which allows for viral attachment to host cells and infect the cells, the monolayers were covered with methylcellulose nutrient medium (370 mL 1.89% Methyl Cellulose, 500 mL 2X EMEM, 10 mL Penicillin/Streptomycin, 10 mL L-glutamine, 10 mL non-essential amino acids, 100 mL Fetal Bovine Serum (FBS)). The progeny infect neighboring cells and create a circular zone of dead cells, or a plaque. The plates were allowed to incubate and were stained with crystal violet stain solution after approximately 166 hours to observe the plaques. The living cells uptake the stain
and appear purple, where plaques are clear circles, each representing one infectious virus particle. A positive control sample was prepared using 500 mL of the supernatant reserved after the initial 10,000 x g virus harvest centrifugation. The viral stock underwent specification testing for sterility, endotoxin levels, presence of mycoplasma, and pH level. Whole genomic sequence comparison was conducted (SeqWright Genomic Services, Houston, TX) to confirm the virus as ECTV-Mos.

2.2 Determination of Potency and LD\(_{50}/LD_{90}\)

2.2.1 Study design

2.2.1.1 Animal Population

Eighty (40M/40F) 6-8 week old BALB/c \textit{Mus musculus} mice were purchased (Charles River Laboratories) and were inspected for signs of ill-health upon arrival and were quarantined for a minimum of three days. All animals were deemed healthy by a certified veterinarian. The animals were tattooed on the tail for identification. Two phases of the first study occurred, the first being a potency study (Phase 1), the second being a study to determine the LD\(_{50}\) and LD\(_{90}\) of ECTV-Mos in the BALB/c mouse model (Phase 2). The potency study involved 16 animals (8M/8F), and the LD\(_{50}/LD_{90}\) study involved 64 animals divided into four groups (16 animals per group, 8M/8F per group). The results from the potency study were used in combination with the phase II results to determine the LD\(_{50}\) and LD\(_{90}\).
2.2.1.2 Challenge Material and Route of Inoculation

The ECTV-Mos working stock was diluted in Dulbecco’s Phosphate Buffered saline (DPBS) to reach the desired viral titer. For study phase 1, the titer of the challenge material was determined to be 700 PFU/animal. For study phase 2, the challenge material was 292.5, 119.5, 27.5, and 2.44 PFU/animal for Groups 1, 2, 3, and 4, respectively. All animals were anesthetized with ketamine/xylazine and inoculated via the intranasal (IN) route of infection with 12.5 µL per nostril [25 µL total volume].

2.2.1.3 Clinical Observations and Weights

All animals were observed twice daily for clinical signs of disease. All symptoms/observations were recorded including, but not limited to, mortality, lethargy, changes in grooming, lacrimation, respiratory distress, and lesion progression. All animals were initially weighed on Study Day 0, the day of challenge, for a baseline weight. Beginning on Study Day 0, the animals were subsequently weighed every other day until they succumb to disease or until Study Day 14, the final day of the study. Terminal body weights were recorded for all animals. The changes in body weights were calculated from the change from baseline weight.

2.2.2 Statistical Determination of LD\textsubscript{50}/LD\textsubscript{90}

The survival outcomes for infected animals were fitted to a probit regression relationship with base-10 logarithm comparing the challenge dose to the time of death for each animal. From this relationship, a median lethal dose was estimated (LD\textsubscript{50}), as well
as a 90th percentile lethal dose (LD$_{90}$). A 95 percent confidence interval was also be
generated for each of the 50th and 90th lethal dose percentiles using Fieller’s method

2.3 Natural History of Disease

2.3.1 Animal Population

BALB/c mice were purchased (Charles River Laboratories) (180 [90M/90F]) and
were inspected for signs of ill-health upon arrival and were quarantined for a minimum of
three days. The animals were implanted with a transponder chip that could confirm
animal identification and measure body temperatures.

2.3.2 Study Design

Animals were randomly divided into 35 groups (Group 1-35) by body weight.
Each group was representative of a time point, or time points, in which samples were
collected via serial sacrifice (Table 7). Animals in Groups 1, 3, 5, 7, 9, 11, 13, and 15
(4M/4F each) were challenged with ECTV-Mos, along with animals in Groups 17-33
(2M/2F each). Group 34 (4M/4F) was challenged with a 125 PFU of ECTV-Mos and
served as a positive control group. Groups 2, 4, 6, 8, 10, 12, and 14 (2M/2F each) were
mock-challenged with DPBS (the carrier control), along with Group 35 (4M/4F), which
served as a negative control group. Four mice (2M/2F) from groups 1, 3, 5, 7, 9, 11, 13,
and 15 and two mice (1M/1F) from groups 2, 4, 6, 8, 10, 12, 14, and 16 were euthanized
per time point, where livers and spleens were removed and oropharyngeal secretion and
blood samples collected. For the Groups 17-32, all animals in each group (2M/2F) were
euthanized per time point. The Group 34 animals were not euthanized unless deemed necessary by euthanasia criteria, or until Study Day 14 (end of study). Animals in Group 35 were not euthanized until Study Day 14. No samples were collected from Groups 34 and 35. Serial euthanasia in each group were randomized by weight.

### 2.3.2.1 Challenge material

ECTV-Mos was diluted in DPBS to obtain a challenge viral titer of 125 PFU per animal. Each animal was anesthetized with kentamine/xylazine and inoculated IN with 12.5 µL per nostril [25 µL total volume]. A sample of the challenge material was used for back-titer analysis by plaque assay techniques.

### 2.3.2.2 Body weights

Baseline body weights of all animals were measured on Study Day 0 prior to challenge. Beginning on Study Day 2, all animals were weighed every other day up until 10 days post-challenge. Subsequently animals were weighed once on both on Study Days 12 and 14 until study completion. Terminal body weights were recorded. Body weight changes following challenge will be calculated as the change from baseline.

### 2.3.2.3 Temperatures

A temperature transponder chip was implanted in the rump/hip area in all animals during quarantine on Day -3. Body temperature was recorded twice daily from Day -3 through Day -1 to establish an average baseline temperature for each animal. Body
temperature was recorded once on Day 0 prior to challenge. Following challenge, body
temperature was recorded twice daily until the end of study.

2.3.2.4 Clinical Observations

All animals were observed twice daily (AM and PM) from arrival to the day of
challenge. Following challenge, the animals were observed for clinical signs of disease
and mortality three times daily through Study Day 10. Beginning on Study Day 11, the
animals were observed twice daily. All symptoms/observations were recorded including,
but not limited to, mortality, lethargy, changes in grooming, lacrimation, respiratory
distress, and lesion progression. Along with all other clinical signs and symptoms, all
animals were specifically monitored for the appearance of a pustular rash.

2.3.2.5 Specimen Collection and Testing

Blood (approximately 1 mL) was collected in EDTA tubes from each animal in
Groups 1-33 in 12 hour increments by intracardiac methods. Four animals (2M/2F) in
Groups 1,3,5,7,9,11, 13, and 15 were euthanized per time point in 12 hour increments, so
that one entire group would be euthanized every 24 hours. Blood collected from these
groups was allocated to qPCR and plaque assay analysis. All animals in Groups 17
through 33 were euthanized in 12 hour increments (one entire group euthanized every 12
hours) starting at hour 0 post-challenge until 192 hours post-challenge for the collection
of blood used for hematology and clinical chemistry analysis. If an animal was found
dead prior to its scheduled collection time point, blood was not taken from that animal.
Oropharyngeal secretions were collected using cotton swabs placed in 1 mL of 1x PBS
after use only from animals in Groups 1-16 at the time of euthanasia, and were analyzed for the presence of virus using qPCR.

2.3.2.6 Hematology

Hematology analysis was conducted (Siemens Advia 120 Hematology Analyzer) for mice in Groups 17-33. The hematology parameters analyzed included: Red Blood Cell Count (RBC, 10^6 cells/mL), Hemoglobin (HGB, g/dL), Hematocrit (HCT, %), Mean Corpuscular Volume (MCV, fL), Mean Corpuscular Hemoglobin (MCH, pg), Mean Corpuscular Hemoglobin Concentration (MCHC, g/dL), Cell Hemoglobin Concentration Mean (CHCM, g/dL), Red Cell Distribution Width (RDW, %), Platelet Count (PLT, 10^3 cells/mL), Mean Platelet Volume (MPV, fL), White Blood Cell Count (WBC, 10^3 cells/mL), Neutrophils (10^3 cells/mL), Lymphocytes (10^3 cells/mL), Neutrophils/Lymphocytes Ratio (N/L Ratio), Monocytes (10^3 cells/mL), Eosinophils (10^3 cells/mL), Basophils (10^3 cells/mL) and Large Unstained Cells (LUC, 10^3 cells/mL).

2.3.2.7 Clinical Chemistry

Clinical Chemistry analysis of sera, collected in SST tubes, was conducted on the Siemens Advia 1200 for mice in Groups 17-33. The clinical chemistry parameters analyzed included: Total Bilirubin (mg/ dL), Aspartate Aminotransferase (AST, U/L), Alanine Aminotransferase (ALT, U/L), Glucose (mg/dL), Blood Urea Nitrogen (BUN, mg/dL), Creatinine (mg/dL), and BUN/Creatinine Ratio.
2.3.2.8 Pathology

Complete gross necropsies were performed on all animals in Groups 1-16 and Groups 34 and 35 that were either found dead or euthanized. For animals that were euthanized, only the spleen and liver was collected and fixed in formalin. The samples were processed for routine hematoxylin-and-eosin staining and examined for abnormalities. A portion of the spleen and liver (1 cm$^3$) from each animal were set aside for vial burden analysis. All microscopic findings were graded semi-quantitatively according to the following scale, with the associated numerical score used to calculate average severity grades for each lesion by group and sex. Minimal (Grade 1) represented the least detectible lesion; mild (Grade 2) represented an easily discernible lesion; moderate (Grade 3) represented a change affecting a large area of the represented tissue; and marked (Grade 4) represented a lesion that approached maximal.

