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EVALUATION OF MONKEYPOX VIRUS INFECTION OF BLACK-TAILED PRAIRIE DOGS (*CYNOMYS LUDOVICIANUS*) USING IN VIVO BIOLUMINESCENT IMAGING

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Abstract

Monkeypox (MPX) is a re-emerging zoonotic disease that is endemic in Central and West Africa, where it can cause a smallpox-like disease in humans. Despite many epidemiologic and field investigations of MPX, no definitive reservoir species has been identified. Using recombinant viruses expressing the firefly luciferase (luc) gene, we previously demonstrated the suitability of in vivo bioluminescent imaging (BLI) to study the pathogenesis of MPX in animal models. Here, we evaluated BLI as a novel approach for tracking MPX virus infection in black-tailed prairie dogs (*Cynomys ludovicianus*). Prairie dogs were affected during a multi-state outbreak of MPX in the USA in 2003 and have since been used as an animal model of this disease. Our BLI results were compared with PCR and virus isolation from tissues collected postmortem. Virus was easily detected and quantified in skin and superficial tissues by BLI before and during clinical phases, as well as in subclinical secondary cases, but was not reliably detected in deep tissues such as the lung. Although there are limitations to viral detection in larger wild rodent species, BLI can enhance the use of prairie dogs as an animal model of MPX and can be used for the study of infection, disease progression, and transmission in potential wild rodent reservoirs.

Keywords

Bioluminescence; black-tailed prairie dog; histopathology; in vivo imaging; *Monkeypox virus*; *Orthopoxvirus*; poxvirus; real time PCR

INTRODUCTION

Monkeypox virus (MPXV), closely related to *Variola virus*, is an orthopox virus that has historically circulated in Central and West Africa. Discovered in captive monkeys from

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West Africa in 1958 (Von-Magnus et al. 1959), human MPX cases were first reported around 1970, as the smallpox eradication campaign lead to increased surveillance (Sejvar et al. 2004; Huhn et al. 2005). Unlike smallpox, MPX is a zoonosis, although the identity of the reservoir species remains elusive. After smallpox vaccination ceased in Africa, human MPX cases were no longer reported in West Africa, but the virus has been isolated from wild-caught animals (Hutson et al. 2007). Serologic evidence suggests that MPXV, or a related orthopox virus, is still circulating in that region (Reynolds et al. 2010; MacNeil et al. 2011). In contrast, human MPX cases continue to occur and have recently surged in Central Africa, especially Democratic Republic of Congo (DRC; Reynolds and Damon 2012). While this might be related to waning population immunity to orthopox viruses following cessation of smallpox vaccination, it is puzzling that human MPX cases occur only in DRC. Alternative theories postulate that the ecology of the reservoir species is changing or that humans are contacting reservoir hosts in new or more frequent interactions. In West Africa, these ecologic changes may not occur, or MPX cases may be under-reported there.

In 2003, MPXV was inadvertently imported from Ghana to the USA via the exotic pet trade. Several African rodents, including rope squirrels (*Funisciurus anerythrus*), Gambian pouched rats (*Cricetomys* spp.) and African dormice (*Graphiurus* spp.) were part of a multi-species importation that resulted in transmission to black-tailed prairie dogs (*Cynomys ludovicianus*) and eventually to humans. During follow-up studies of this outbreak, MPXV DNA was detected in 33 animals of five species (Hutson et al. 2007). Infection trials in potential native hosts would help characterize the disease in wild rodents and help determine which species may play important roles in the natural ecology of MPXV.

In vivo bioluminescent imaging (BLI) is used to detect luminescent marked targets (pathogens, antibodies, cancer cells) in live animals through time. This technique has been used to study various bacterial and viral pathogens in mice (Cook and Griffin 2003; Luker and Luker 2009, 2010; Ozkaya et al. 2012) to track the dissemination of pathogens between and within internal organs and to quantify and compare relative pathogen tissue load. In a previous study, we demonstrated that BLI was effective in tracking MPXV infection in immunocompetent and immunodeficient mice (Osorio et al. 2009), and that MPXV expressing the luciferase enzyme displayed no difference in pathogenicity compared to wild-type MPXV. This approach could be useful for the study of MPXV in other animal models and in its potential host species to predict sites of viral shedding and to detect viral replication in the absence of clinical disease. In this study, we used BLI to track MPXV replication in four black-tailed prairie dogs and compared our observations to PCR results and virus isolation from tissues collected postmortem.