2.3.2.9 Viral Burden

Spleen and liver samples collected from animals in Groups 1-16 were homogenized in 1 mL of lysis buffer or 1x PBS and processed for the determination of viral load using qPCR and plaque assay analysis. Viral load from oropharyngeal secretions (collected via cotton swabs and placed into 1 mL 1x PBS) was analyzed by qPCR. Blood collected from animals in Groups 1-16 was processed and viremia was
determined by qPCR and plaque assay analysis. In the event that a small amount of 
blood was obtained from an animal, preference was placed first on qPCR analysis, and 
second on plaque assay analysis.

2.3.2.9.1 Real-time polymerase chain reaction (PCR) Parameters

Each nucleic acid sample was assayed in duplicate by qRT-PCR for 
detection of a portion of the hemagglutinin [HA(J7R)] gene using a 7900HT real-
time PCR system (Applied Biosystems, Life Technologies Corp., Carlsbad, CA). 
Each 25 µL reaction contained 5µL of sample with the remaining volume 
consisting of TaqMan Gene Expression Master Mix, sterile water, and a custom 
gene expression assay consisting of primers and a 3’-minor groove binding probe 
specific for a portion of the HA(J7R) gene (Forward primer: 5’-
GATGATGCAACTCTATCATGTA-3’, Reverse primer: 5’-
GTATAATTATCAAAATACAAGACGTC-3’).

2.4 Animal Care and Husbandry

All animals were group housed in filter top disposable cages in temperature 
controlled rooms functioning under a light/dark cycle of approximately 12 hours each per 
day. Water and rodent chow were available *ad libitum*. Animals were released from 
quarantine by the study veterinarian prior to placement on study. Euthanasia of an 
animal prior to its scheduled euthanasia time was determined by moribundity/persistent
prostration and unresponsive to stimuli, and any animal that exhibits ocular changes that includes lacrimation, discharge, blepharitis, and/or bupthalmos in combination with dehydration, and/or consistent weight loss observed for 48 hours. Euthanasia was carried out by exsanguination via intracardiac blood draw under kentamine/xylazine anesthesia, or cervical dislocation under anesthesia.

2.5 Analytical and Statistical Plan

Sample size was determined for adequate statistical power. Survival rates were summarized by group and plotted on Kaplan-Meier curves. For hematology parameters, clinical chemistry parameters, qPCR, plaque assay, body temperature, body weight and clinical observation data, descriptive statistics were produced for each animal at each sample collection time. Baseline data was used in the assessment of these endpoints, with each animal serving as its own control. Mean changes in hematology parameters, clinical chemistry parameters, temperature and weight data were compared to baseline using analysis of variance (ANOVA) models, to evaluate any change in health status and estimate a time at which the parameter was abnormal. For qPCR and plaque assay data, and clinical observations a threshold was set to assess change in health status (i.e. if animals were positive or negative for vDNA or infectious virus). For each of these measures, the time to onset of illness was calculated. The time to disease onset for each parameter was then compared to the time-to-death. Paired t-tests and correlation coefficients were used in the analysis and log-rank tests or other methods that account for censoring were considered. In addition, the proportion of animals with an abnormal value
for each parameter was compared to the proportion of animals that died to determine correlates of mortality and survival. The data from all challenged groups were combined to assess the consistency of clinical signs and disease progression over time among the groups.

2.5.1 Clinical Parameter Analyses

The data for hematology, clinical chemistry, qPCR, body weight, and temperature was statistically analyzed. For each endpoint of each animal, either the raw measurement (if no baseline exists) or the change from baseline was tabulated. These endpoints, or change from baseline endpoints, were summarized to include a measure of central tendency (e.g., mean or median) and of variability (e.g., 95 percent confidence interval or range) by group and time point. The data was further fit to analysis of variance models and statistical comparisons were made between infected and sham infected groups (where data was collected from both types of groups) with the same data collection schedule. The output of all statistical models was examined to make sure that necessary assumptions such as normality, constant variance, or lack of outliers, were satisfied. If violations were found, remedial measures were taken such as data transformations (e.g., log transformation), performing the analysis both with and without outliers included, or utilizing a nonparametric test.

2.5.2 Time-to-Abnormal Analysis

For each endpoint, a further analysis was conducted based on the sham-infected animals to establish 95 percent population bounds for expected responses in a non-
infected animal. The data for the infected animals were compared to these limits and the post-challenge time of the first data point that exceeds the limits was identified as a "time to abnormal". (Note: This time actually exists on an interval from the previous observation where the value did not exceed the normal range. Recognition of the values falling within these intervals was incorporated into the subsequent statistical analysis). The time to abnormal data was summarized for all endpoints. These data may include a mixture of observations that fail to ever reach an abnormal state as well as measured times to the first abnormal observation. Therefore, the characterization of each of the times associated with the endpoint to abnormal response incorporate both of these factors. These analyses helped to establish which endpoints are the earliest and most reliable to appear, and hence could serve as triggers-for-treatment in the animal model.

2.5.3 Time to Abnormal Correlated with Time to Death Analysis

The time-to-abnormal for each endpoint may correlate with time-to-death in those animals that succumb. To evaluate the data, regression analysis was performed with time-to-death as the response and time to abnormal as the predictor. Those statistically significant relationships were reported.

2.5.4 Clinical Parameters Correlated with Challenge Survival Analysis

Endpoint data at each time point was summarized and compared between the aggregate group of infected survivors and infected non-survivors, assuming both
outcomes are observed. Where statistically significant differences were observed, if any, lies evidence for clinical parameters that correlate with challenge survival.
Chapter 3: Results

3.1 Certified Titer of Harvested ECTV-Mos Working Stock

Propagated purified ECTV-Mos working stock was titrated using Vero E6 African Green Monkey kidney epithelial cells. The resulting ECTV-Mos working stock titer was $4.91 \times 10^6$ PFU/mL. No growth was detected in sterility testing, endotoxin levels were determined to be 0.611 EU/mL (passed acceptance criteria), the stock was negative for *Mycoplasma*, and the pH was 7. DNA sequencing confirmed 100% homology between the target sequence of the stock and the GenBank reference ECTV Moscow Strain, 846 bases (HA gene region). The positive control sample was titered and certified, yielding a final concentration of $4.85 \times 10^3$ PFU/mL. Certification of the positive control and viral stock titers consisted of performing three plaque assays in duplicate of three different vials of both viral samples, where each of the six total plaque assays (6 positive control, 6 viral working stock) yielded results within a 0.5 log range of one another.

3.2 In-vivo Study Part One: Potency and LD$_{50}$/LD$_{90}$

3.2.1 Mortality and LD$_{50}$/LD$_{90}$ Determination

The objective of study phase one was to evaluate the potency of the propagated ECTV-Mos viral working stock. Sixteen mice were inoculated via the IN route of infection with a target dose of 1,000 PFU per animal (Group 5). A titration of the
challenge material post-inoculation (back-titer) of the animals revealed an actual challenge dose of 700 PFU per animal. All animals succumbed to disease, resulting in an estimated mortality rate of 100%, proving that propagated virus was lethal in the BALB/c mouse model.

The objective of study phase 2 was to determine the LD$_{50}$ and LD$_{90}$ of the propagated virus. After the ECTV-Mos working stock was determined to be potent by producing 100% mortality in Group 5 (700 PFU), the LD$_{50}$ and LD$_{90}$ of the virus was evaluated by creating target challenge doses for Groups 1 through 4 of 5, 50, 300, and 500 PFU per animal, respectively. Back titrations of the challenge material via plaque assay from the four animal groups revealed actual challenge doses of 2.44, 27.5, 119.5, and 292.5 PFU per animal, respectively. Groups 1 through 4 challenged with 2.44, 27.5, 119.5, and 292.5 PFU per animal produced mortality percentages of 63.0%, 88.0%, 94.0% and 100% respectively (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Dose (PFU)</th>
<th>Actual Dose (PFU)</th>
<th>Number of Animals Succumbed / Total Number of Animals</th>
<th>Mortality Proportion (Binomial Exact 95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>2.44</td>
<td>10/16</td>
<td>0.63 (0.35, 0.85)</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>27.5</td>
<td>14/16</td>
<td>0.88 (0.62, 0.98)</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>119.5</td>
<td>15/16</td>
<td>0.94 (0.70, 1.00)</td>
</tr>
<tr>
<td>4</td>
<td>500</td>
<td>292.5</td>
<td>16/16</td>
<td>1.00 (0.79, 1.00)</td>
</tr>
<tr>
<td>5</td>
<td>1,000</td>
<td>700</td>
<td>16/16</td>
<td>1.00 (0.79, 1.00)</td>
</tr>
</tbody>
</table>

Table 1. Group mortality results for the Potency (Group 5) and LD$_{50}$/LD$_{90}$ Study
A Kaplan Meier plot was produced (Figure 1) to illustrate the mortality data for each group over time. All animals that succumbed were found dead or euthanized between five and thirteen days post-challenge. The earliest death occurred on Day 5 post-challenge in the group challenged with 292.5 PFU (Group 4), and the latest death occurred on Day 13 post-challenge in the group challenged with 2.44 PFU (Group 1).

![Kaplan-Meier Plot for groups in the potency and LD50/LD90 studies.](image)

**Figure 1.** Kaplan-Meier Plot for groups in the potency and LD50/LD90 studies.

A probit dose-response model was fitted to the data from all five groups (Figure 2). LD$_{50}$ and LD$_{90}$ values were estimated, and 95 percent confidence intervals were calculated. Due to the fact there were no dose groups with less than fifty percent mortality, the estimated LD$_{50}$ value was below the challenge dose of the lowest dose group (Group 1, 2.44 PFU). Because of this, the exact LD$_{50}$ value could not be
calculated, but can be estimated to be some value less than the 2.44 PFU challenge dose from group 1. The estimated LD$_{90}$ value was calculated to be 32.10 PFU (Table 2).

**Figure 2.** Estimated Probit Regression Curve and 95 Percent Confidence Bounds with Points Showing Mortality Outcomes by Group.

<table>
<thead>
<tr>
<th>50th Lethal Inhaled Dose Percentile (PFU/animal)</th>
<th>90th Lethal Inhaled Dose Percentile (PFU/animal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD$_{50}$</td>
<td>LD$_{90}$</td>
</tr>
<tr>
<td>&lt;2.44</td>
<td>32.10</td>
</tr>
<tr>
<td>95% Confidence Bounds</td>
<td>95% Confidence Bounds</td>
</tr>
<tr>
<td>(0.02, 4.43)</td>
<td>(10.63, 227.47)</td>
</tr>
</tbody>
</table>

**Table 2.** Estimates of the 50$^{th}$ and 90$^{th}$ lethal inhaled dose percentiles along with the 95$^{th}$ percent confidence bounds.
3.2.2 Body Weights

All animals were weighed on study day 0 (before challenge) to determine baseline weight, and subsequently thereafter weighed every other day until they succumb to disease or study day 14 (end of study). The group mean weight over time (Figure 3) and group mean change from baseline weight as a percentage of baseline weight over time (Figure 4) were evaluated. Change from baseline ANOVA least-square mean estimates and group comparisons of weight data were also evaluated (Table 4). For study days when either zero or one animals survived, statistical significance could not be determined, and therefore a mean change from baseline was not presented. Significant decreases in weight began to occur on study day 4 (Average (Avg): -0.5 g) in the Group 5 animals (highest challenge dose) and continued through day 8 (Avg. -2.1 g), with steady weight declines occurring until the death of all Group 5 animals. Group 4 animals (292.5 PFU challenge dose) began to lose weight between days 6 and 8 post-challenge (Avg. -1.2 g) and continued to steadily lose weight until all animals had succumbed to disease. Similarly, animals in Group 3 (119.5 PFU challenge dose) also began to lose weight between days 6 and 8 post-challenge (Avg. -0.8 g), but with less dramatic weight loss following initial weight decline. Group 2 animals also began to lose weight between study days 6 and 8, although not significant until study day 10 (Avg. -1.4 g), and continued to show steady decreases collectively. Significant weight loss was never experienced in Group 1 animals, although a slight decrease in group mean weight was seen between study days 9 and 11. The appearance of a large increase in the weight average of Group 3 after study day 10 can be attributed to one surviving animal that never experienced weight loss, but instead continued to gain weight after challenge. Of
the two surviving animals in Group 2, one animal continued to lose weight, while the second animal gained weight throughout the study, in turn creating an apparent increase in group mean weight on study day 12. Out of the seven surviving animals across groups, only one animal experienced weight loss relative to baseline at the end of the study. Overall, mice infected with ECTV-Mos experience weight loss prior to death, although the weight loss is not considered ‘extreme’.

Figure 3. Group Mean Weight over Time.
3.2.3 Clinical Observations

All challenged animals across all five groups consistently exhibited adverse clinical signs of disease. Lethargy, ruffled fur, hunched posture, lacrimation, and occasional lesions were noted in most animals. The rare presence of possible lesions was mostly noted at the base of the tail and the ears, where lesions on the body were difficult to detect due to the fur. Animals in Group 5 (700 PFU) began displaying clinical signs of disease four days after challenge, the first sign usually being lethargy. Ruffled fur and hunched posture followed, occurring at approximately 7 days post challenge. Lacrimation and other ocular changes occurred 7 to 9 days post challenge in some animals. Suspect lesions were noted in 15% of animals (Groups 1-5) and appeared
mostly on the tail and ears after 8 days post-challenge. Groups 4 and 5 contained 7 of the 12 animals that presented with lesions. No lesions were noted on any animals that succumbed to disease prior to 8 days post-challenge. Animals in Groups 4 and 3 (292.5 and 119.5 PFU) began to demonstrate clinical signs of disease similar to those observed in Group 5 between days 5 and 8 post-challenge, first displaying signs of lethargy and ruffled fur, followed by hunched posture and lacrimation. Animals in Group 2 (27.5 PFU) and Group 1 (2.44 PFU) took slightly longer to display signs of disease, with lethargy typically appearing 8-10 days post-challenge. Clinical signs of disease in Groups 1 and 2 were more varied compared to the higher titer groups, with some animals succumbing to disease before any clinical signs of disease were observed or within 12 hours of the first display of clinical signs. Respiratory abnormalities were noted in some animals across all groups, appearing in animals that survived until day 12 post-challenge in low dose groups, where high dose groups presented sooner, about 8-9 days post-challenge.

3.3 Natural History of Disease

3.3.1 Mortality

Animals in Groups 1 through Group 33 were serially euthanized at scheduled time point post-challenge and were therefore not considered for mortality data. Mortality statistics were only performed on Groups 34 and 35 (control groups to demonstrate the model was consistent with the previous results), where Group 34 was challenged with 200 PFU ECTV-Mos and monitored until animals succumb to disease or were euthanized following the display of serious clinical disease, and Group 35 was mock-challenged with
PBS. All animals in Group 34 succumbed to disease between 7 and 10 days post-challenge, resulting in a 100% estimated mortality rate. The median time-to-death for Group 34 was 8.94 days post-challenge. All of the mock-challenged animals in Group 35 survived until the end of study (Table 3, Figure 5). To determine if the sex of the animal influences time to death, a log-rank test was conducted comparing time to death of the female mice in Group 34 with that of the male mice in Group 34 (data not shown). There was no significant difference in time-to-death for female versus male animals.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sex</th>
<th>Number of Animals Succumbed / Total Number of Animals</th>
<th>Mortality Proportion (Binomial Exact 95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 (ECTV)</td>
<td>Combined</td>
<td>8/8</td>
<td>100 (0.63, 1.00)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4/4</td>
<td>100 (0.40, 1.00)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4/4</td>
<td>100 (0.40, 1.00)</td>
</tr>
<tr>
<td>35 (Sham)</td>
<td>Combined</td>
<td>0/8</td>
<td>0 (0, 0.37)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>0/4</td>
<td>0 (0, 0.60)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>0/4</td>
<td>0 (0, 0.60)</td>
</tr>
</tbody>
</table>

Table 3. Mortality Results for Groups 34 (ECTV) and 35 (Sham)
3.3.2 Body Weights

Animals were weighed prior to challenge on Study Day 0 to determine a baseline weight. They were subsequently weighed every other day until Study Day 14 or until the animals succumbed to disease. Only the weights from animals in Groups 34 (ECTV challenge) and 35 (mock-challenge) were analyzed. Group mean weight over time (Figure 6) and group mean change from baseline weight as a percentage of baseline weight over time (Figure 7) were evaluated. Change from baseline ANOVA least-square mean estimates and group comparisons of weight data were also evaluated (Table 8). Animals in Group 34 steadily gained weight until 8 days post-challenge where significant weight loss occurred. All animals succumbed to disease before the next scheduled weigh
in on day 10 post-challenge. Sham infected animals experienced significant weight loss until day 4 post-challenge, then subsequently gained weight steadily until study day 14 (end of study). Significant weight gain was noted for sham infected animals from day 12 post-challenge until day 14.

Figure 6. Mean weight over time for Groups 34 (ECTV) and 35 (Sham)
Figure 7. Group mean change from baseline weight (as a percentage of baseline weight) over time.

<table>
<thead>
<tr>
<th>Days Post-Challenge</th>
<th>ECTV</th>
<th>Sham</th>
<th>Group Effect P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-0.03</td>
<td>-0.38 ↓</td>
<td>0.0002 *</td>
</tr>
<tr>
<td>4</td>
<td>0.04</td>
<td>-0.33 ↓</td>
<td>0.0039 *</td>
</tr>
<tr>
<td>6</td>
<td>0.18</td>
<td>0.16</td>
<td>0.9127</td>
</tr>
<tr>
<td>8</td>
<td>-0.97 ↓</td>
<td>0.08</td>
<td>0.0029 *</td>
</tr>
<tr>
<td>10</td>
<td>N/A</td>
<td>0.23</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>N/A</td>
<td>0.65 ↑</td>
<td>N/A</td>
</tr>
<tr>
<td>14</td>
<td>N/A</td>
<td>0.69 ↑</td>
<td>N/A</td>
</tr>
</tbody>
</table>

↑, ↓: Indicates that the difference between baseline and study day means was significantly different from zero (at the 0.05 level); “↑” indicates that the mean at the study day was greater than baseline, while “↓” indicates that the mean at the study day was less than baseline.