MATERIALS AND METHODS

Animals

Adult black-tailed prairie dogs were wild-caught in South Dakota and transferred to a BSL-3 animal facility at the US Geological Survey, National Wildlife Health Center (NWHC), in Madison, Wisconsin. The animals were dusted with carbaryl prior to shipment, and, upon arrival at NWHC, they were inspected for external parasites (none were found), injected with an anthelmintic (200 µg/kg of Ivermectin; Merck and Co., Inc., West Point,

Pennsylvania, USA), then treated with 200 µL of Advantage flea control (Imidacloprid; Bayer HealthCare, Shawnee Mission, Kansas, USA), via external application to the skin on the back of the neck. They were group-housed as described by Rocke et al. (2010). Weights before infection ranged from 774 to 1,036 g.

Infection

Two adult male (PD54 and PD1) prairie dogs and one adult female (PD19) were infected via intranasal (IN) inoculation (25 µL each nostril) with 10^4 plaque forming units (pfu) of MPXV-USA-2003/LUC. This recombinant virus expresses firefly luciferase. It has been shown to be stable through multiple passages in cell culture and comparable to the parental strain in growth rate in vitro and virulence in vivo (Osorio et al. 2009). The dose chosen was based upon previous studies as likely to cause morbidity in all animals (Hutson et al. 2010). Viral titration was confirmed by back-titration using 50% tissue culture infectious dose method described below. One uninfected male animal (PD24) was cohoused with infected animals to act as a sentinel for contact transmission. Animals were monitored daily for clinical signs of disease and were euthanized in the event of severe weight loss, inability to eat or drink, or difficulty breathing.

In vivo BLI and sampling

We performed BLI on days 1, 3, 5, 7, 9, 11, 15, 18, and 25 after infection using an IVIS® 200 series in vivo imager (Perkin Elmer [formerly Caliper Life Sciences], Alameda, California, USA). Briefly, animals were injected intraperitoneally with 125 mg/kg D-luciferin potassium salt (Gold Biotechnology, St. Louis, Missouri, USA) and anesthetized with isoflurane, before being imaged with a highly sensitive Charged-coupled device (CCD) camera that is used to detect luminescence. Anesthesia nose cones were disinfected by submerging them in 70% ethanol between imaging periods, and the sentinel animal was always imaged first. Animals were imaged in dorsal and ventral views to maximize luminescence detection in this large rodent species. Images were collected and analyzed using Living Image version 3 and 4.2, respectively (Perkin Elmer, Alameda, California, USA). Region of interest (ROI) analysis was conducted using rectangular ROIs including the whole animal in the ventral view and all except those portions rostral to the eyes in the dorsal view. This was done to prevent double counting of luminescence in the nasal and oral cavities, which is visible in both dorsal and ventral views. Measurements from dorsal and ventral ROIs were added to calculate a total luminescence in radiance (photons/sec persquare centimeter per steradian).

Animals were also weighed, examined for lesions, and sampled every other day during anesthesia on the days they were imaged. Blood samples were collected via the saphenous vein into tubes containing calcium ethylenediaminetetraacetic acid. Swabs were taken of oral, nasal, conjunctival, and rectal mucosa using sterile Dacron swabs. Blood samples and swabs were frozen at -80°C without diluent.

Necropsy and sample processing

At the end of the experiment, all animals were necropsied. Tissues were trimmed with a portion frozen at -80°C and the remnants fixed in 10% neutral buffered formalin, embedded

in paraffin, sectioned at 5 µm and stained either with hematoxylin and eosin, or using immunohistochemistry following techniques previously described (Xiao et al. 2005; Osorio et al. 2009). Later, frozen tissues were sectioned, weighed, and ground in liquid nitrogen with sterilized mortar and pestles and tissue powders were added to 500 µL of phosphate-buffered saline. Tissue homogenates were sonicated before being frozen at -80 C for later viral titration and DNA extraction. Viral DNA was extracted from tissues and swabs using a QiaAMP DNA mini kit (Qiagen, Hilden, Germany).