N/A: No animals in the group had weight measurements after study day 8.

Table 4. Results of Change from Baseline (Study Day 0) ANOVA Comparison and Least Square Mean Estimates of Body Weight.
3.3.3 Temperatures

Body temperatures were taken twice daily from four days prior to challenge through study day 0 (challenge day) via an implanted telemetry transponder chip to determine a baseline temperature for each animal (Figure 8). After challenge, body temperatures were recorded three times a day until the animal were serially euthanized, succumbed to disease, or study day 14 (end of study). Temperature data from animals in Groups 34 (ECTV challenged) and Group 35 (mock-challenged) was analyzed. Temperatures of animals in Group 34 remained fairly stagnant and were maintained within a small range until immediately prior to death when euthanized. Animals experienced significant drops in body temperature around 9 days post-challenge, and were euthanized. There were no significant findings in the control Group 35 animals.

Figure 8. Group Mean Temperatures (F) with 95 Percent Confidence Intervals by Group and Study Day.
3.3.4 Clinical Observations

Clinical observations were recorded three times daily following challenge from Study Days 1 through 10 and twice daily from Study Days 11-14. Observed adverse clinical signs of disease were first observed between 4 and 5 days post-challenge and mimicked those of animals in the potency and LD50/LD90 study. Signs included lethargy, ruffled fur, hunched posture, lacrimation, ocular abnormalities, and a low presence of lesions located mainly at the base of the ears and tails. Lethargy and ruffled fur were the first and most common signs of disease, with lacrimation occurring around day 7 post-challenge. A scoring system was created to analyze disease progression (Table 5).

<table>
<thead>
<tr>
<th>Severity of condition</th>
<th>Clinical condition / Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General Appearance</strong></td>
<td></td>
</tr>
<tr>
<td>Mild (1)</td>
<td>1 of 3 of the following signs: Lethargy, Hunched Posture, Ruffled Fur</td>
</tr>
<tr>
<td>Moderate (2)</td>
<td>2 of 3 of the following signs: Lethargy, Hunched Posture, Ruffled Fur</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>3 of 3 of the following signs: Lethargy, Hunched Posture, Ruffled Fur or Lesions present</td>
</tr>
<tr>
<td><strong>Dyspnea</strong></td>
<td></td>
</tr>
<tr>
<td>Mild (1)</td>
<td>Undefined</td>
</tr>
<tr>
<td>Moderate (2)</td>
<td>Demonstrates respiratory abnormalities</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>Demonstrates labored breathing</td>
</tr>
<tr>
<td><strong>Ocular Abnormalities</strong></td>
<td></td>
</tr>
<tr>
<td>Mild (1)</td>
<td>Clear discharge from one or both eyes</td>
</tr>
<tr>
<td>Moderate (2)</td>
<td>Clear discharge from one or both eye; Partial closure of one eye</td>
</tr>
<tr>
<td>Severe (3)</td>
<td>Clear discharge from one or both eyes; and/or partial closure of both eyes</td>
</tr>
</tbody>
</table>

Table 5. Clinical scoring system for ECTV infected animals
The severity of disease was recorded for appearance, dyspnea, and ocular abnormalities. A score of “0” represents a normal observation; a score of “1” represents a mild disease condition; a score of “2” represents a moderate disease condition; and a score of “3” represents a severe disease condition. The overall clinical observation score per animal per time point was reported as the average of the four condition scores. Animals exhibited mild clinical signs in a median TTA of 4 days (4.00, 4.33). Moderate clinical signs were displayed in a median TTA of 7 days, and severe clinical signs were soon after displayed in a median TTA of 7.66 days (Table 6).

<table>
<thead>
<tr>
<th>Severity Score of Any Clinical Sign</th>
<th>Median TTA in Days (95% Confidence Interval) for ECTV-Infected Animals</th>
<th>Median TTA in Days (95% Confidence Interval) for Sham-Infected Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>4.00 (4.00, 4.33)</td>
<td>-- (5.66, --)</td>
</tr>
<tr>
<td>Moderate</td>
<td>7.00 (--</td>
<td>-- (--</td>
</tr>
<tr>
<td>Severe</td>
<td>7.66 (7.00, 7.66)</td>
<td>-- (5.66, --)</td>
</tr>
</tbody>
</table>

-- Not enough animals became abnormal or there was not enough variability to estimate the value. In some cases, only a lower bound could be estimated (i.e. Mild severity score median).

Table 6. Clinical Observation Kaplan Meier Results.

Additionally, general appearance and ocular abnormalities as a whole were scored for each of the surviving animals in Groups 1 through 33 on a scale consisting of four levels: condition not present (0), mild (1), moderate (2), and severe (3). Estimated odds ratios
and corresponding confidence intervals were calculated via logistic models using a 0.05 level of significance testing the differences in each clinical parameter between ECTV and sham-infected animals. Significant differences in general appearance scores between ECTV and sham-infected animals were first seen four days post challenge (Odds Ratio (OR): 1.796; p-value = 0.0057) and continued to be significant until the end of the study when animals were considered to be displaying mild or stronger signs. Significant differences in ocular abnormalities were seen even sooner in disease progression, where ECTV-infected animals were 1.701 times as likely to display mild or stronger ocular abnormalities than sham-infected animals three days post-challenge (p-value: 0.0190).

### 3.3.5 Hematology

The proportion of animals that displayed abnormal parameter levels, the median TTA in days for all animals, and the median TTA in days for abnormal animals only were summarized (Table 9). Several measured hematology parameters yielded abnormal results in the majority of evaluated animals (50% or greater), including RBC count, lymphocyte count, monocyte count, basophil count, and large unstained cell count (LUC), all reaching a median TTA from four and a half to six days post-challenge. Although these parameters were abnormal for the majority of sampled animals, not all had a distinguishable trend. Mean RBC counts remained relatively constant over days post-challenge with a median TTA of six days (CI: 5.00, 7.00).
3.3.6 Clinical Chemistry

The proportion of animals that displayed abnormal parameter levels, the median TTA in days for all animals, and the median TTA in days for abnormal animals were calculated (Table 10). BUN and BUN/creatinine ratio levels yielded abnormal results in the majority of evaluated animals (50% or greater), where levels were abnormally high (normal BUN levels, 17.0 – 60.0 mg/dL). The median TTA for abnormally high BUN levels was five days post-challenge (Confidence Interval (CI): 3.50, 6.00 days), although abnormal levels remained relatively uniform over time. The median TTA for abnormally high BUN/creatinine ratio was also five days post-challenge (CI: 1.50, 6.00 days), with levels generally steadily increasing per time point after challenge (Figure 10). Although a majority of animals did not show abnormal AST and ALT levels (normal levels being 24.0-475 U/L and 15.0 – 200.0 U/L respectively), common trends could be seen across hours post-challenge. AST levels showed an increasing trend, where levels slowly began to rise after 2 days post-challenge, and continued to rise until 8 days post-challenge. Mean abnormal levels of AST were first detected 1.5 days post-challenge, with extremely high levels (Geometric mean of 1,157.5 to 3,581.6 U/L) occurring from 6.5 through 7.5 days post-challenge. ALT levels (normal levels: 15.0 – 200.0 U/L) were similarly expressed with a general rise in levels starting two days post-challenge. Mean abnormal levels were first detected 6.5 days post-challenge (GM 1,161.9 U/L), and continued until 7.5 days post-challenge (GM 2,784.5 U/L). Although only one animal experienced abnormally high CRP levels, a general increasing trend in CRP levels can be seen over time starting around 4 days post-challenge.
3.3.7 Pathology

Gross lesions were noted at the time of necropsy, although rare and limited to animals that succumbed to disease six days (144 hours) post-challenge and after, and included lesions on the tail and discoloration of the spleen and liver. Microscopic signs of disease in the livers and spleens of ECTV-infected mice were absent until five days (120 hours) post-challenge (Table 11). Hepatocellular viral cytoplasmic inclusions and hepatocellular necrosis was detected at minimal levels in the livers of infected animals 120 hours post-challenge, and continued to be detected, progressing to mild levels until 192 hours post-challenge. Fibrin, inflammation, and lymphoid necrosis were first detected in the liver at minimal and mild levels 120 hours post-challenge. Fibrin levels progressed from mild to moderate 168 hours post-challenge and were considered marked by 192 hours post-challenge. Inflammation of the liver tended to be minimal across all time points. Lymphoid necrosis was first detected at mild levels 120 hours post-challenge and shortly progressed to showing moderate and marked levels at 144 and 156 hours post-challenge, respectively. Hemorrhage and red pulp necrosis of the liver was first detected at 168 hours post-challenge at moderate and marked levels, respectively. Both parameters continued to be present until 192 hours post-challenge at mostly moderate levels. There were no findings unrelated to ECTV-Mos infection in the liver and spleen examined.
3.3.8 Viral Burden

3.3.8.1 Viremia

ECTV vDNA was first detected by qPCR in the blood 84 hours post-challenge, although detected levels were below the limit of quantification (LOQ). The first quantifiable levels of vDNA (1.180 x 10^5 copies/mL) were detected 108 hours post-challenge and continued to increase until the end of study (Figure 9). Plaque assay analysis was conducted to determine the infectious viral load in the liver, spleen, and blood samples, as opposed to non-infectious vDNA. Infectious virus in the blood was first detected via plaque assay 108 hours (4.5 days) after challenge, where viral titers measured 4.47 x 10^3 PFU/mL. Titers continued to increase until the end of study, reaching maximum levels of 1.36 x10^6 PFU/mL (CI: 3.55 x10^4, 5.19 x 10^7 PFU/mL) at 180 hours post-challenge. (Figure 10)
Figure 9. Plot of Individual Animal qPCR in Blood over Time

Figure 10. Plot of Individual Animal Plaque Assay Levels in Blood over Time
3.3.8.2 Oropharyngeal Swabs

Viral DNA was not detected in the oropharyngeal swabs (by qPCR) from animals at any time point.

3.3.8.3 Spleen and Liver Tissues

ECTV vDNA was first detected in the liver and spleen 60 hours post-challenge, although levels detected were below the LOQ. Quantifiable vDNA levels were first detected 84 hours post-challenge and continued to increase until the end of study. Although virus was detectible at the same time points in the liver and spleen samples, levels of vDNA in the spleen became higher at a faster rate than that of vDNA levels in the liver (Figure 11 and Figure 12). Infectious virus was first detected in both spleen and liver samples via plaque assay 84 hours (3.5 days) post-challenge, with titers equaling $1.34 \times 10^4$ and $3.30 \times 10^5$ PFU/mL, respectively. Following the same trend seen in qPCR data analysis, viral titers in spleen tissues became higher at a faster rate and remained slightly higher than that of viral titers in liver tissues in the hours post-challenge (Figure 13 and Figure 14).
Figure 11. Plot of Individual Animal qPCR in the Liver over Time

Figure 12. Plot of Individual Animal qPCR in Spleen Tissue over Time
Figure 13. Plot of Individual Animal Plaque Assay Levels in Liver over Time.

Figure 14. Plot of Individual Animal Plaque Assay Levels in Spleen over Time.
Chapter 4: Discussion

VARV is the causative agent of smallpox and it is believed that there is a possibility that it could be utilized in a bioterrorism attack in the future. Because smallpox has been eradicated, there are no human subjects that could be employed in anti-orthopoxvirus biological countermeasure efficacy testing. The FDA developed the ‘Animal Rule’ to allow the use of controlled animal efficacy data to support the licensure of biological products to combat or prevent diseases that are either not feasible or unethical to test in the human population. Because of this, the development of a surrogate animal model of disease that most closely resembles the desired disease pathology and outcomes as the pathogen at hand in an infected human is required. In order to develop a surrogate animal model of respiratory smallpox disease in the human, BALB/c mouse were infected IN with ECTV-Mos to define the clinical parameters that provide the earliest indication of disease onset following challenge and that correlate with time-to-death and survival for the future use in anti-orthopoxvirus drug efficacy evaluation.
4.1 Comparison of Disease Pathogenesis after Infection with ECTV Through Various Inoculation Routes in the BALB/c Mouse Model

The BALB/c mouse model has previously been proven highly susceptible to ECTV infection. Although very few ECTV disease pathogenesis studies have been published using any of the studied mouse models, previous studies that utilize the ECTV infected BALB/c mouse model have employed the footpad scarification and subcutaneous routes of exposure as well as IN inoculation and demonstrated high mortality rates with low viral inoculation titers (Jacoby & Bhatt, 1987; Xiao, Aldaz-Carroll et al., 2007; Krzyzowska, Polanczyk et al., 2005; Martyniszyn, Szulc-Dabrowska et al., 2013). Footpad scarification mimics what is believed to be the natural route of ECTV transmission, but does not produce disease that includes respiratory involvement. Utilizing an IN route of exposure more closely resembles the disease pathogenesis of a human after aerosol exposure to VARV during a bioterrorism attack, and subsequently the natural infection of the susceptible population via person-to-person liquid droplet respiratory exposures after initial infection of individuals at the primary source of exposure. Few studies that utilize BALB/c mice along with the IN route of exposure to ECTV-Mos have been performed. These reports have reported LD$_{50}$ values ranging from 1 to 5 PFU, similar to the results of the current study (estimated LD$_{50}$ of $> 2.44$ PFU) (Xiao Y., Aldaz-Carroll L. et al., 2007; Israely, Paran et al., 2012). The reported LD$_{50}$ values for studies utilizing footpad scarification tends to be lower than IN infected animal LD$_{50}$ values, with calculated estimated values below one PFU (Martyniszyn, Szulc-Dabrowska et al., 2013). Because of the standard error associated with plaque assay analysis (used to determine the viral titer of the challenge material), it is widely regarded
that any value below 10 PFU is statistically similar. Thus, the LD$_{50}$ of ECTV-Mos in the BALB/c mouse via IN or footpad routes of exposure is likely to be the same. Based on several pieces of evidence the infectious dose of VARV is believed to be very low, therefore the low infectious dose needed to cause disease in the BALB/c model is an ideal characteristic for a surrogate animal model for VARV infection (Fenner, Henderson et al., 1988). Combining data from the potency, LD$_{50}$/LD$_{90}$, and natural history studies, animals succumbed to disease between five and fourteen days post-challenge. The median times-to-death were 8.00, 8.80, 8.81, 9.83, and 10.84 days for animals infected with 700, 292.5, 119.5, 27.5, and 2.44 PFU ECTV-Mos, respectively. Similar studies reported median times-to-death of 8 to 9 days after IN challenge of BALB/c mice with approximately 15 PFU and 1000 PFU of ECTV-Mos (Xiao Y., Aldaz-Carroll L. et al., 2007; Parker, Siddiqui et al., 2009; Israely, Paran et al., 2012). The time-to-death in the ECTV model is relatively shorter to that in VARV-infected humans, where death in ordinary smallpox cases occurs approximately 18 to 22 days post infection (Fenner, Henderson et al., 1988). One of the important factors when developing an animal model for the efficacy testing various drugs against human diseases is developing a model that is clinically relevant to human disease, including mortality rates. Because smallpox has been eradicated and reemergence would be attributed to a release of VARV via a bioterrorism attack, decreasing the mortality rates of our current almost entirely susceptible population would be one of the most pressing objectives. Although smallpox has an estimated mortality rate of 30-40%, utilizing a mouse model with upwards of 100% mortality, such as the BALB/c mouse, can be beneficial when evaluating the
efficacy of potential vaccines and therapeutics, as the model would better amplify differences in the effectiveness of various biologics when the desired outcome is survival.

Similar to the findings in the current study, ECTV-infected (1,000 PFU/animal) control animals in a smallpox vaccine efficacy study that evaluated body weight did not experience weight loss below 20% of their baseline weight after IN infection, although certain control animal outliers lost upwards of 25% of their baseline weight (Xiao, Aldaz-Carroll et al., 2007). Published data pertaining to weight loss after ECTV infection is largely limited to drug efficacy studies, with only one small control group for data comparison to the data obtained from the natural history study. Animals who were infected and subsequently treated tended to lose a higher percentage of weight than infected control animals, where the majority of the increased weight loss percentages occurred after the death of the control animals. The differences in the observed weight loss could also potentially be contributed to the cage effects of maintaining animals in a group housing situation, where animals could potentially utilize huddling more easily and thereby reduce heat loss and caloric expenditure (Chvedoff et al. 1980).

Liver and spleen samples were harvested in the current study to assess the degree of their involvement in disease pathogenesis, as the ECTV model is known to develop high viral titers and major tissue damage in these organs, unlike human smallpox infections (Chapman J.L., 2010). A study that evaluated the effects of ECTV strain NIH-79 infection on the spleen found lesions that consisted of massive necrosis of lymph follicles, congestion of the red pulp and fibroses, and necrosis of the spleen by 7 days post-infection (Martyniszyn, Szulc-Dabrowska et al., 2013). Similarly, we reported
microscopic abnormalities first appearing in the spleen two days earlier, with symptoms that included inflammation, fibrin, and lymphoid necrosis, followed by red pulp necrosis and hemorrhage around 7 days post-infection. In the same study, reports of infectious virus and viral antigen were widely distributed in the BALB/c, with virus detected in the respiratory tract, genital tract, oral tissues, bone marrow, and intestines (Bhatt, Jacoby et al., 1988; Parker, Siddiqui et al., 2009). Overall, our findings of disease pathogenesis in the BALB/c model coincide with what little data is available pertaining to IN and other methods of inoculation of ECTV in the BALB/c mouse model.