Quantitative (Q) PCR

Viral DNA was detected using Q-PCR to detect the E9L gene of European orthopoxviruses as described by Li et al. (2006) with minor modifications: Amplitaq Gold® (Applied Biosystems, Foster City, California, USA) was used in the DNA Engine Opticon® (Bio-Rad, Hercules, California, USA). DNA standards were made by extracting DNA from purified MPXV Congo, measuring DNA concentration with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA), and making 10-fold serial dilutions in molecular grade water, resulting in standards ranging from 0.2 to 2×10^{-6} ng. The cutoff was chosen as any fluorescence value two standard deviations above background measured during cycles 3–10. Samples that surpassed the cutoff by 40 cycles were considered positive. All assays were sensitive enough to detect 2×10^{-4} ng of MPXV DNA, approximately 125 viral genomes.

Virus titration

To quantify viral particles, tissue homogenates or purified viral stocks were thawed and serially diluted in cell culture medium. Each dilution (100 µL) was incubated in eight wells each of a 96-well plate with Vero cells grown to 90% confluency. Plates were incubated for 3 days at 37 C, 5% CO₂ before cells were fixed using 1.5% crystal violet in 10% buffered formalin. The Reed and Muench (1938) method was used to calculate viral titer, and titer was normalized to the weight of tissue in each homogenate (pfu/g).

RESULTS

Clinical findings

All three infected prairie dogs were clinically normal until day 11. PD54, a male, displayed distended abdomen, diarrhea, and vesicles on his mouth beginning on day 11; he died on day 14. PD1, also a male, had a distended abdomen beginning on day 11 and maculopapular skin lesions visible by day 12. On day 16 he was euthanized due to severe weight loss (24% of body weight). PD19, the lone female, displayed distended abdomen, ocular discharge, and skin lesions beginning on day 11. Her eyes became red and swollen on day 12. She was euthanized on day 15 due to malaise and unwillingness to move. The sentinel animal, PD24, experienced very mild weight loss (2.5%). No other clinical signs were observed. He was euthanized on day 29 at the end of the study.

In vivo BLI

The luciferase enzyme expressed during MPXV replication has a half-life of 3 hr and the luciferin substrate is metabolized in approximately 1 hr (Virostko and Jansen 2009).

Therefore, luminescence is indicative of recent viral replication. No luminescence was detected 1 day postinoculation (dpi) in any of the animals. Luminescence was first detected at 3 dpi in the oronasal area of the prairie dogs inoculated IN with MPXV (Figs. 1 and 2). At 5 dpi it was present in the ventral neck region (Fig. 2), at the site of the superficial cervical lymph nodes (Dearden 1953). Luminescence was detected in the skin in one animal at 7 dpi and in all three infected animals by 9 dpi. Total luminescence peaked by day 9 in PD1 and decreased until euthanasia at 16 dpi. Total luminescence increased in PD54 until death 14 dpi and in PD19 until it was euthanized at 15 dpi (Fig. 3). In the sentinel animal (PD24), luminescence was first detected in the dorsal skin at 9 dpi (Fig. 4), although no lesions were detected on external exam. A small amount of luminescence was detectable in the ventral neck on day 13 and 15. On day 15 the oronasal region of the sentinel, PD24, showed luminescence. No luminescence was detectable in this animal on day 25, indicating that the animal had cleared the infection or viral replication was below detectable levels.