4.2 Comparison of the BALB/c Model to Other IN ECTV Mouse Models

Other than the BALB/c mouse strain, the A/Ncr and C57BL/6 mouse strains are the two strains other than BALB/c mice that have been moderately studied as surrogate animal models for human smallpox disease. While the BALB/c and A/Ncr models are considered to be highly susceptible to ECTV infection, the C57BL/6 model is largely considered to be a resistant mouse strain (Parker, Siddiqui et al., 2009; Bhatt, Jacoby et al., 1988; Fenner, 2000). The C57BL/6 mouse is highly resistant to ECTV infection via the footpad scarification route, but is in fact susceptible to ECTV-Mos infection through IN inoculation at high inoculation titers, with a reported LD$_{50}$ of 90 PFU (Parker, Siddiqui et al., 2009). The LD$_{50}$ of the A/Ncr mouse infected via footpad scarification and IN infection have been estimated to be <.009 and 0.3 PFU respectively, making it equally as, or possibly more highly susceptible to ECTV infection than the BALB/c mouse (estimated LD$_{50}$ of < 2.44 PFU) (Parker, Siddiqui et al., 2009). After IN infection of 1,500 PFU ECTV-Mos, virus particles were detected in liver samples 2 and 3 days post-infection in the A/Ncr and C57BL/6 mice, respectively, and liver titers increased
thereafter. Viral titers in the livers of A/Ncr mice were approximately two logs greater than those of C57BL/6 mice 6 days post-infection. Viral titers were also detected in the spleens of both strains, with A/Ncr mice consistently producing higher titers several days earlier than the C57BL/6 mice. Along with evaluating viral load in the spleen and liver, levels of AST and ALT in the blood were measured as biomarkers of acute liver damage. AST and ALT levels are reported to be consistently elevated 6 days post-infection in A/Ncr mice, where AST and ALT levels in the C57BL/6 mice tended to take approximately 7 days to become abnormal with highly variability. Similar to our results in the BALB/c mouse, significant weight loss was displayed 7 days post-challenge in both mouse strains, along with the display of clinical signs of disease such as ruffled fur, conjunctivitis, and lethargy 6 days post-challenge (Parker, Siddiqui et al., 2009). The same study reported the average time to death for A/Ncr and C57BL/6 mice being 7.2±0.2 and 9.2±0.2 days post-challenge, respectively (Parker, Siddiqui et al., 2009). An estimated mean time to death for IN ECTV-Mos infected (800 PFU) C57BL/6 mice from a different study was reported at 11±0.6 days post-infection (Parker, Chen et al., 2012).

Because smallpox is believed to have a low infectious dose, it is important that a surrogate animal model requires a low infectious dose to induce disease pathogenesis. The C57BL/6 mouse strain requires a high infectious dose to cause disease and consequently might not be the best model for smallpox drug efficacy testing. Furthermore, C57BL/6 mice exhibit Th1 biased immune responses that allow for a more robust immune system that is more efficient at fighting highly infectious diseases, ultimately making the animals less susceptible to ECTV infection (Parker, Siddiqui et al., 2009). When testing anti-orthopoxvirus drug products, it is important to establish clear
primary and secondary endpoints. Primary endpoints indrug efficacy studies pertaining to high mortality bioterrorism-class infectious agents tend to revolve around the survival of animals, where secondary endpoints such as the reduction of viremia, and the better regulation of body temperature and body weight could also be analyzed in terms of the reduction of disease progression by a biological product. If an animal is known to possess an unusually robust immune system, it is harder to determine if the reduction in disease severity or the survival of the animal can be attributed to the drug product at hand, or the natural immune defenses of the animal, potentially causing uncertainties about the effectiveness of the product at hand. For these reasons, the use of the C57BL/6 mouse strain may not be the best option when evaluating the effectiveness and efficacy of anti-orthopoxvirus vaccines and therapeutics. Like the BALB/c mouse model, the A/Ncr mouse has been shown to be naturally highly susceptible to IN ECTV infection and shows similar clinical signs of disease. This being said, the A/Ncr mouse strain is an outbred immunodeficient nude mouse that is lacking a thymus, making it unable to produce T cells (Charles River Laboratories, 2014). This opposite extreme is also not ideal for drug efficacy testing due to the fact that it too, along with the C57BL/6 strain, has a susceptibility threshold to disease that might differ too greatly when compared to the majority of human Orthopoxvirus infections (U.S.Food and Drug Administration, 2009). The naturally high susceptibility to ECTV infection and extensive knowledge pertaining to its genetics, along with a disease progression similar to that that is known for smallpox pathogenesis in humans renders the BALB/c mouse model a good rodent candidate for smallpox drug efficacy testing.
4.3 Highly Variable Hematology and Clinical Chemistry Data

Hematology and clinical chemistry parameters can and have been used as biomarkers of various diseases, but pose many hurdles when it comes to the evaluation of these parameters in certain surrogate animal models of disease. Human data, normal or “expected” hematology and clinical chemistry values for specific small animal models of disease are difficult to obtain for many reasons. Differences in assay procedure conditions and the specifications of the assayed animals are seldom the same between laboratories and potential variation due to genetic drift in animal stocks and small sample sizes contributes to varied values (Giknis M. & Clifford C.B., 2006; Parker, Siddiqui et al., 2009). Even after the close consideration of these variables, obtaining meaningful data on hematology and clinical chemistry parameters is often difficult for reasons including the difficulty of obtaining accurate published control group data and complete methodology, along with varying reported methods of blood collection, which can affect parameter outcomes (Dameron, Weingand et al., 1992; Smith, Neptun et al., 1986; Weingand, Odioso et al., 1992). Therefore, historical data collected within a single laboratory or research institute is considered the most reliable when comparing and analyzing hematology and clinical chemistry parameters. Unfortunately, the hematology and clinical chemistry obtained from the natural history portion of the study was of poor quality. High levels of variability were observed and can be attributed to a number of factors. These factors include not being able to collect enough blood sample from certain animals, or not being able to collect any blood sample from animals, along with issues with collected sample, including clotting and the presence of hemolysis. Most of these factors can be attributed to the method of blood collection, that being intra-cardiac, and
the size of the BALB/c mice. Because the BALB/c mouse is relatively small (18 – 22 grams in weight), targeting the heart without causing extensive tissue damage to obtain what little blood is present proves difficult even for highly trained technical staff.

Obtaining blood via the orbital sinus was tested and resulted in lower blood volume being obtained and was a more difficult method to perform, overall. Also, because the number of animals that were serially sacrificed at each time point was low (4 ECTV-infected animals every 12 hours), removing even one animal from group statistical analysis resulted in large confidence intervals. Because of these factors, one should take caution when interpreting the obtained hematology and clinical chemistry data from the ECTV natural history study and these parameters will not be considered in the determination of a trigger for intervention. It is likely that if any of these parameters were to be considered as triggers for intervention in the case of having obtained more statistically sound data, elevated AST and ALT levels along with abnormal BUN levels would be the most likely parameters to be chosen. BUN analysis measures the amount of nitrogen that comes from urea in the blood and is indicative of both kidney and liver damage, along with heart failure and dehydration. Abnormally high levels of BUN can be indicative of kidney and heart failure and dehydration, where abnormally low levels of BUN can be indicative of liver damage. Abnormal BUN levels were expressed in 47/60 ECTV-infected animals with a median time to abnormal of 5 days. Because elevated levels of AST and ALT are indicative of acute liver damage and hepatic disease, it is likely that elevated levels of AST and ALT could be seen as early as 120 hours (5 days) post-challenge, as this is the time point in which liver damage is first observed via histopathology analysis. Similar studies found significantly elevated levels of AST and ALT by day 6 and 7 post-infection
in A/Ncr and C57BL/6 mice, respectively, which were shown to correlate closely with the average time to death (Parker, Siddiqui et al., 2009). Previous studies performed by the same laboratory found ALT levels to be less variable and better indicators of disease progression compared to AST levels (Parker, Schriewer et al., 2008). Data obtained from the 2003 Monkeypox outbreak in the United States revealed that AST and ALT levels were abnormally high in 50% and 59% of analyzed individuals, respectively (Parker, Siddiqui et al., 2009; Huhn, Bauer et al., 2005). Although there are no reports of liver involvement in smallpox cases and the general belief of an absence of liver and spleen involvement, high AST and ALT levels in patients infected with MPXV can raise speculation about the validity of this claim. MPXV is in the same genus as VARV and its disease presentation is largely similar to that of smallpox, although less virulent. One major difference in the pathophysiology of ECTV vs. VARV is the presence spleen and liver involvement, and if liver involvement is present in smallpox patients as it has proven to be in monkeypox patients, the ECTV surrogate mouse model could prove to be an even better disease model for smallpox infection. Evaluation of AST, ALT, and BUN levels after ECTV-challenge should be conducted with larger sample size, a more reliable method of blood collection, or a combination of both to obtain more accurate data if it is to be considered a potential trigger for intervention.
4.4 Determining a Trigger for Intervention for Drug Efficacy Testing in the BALB/c Model

One critical issue when utilizing animal models to test the efficacy of a biological product for licensure is the fact that the pathophysiology in the experimental animal model may not completely summarize human disease. This being said, although the exact disease pathophysiology of human smallpox disease is not attained in the ECTV model, mousepox is arguably the best respiratory challenge surrogate rodent model for smallpox (Parker, Chen et al., 2012). Due to the disease differences of smallpox compared with mousepox and the fact that there were very few disease-defining manifestations measured in smallpox victims beyond rash presentation and viral infectivity, it is difficult to determine an appropriate trigger for therapeutic intervention in ECTV-infected mice. A “disease-defining manifestation” and/or a “biological parameter/biomarker” are two categories of triggers for intervention that are supported by the FDA under the Animal Rule (U.S. Food and Drug Administration, 2009). Because the appearance of the characteristic pustular rash was critical in the diagnosis of smallpox during its existence, the presence of a rash, or lesions, is considered a “disease-defining manifestation” and would be the ideal trigger for intervention in the mouse model.

Lesions were noted in a small number of mice throughout all three studies, mainly found on the base of the tails and the ears. It is unclear whether or not the noted lesions were truly lesions, as it is more likely that the small scabbing regions on the tails and ears of infected animals were bites marks from aggressive cage-mates. This argument becomes even more likely when evaluating the reported lesion counts of sham-infected animals, which were also reported as having similar ‘lesions’ on their tails. Previously conducted
studies report presence of lesions on animals whose hair had been removed via waxing. They concluded that the reported ‘lesions’ were more consistent with folliculitis and other consequences of forceful removal of the hair as opposed to an ECTV-associated rash (Parker, Chen et al., 2012). Unfortunately, it has been determined by our findings and the findings of others that the appearance of lesions is rare and highly variable, making it a poor trigger for intervention (Parker, Chen et al., 2012; Cespedes, Toka et al., 2001).

Signs of clinical disease such as lethargy and ruffled fur were first observed 4 to 5 days post-infection, potentially early enough in disease progression to be a possible trigger. Due to the fact that clinical signs of disease are largely objective and can be interpreted differently by various individuals, they typically should not be solely used as a trigger for intervention, but rather paired with a more concrete biomarker of infection if possible. The presence of mild or stronger ocular abnormalities, such as lacrimation and the partial or complete closure of one or both eyes is one of the most promising triggers for intervention. Ocular abnormalities are seen in ECTV-infected animals due to viral replication in the mucosal tissues surrounding the eyes, causing tissue damage, inflammation, and fluid drainage as a result of innate immune responses (source). With ECTV-infected animals being 1.7 times as likely to display ocular abnormalities as sham-infected animals three days post-infection (p-value: 1.0190), this clinical parameter is the first measurable sign of clinical disease that is displayed by the infected animals. Although most clinical signs of disease, such as lethargy and ruffled coat, are highly variable and subjective depending on the individual completing animal evaluation, ocular abnormalities are relatively objective and are easy to discern. The determination of ocular abnormalities is non-invasive and requires no turn-around time, as results can be recorded
as they are examined, an advantage when trying to treat an animal as quickly as possible after disease onset.

Weight loss and presence of a ‘fever’ are two non-specific disease manifestations that could be used as potential triggers for intervention. Significant weight loss first occurred 4 days post-challenge in animals infected with a high titer, where it did not occur in the subsequent lower challenge groups (292.5 and 119.5 PFU/animal) until 8 days post-challenge. With a median time-to-death of 8.94 days for animals infected with a mid-range viral titer, it is safe to assume that most animals would be too far advanced in their disease progression by the time significant weight loss was displayed to be saved by a medical intervention, making weight loss a poor trigger for intervention. Further proving that weight loss is highly variable and a poor indicator for disease progression, sham infected animals were reported as losing significant amounts of weight during the beginning days of the natural history study, when ECTV-infected animals did not display significant levels of weight loss as would be expected. Another potential explanation for why high degrees of weight loss were not seen in the study is the fact that the animals are still considered to be very young and are still at a point in their development when they should be steadily gaining weight. Because of this, older animals could potentially display more extreme levels of weight loss, although most likely still at a point where disease progression is too far advanced to be saved by any therapy. Body temperature data showed no clear pattern, with relatively stagnant temperatures after challenge in both ECTV- and sham-infected animals. In fact, sham-infected animals experienced rises in temperature at an earlier time point and had generally higher body temperatures the majority of the study when compared to ECTV-infected animals.
Viral DNA was first detected in the blood by qPCR 84 hours post-challenge, albeit at levels below the limit of quantification. After the initial time point of viremia detection, approximately 78% (29/30) of animals in the 96 to 192 hour euthanasia time points had detectible blood-virus levels either below the limit of quantification, or at quantifiable viral levels. Detection of viral load in the liver and spleen were also early in disease progression, where virus was detected in both organs after only 60 hours post-challenge at levels below the limit of quantification. Quantifiable virus levels were detectible at 84 hours post-challenge in both liver and spleen tissue, where viral titers in the spleen became higher at a faster rate than in the liver, although titers were relatively high in both organ tissues. Although virus was detected in the spleen and liver tissues 24 hours before it was detected in the blood, the use of spleen and liver viral load as a trigger for intervention would be challenging due to the fact that the animals must be euthanized and organs harvested and processed to obtain results. The main drawback of utilizing viremia as a trigger for intervention is the amount of blood that is needed to run qPCR and plaque assay analysis, along with the length of time needed to complete one plaque assay, as previously described. Depending on the method of blood collection, animals do not need to be euthanized to collect blood samples to be evaluated for the presence of virus. As an alternative approach, one or two animals per time point group could be chosen at random to be sacrificed to undergo blood collection for the analysis of viremia via qPCR. Results from that single animal could be generalized over the group of animals as a whole, and the group of animals could be treated if the sacrificed animal revealed positive viremia, or the group could remain untreated if viremia was not displayed. This method would reduce the amount of animals that would need to undergo
potentially life-threatening blood draws while still yielding results allowing the treatment, or non-treatment, of the corresponding group of infected animals.

Another parameter that was evaluated as a potential trigger for intervention was the presence of viral DNA in oropharyngeal secretions. The detection of virus in saliva is convenient and non-invasive, making it an ideal method of disease detection and diagnosis. PCR has been used previously in the detection of several different viruses in the saliva and can be used as an early diagnostic test for infection (Balamane, Winters et al., 2010; Bilder, Machtei et al., 2011; Belec & Brogan, 2011; Boppana, Ross et al., 2011; Magalhaes, Martins et al., 2011). The presence of vDNA in oropharyngeal secretions has been shown to be a consistent early biomarker of virus replication in other Orthopoxvirus animal models, having been detected via PCR as early as four days p.i. in NHP models after MPXV aerosol challenge and four to six days p.i. in MPXV infected prairie dogs (Magalhaes, Martins et al., 2011; Smith, Self et al., 2011). Unfortunately, the presence of virus in the oropharyngeal secretions was not detected, although virus has been proven to be present in the oropharyngeal secretions of other mouse models as early as two days after IN infection with ECTV (Parker, Chen et al., 2012).

Overall, based on the data obtained from these studies, the best trigger for intervention would have to be the detection of ECTV DNA in the blood and/or the presence of mild or stronger ocular abnormalities. Although the ideal trigger for intervention would be the presentation of a characteristic pustular rash, the presence of ocular abnormalities and the detection of vDNA in the blood occur at an earlier stage of disease progression, allowing the earlier initiation of therapeutic intervention and in turn translating into higher degrees of treatment efficacy. Also, in a real-world situation there
are other clinical rash-causing diseases such as chickenpox and monkeypox that have been misinterpreted as smallpox infections (Fenner, Henderson et al., 1988; MacNeil, Reynolds et al., 2009; Parker, Siddiqui et al., 2009). Because smallpox has been eradicated, misdiagnosis of a rash-presenting disease that is not smallpox as a smallpox infection would result in public panic. Therefore, using DNA detection assays provides a higher level of certainty that a questionable *Orthopoxvirus* infection is in fact caused by an *Orthopoxvirus*, or that it is a completely unrelated disease. Reversely, monkeypox infections can be misdiagnosed as chickenpox based on rash presentation alone (Meyer, Perrichot et al., 2002; Rimoin, Kisalu et al., 2007). For these reasons, the detection and diagnosis of *Orthopoxvirus* infections in real-world scenarios is most accurate, and can occur at the fastest rate via the use of DNA signature detection assays. Therefore, the trigger for intervention to obtain the highest levels of drug efficacy when evaluating disease endpoints in the surrogate IN ECTV-challenged BALB/c animal model should be the detection of vDNA in the blood paired with the presence of ocular abnormalities.
References


Appendix A: Tables

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge</th>
<th>No. of Animals (M/F)</th>
<th>Oropharyngeal Secretions (Swabs) (hours)</th>
<th>Clinical Data</th>
<th>qPCR/Plaque Assay (Hours)</th>
<th>Hematology/Clinical Chemistry (Hours)</th>
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1 Oropharyngeal secretions (swabs) will be analyzed by qPCR.
2 Clinical data includes: monitoring for rash, body temperature, and clinical observations. Body weights will be recorded for all animals on study.
3 The blood draws will occur ±1 hour with respect to each designated time point.

Table 7. Study design for the Natural History of Disease Study
<table>
<thead>
<tr>
<th>Study Day</th>
<th>Mean Change from Baseline Weight (grams), by Group</th>
<th>Group Effect</th>
<th>Estimated Difference (Relationship)</th>
<th>Tukey's P-value</th>
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<td>Group 3</td>
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<td>0.4 ↑</td>
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# Cells contain all pairwise comparisons that are statistically significant at the adjusted 0.05 level. The format within each cell is: (1) the difference in shifts, (2) the relationship between the corresponding pair of group mean shifts shown in parentheses, and (3) the Tukey-adjusted p-value.

↑, ↓ “↑” indicates the group mean at the study day was significantly greater than that at pre-treatment baseline; “↓” indicates the mean at the study day was significantly less than that at pre-treatment baseline (at the 0.05 level).

* Group effect was significant at the 0.05 level.

NA These shifts were not calculated due to the small sample size of either one or zero surviving animals on this study day.

Table 8. Results of Change from Baseline ANOVA Least-Square Mean Estimates and Group Comparisons of Weight Data
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<th>Parameter</th>
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<th>Median TTA in Days (95% Confidence Interval)</th>
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<td>RBC</td>
<td>38/60</td>
<td>0.63 (0.50, 0.75)</td>
<td>6.00 (5.00, 7.00)</td>
<td>4.88 (2.45)</td>
</tr>
<tr>
<td>HGB</td>
<td>23/60</td>
<td>0.38 (0.26, 0.52)</td>
<td>7.00 (6.50, 7.50)</td>
<td>5.46 (2.18)</td>
</tr>
<tr>
<td>HCT</td>
<td>27/60</td>
<td>0.45 (0.32, 0.58)</td>
<td>6.50 (6.00, 7.50)</td>
<td>5.48 (2.14)</td>
</tr>
<tr>
<td>MCV</td>
<td>10/60</td>
<td>0.17 (0.08, 0.29)</td>
<td>8.00 (6.50, 8.00)</td>
<td>5.40 (2.38)</td>
</tr>
<tr>
<td>MCH†</td>
<td>18/60</td>
<td>0.30 (0.19, 0.43)</td>
<td>7.50 (6.50, 8.00)</td>
<td>5.19 (2.25)</td>
</tr>
<tr>
<td>MCHC†</td>
<td>56/60</td>
<td>0.93 (0.84, 0.98)</td>
<td>4.50 (3.50, 5.50)</td>
<td>4.36 (2.29)</td>
</tr>
<tr>
<td>CHCM†</td>
<td>8/60</td>
<td>0.13 (0.06, 0.25)</td>
<td>-- (7.50, --)</td>
<td>4.81 (2.19)</td>
</tr>
<tr>
<td>RDW†</td>
<td>3/60</td>
<td>0.05 (0.01, 0.14)</td>
<td>-- (--, --)</td>
<td>2.83 (3.18)</td>
</tr>
<tr>
<td>PLT</td>
<td>22/60</td>
<td>0.37 (0.25, 0.50)</td>
<td>-- (4.00, --)</td>
<td>3.09 (1.12)</td>
</tr>
<tr>
<td>MPV†</td>
<td>3/60</td>
<td>0.05 (0.01, 0.14)</td>
<td>-- (7.50, --)</td>
<td>5.17 (3.21)</td>
</tr>
<tr>
<td>WBC†</td>
<td>26/60</td>
<td>0.43 (0.31, 0.57)</td>
<td>7.00 (6.00, 7.50)</td>
<td>4.83 (2.25)</td>
</tr>
<tr>
<td>Neutrophil Count†</td>
<td>26/60</td>
<td>0.43 (0.31, 0.57)</td>
<td>7.00 (6.00, 7.50)</td>
<td>5.58 (1.85)</td>
</tr>
<tr>
<td>Lymphocyte Count†</td>
<td>36/60</td>
<td>0.60 (0.47, 0.72)</td>
<td>6.00 (4.50, 7.00)</td>
<td>4.53 (2.29)</td>
</tr>
<tr>
<td>N/L Ratio†</td>
<td>27/60</td>
<td>0.45 (0.32, 0.58)</td>
<td>7.00 (5.50, 7.50)</td>
<td>5.39 (2.04)</td>
</tr>
<tr>
<td>Monocyte Count</td>
<td>36/60</td>
<td>0.60 (0.47, 0.72)</td>
<td>5.50 (4.50, 7.00)</td>
<td>4.44 (2.06)</td>
</tr>
<tr>
<td>Eosinophil Count†</td>
<td>0/60</td>
<td>0.00 (0.00, 0.06)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Basophil Count†</td>
<td>38/60</td>
<td>0.63 (0.50, 0.75)</td>
<td>6.00 (5.00, 7.00)</td>
<td>5.29 (2.15)</td>
</tr>
<tr>
<td>LUC†</td>
<td>58/60</td>
<td>0.97 (0.88, 1.00)</td>
<td>4.50 (3.50, 5.50)</td>
<td>4.41 (2.19)</td>
</tr>
</tbody>
</table>

Table 9. Descriptive Statistics for Proportions of Abnormal ECTV-Infected Animals and Time to Abnormal by Hematology Parameter.

TTA Time to abnormal.
SD Standard deviation.
a Estimates include only those animals that became abnormal.

Continued
Table 9 Continued
-- Not enough animals became abnormal or there was not enough variability to estimate the value. In some cases, only a lower bound could be estimated (i.e. CHCM median TTA).
NA Could not be calculated since one or fewer animals became abnormal.
† Distribution was log-normal for this parameter.
1 N does not include observations taken at Study Day 0 and there were four animals (119, 141, 166, and 194) with missing hematology values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number Abnormal /N↑</th>
<th>Proportion Abnormal (Clopper-Pearson 95% Confidence Interval)</th>
<th>Median TTA in Days (95% Confidence Interval)</th>
<th>Mean TTA in Days for Abnormal Only (SD)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST↑</td>
<td>9/59</td>
<td>0.15 (0.07, 0.27)</td>
<td>8.00 (7.00, 8.00)</td>
<td>4.94 (2.55)</td>
</tr>
<tr>
<td>ALT↑</td>
<td>2/60</td>
<td>0.03 (0.00, 0.12)</td>
<td>-- (7.00, --)</td>
<td>7.25 (0.35)</td>
</tr>
<tr>
<td>BUN↑</td>
<td>47/60</td>
<td>0.78 (0.66, 0.88)</td>
<td>5.00 (3.50, 6.00)</td>
<td>4.11 (2.34)</td>
</tr>
<tr>
<td>Creatinine↑</td>
<td>10/23</td>
<td>0.43 (0.23, 0.66)</td>
<td>7.50 (3.50, 8.00)</td>
<td>3.65 (2.67)</td>
</tr>
<tr>
<td>BUN/CreatinineRatio↑</td>
<td>17/20</td>
<td>0.85 (0.62, 0.97)</td>
<td>5.00 (1.50, 6.00)</td>
<td>4.35 (2.50)</td>
</tr>
<tr>
<td>CRP↑</td>
<td>1/21</td>
<td>0.05 (0.00, 0.24)</td>
<td>-- (7.50, --)</td>
<td>7.50 (--⁠)</td>
</tr>
</tbody>
</table>

TTA Time to abnormal.
SD Standard deviation.
a Estimates include only those animals that became abnormal.
-- Not enough animals became abnormal or there was not enough variability to estimate the value. In some cases, only a lower bound could be estimated (i.e. ALT median TTA).
1 N does not include observations taken at Study Day 0 or any missing values.

Table 10. Descriptive Statistics for Proportions of Abnormal ECTV-Infected Animals and Time to Abnormal by Clinical Chemistry Parameter.
Table 11. Incidence and Average Severity\(^1\) of Microscopic Findings in the Liver and Spleen of Mice Between 120 and 192 Hours Post-Challenge

<table>
<thead>
<tr>
<th>Termination (Hours Post-Challenge)</th>
<th>120</th>
<th>132</th>
<th>144</th>
<th>156</th>
<th>168</th>
<th>180</th>
<th>192</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Animals Examined</td>
<td>2M/2F</td>
<td>2M/2F</td>
<td>2M/2F</td>
<td>2M/2F</td>
<td>2M/2F</td>
<td>1M/2F</td>
<td>2M/2F</td>
</tr>
<tr>
<td><strong>Hepatocellular Viral Cytoplasmic Inclusions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>1M/0F</td>
<td>0M/1F</td>
<td>0M/1F</td>
<td>0M/0F</td>
<td>2M/2F</td>
<td>0M/2F</td>
<td>2M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Hepatocellular Necrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>1M/0F</td>
<td>0M/1F</td>
<td>0M/1F</td>
<td>0M/0F</td>
<td>2M/2F</td>
<td>0M/2F</td>
<td>2M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>1.0/NA</td>
<td>NA/1.0</td>
<td>NA/1.0</td>
<td>NA</td>
<td>1.0/2.0</td>
<td>NA/1.5</td>
<td>1.5/1.5</td>
</tr>
<tr>
<td><strong>Spleen Abnormalities</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Fibrin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>2M/1F</td>
<td>0M/2F</td>
<td>1M/1F</td>
<td>1M/1F</td>
<td>2M/2F</td>
<td>1M/2F</td>
<td>2M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>2.0/1.0</td>
<td>NA/1.5</td>
<td>1.0/3.0</td>
<td>1.0/3.0</td>
<td>3.0/3.5</td>
<td>1.0/3.5</td>
<td>3.5/4.0</td>
</tr>
<tr>
<td><strong>Hemorrhage</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Incidence</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>0M/2F</td>
<td>0M/2F</td>
<td>1M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA/3.0</td>
<td>NA/1.5</td>
<td>3.0/2.0</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>2M/1F</td>
<td>0M/2F</td>
<td>0M/1F</td>
<td>0M/1F</td>
<td>2M/0F</td>
<td>0M/1F</td>
<td>2M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>1.0/1.0</td>
<td>0M/1.0</td>
<td>0M/2.0</td>
<td>NA/1.0</td>
<td>2.0/NA</td>
<td>NA/1.0</td>
<td>1.0/1.0</td>
</tr>
<tr>
<td><strong>Lymphoid Necrosis</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Incidence</td>
<td>2M/1F</td>
<td>0M/2F</td>
<td>1M/1F</td>
<td>1M/1F</td>
<td>2M/2F</td>
<td>1M/2F</td>
<td>2M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>2.0/2.0</td>
<td>NA/2.0</td>
<td>1.0/3.0</td>
<td>1.0/4.0</td>
<td>3.5/4.0</td>
<td>1.0/3.5</td>
<td>3.5/4.0</td>
</tr>
<tr>
<td><strong>Red Pulp Necrosis</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Incidence</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>0M/0F</td>
<td>1M/2F</td>
<td>0M/1F</td>
<td>1M/2F</td>
</tr>
<tr>
<td>Severity Score</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.0/4.0</td>
<td>NA/3.0</td>
<td>3.0/3.0</td>
</tr>
</tbody>
</table>

\(^1\) Average severity was calculated as the sum of the severity scores for each affected animal divided by the number of animals affected.