Histopathology

Tissues that were examined histologically and the presence or absence of lesions are summarized in Table 1. Discrete foci of subacute, necrotizing dermatitis were present in three prairie dogs. This focus was severe in PD1 and PD19 with vesicles and syncytial cells within the epidermis, and neutrophils, macrophages, and necrotic debris in the dermis (Fig. 5A). The uninoculated sentinel prairie dog had an ulcerative subacute dermatitis. No viral inclusions were seen in any of the sections of skin. Focally severe acute necrosis of lymphoid tissue and fibrinoid necrosis of blood vessels were present in the thymus and tonsil. In addition, thrombosis (PD1), syncytial cell formation, and neutrophils (PD54) were seen in sections of tonsil. Lymph node lesions ranged from focal necrosis and fibrosis (PD19) to extensive necrosis (PD54) with abundant syncytial cells and dense infiltrates of subcapsular neutrophils and macrophages (Fig. 5B). Lungs of all prairie dogs had multifocal lymphoplasmacytic interstitial pneumonia that was mild in the sentinel and moderate to severe in the intranasally inoculated animals with syncytia and areas of acute necrosis (Fig. 5C). Lung mites were present in PD19 but were not associated with inflammation. Mild tracheitis with lymphocytes, plasma cells, macrophages, eosinophils and neutrophils was present in an inoculated prairie dog (PD19) and the uninoculated sentinel (PD24). The squamous portion of the stomach of PD1 and PD54 had lymphoplasmacytic submucosal inflammation with ballooning of cells on the mucosal surface and superficial cell sloughing. There was mild valvular endocarditis in heart sections of PD1 and PD19. The brain of PD1 had occasional arterioles with vacuolation of endothelium, karyomegally of cells in the tunica media, and increased perivascular spaces suggesting edema.

Immunohistochemistry detected MPXV antigen in skin lesions (3/3), kidney (2/2), tonsil (1/2), thymus (1/2), lymph node (1/4), lung (1/4) (Fig. 5D), and intestine (1/4). No MPXV antigen was detected in brain (0/4), heart (0/3), liver (0/4), spleen (0/3), tongue (0/2), stomach (0/3), or testes (0/2).

PCR and virus isolation

The results of PCR and viral plaque assays are shown in Table 2 and Figure 6. The superficial cervical lymph node was positive by viral isolation and real time PCR in two

animals, PD54 and PD19. These animals still had active luminescence in the final image before euthanasia 15 dpi. The lymph node of PD24 was positive by PCR but not by plaque assay. This animal last showed luminescence in the ventral neck 14 days before necropsy. In the three experimentally infected animals, virus shedding detected in oral, nasal, fecal, and ocular swabs peaked between days 13 and 15, coinciding with the highest luminescence and most severe clinical signs. Peak oral titers ranged from 5×10^5 to 3.5×10^6 pfu/ml in experimentally infected animals (Fig. 7). Peak nasal titers ranged from 7.5×10^6 to 4×10^7 pfu/ml. Peak fecal and ocular titers ranged from 250 to 2×10^7 pfu/ml and 7.5×10^5 to 1.5×10^7 pfu/ml, respectively. Blood titers were positive on days 7 and 9 for PD1 and PD54, but only on day 7 for PD19. Blood titers ranged from 75 to 7.5×10^5 pfu/ml. All titers for the sentinel PD24 were later and lower in titer. Blood titer for the sentinel animal was 250 pfu/ml on day 15, but virus shedding in oral, ocular and nasal swabs was detected on day 13. PCR was not performed on swab or blood samples, because there was a small amount of material and viral titration was prioritized over PCR.

DISCUSSION

Previous studies have demonstrated that the prairie dog is a suitable animal model for studying human MPX as they display most of the clinical signs commonly found in humans, including those affecting skin, oral mucosa, and lung (Guarner et al. 2004; Hutson et al. 2010). This model is enhanced by BLI because it allows the use of fewer animals and the ability to quantify luminescence, indicative of viral replication, in the same animal over time (Xiao et al. 2005; Osorio et al. 2009). Experiments with large numbers of prairie dogs are difficult to perform because of their wild nature and husbandry requirements. In wild animals with greater genetic diversity than laboratory rodents, BLI offers an advantage over traditional timed sacrifice studies that may miss host differences in disease progression by sacrificing individual animals before these differences can be recognized.

Using BLI, we were able to detect luminescence indicative of MPXV replication in prairie dogs after both experimental infection and secondary contact transmission to an uninoculated prairie dog. This allowed us to target specific tissues for virus isolation that are not commonly saved in routine infection trials, including superficial cervical lymph nodes. It also allowed the detection of virus in tissues with replicating virus early during infection that were no longer sites of active replication or had low titers at the time of death. For example, luminescence was evident in the region of the superficial cervical lymph nodes in PD1 and PD24 on days 5–9 and 13–15, respectively; it was not present at the time of death and no virus was isolated from the tissues. In addition to targeting specific tissues for collection, BLI helped detect natural infection, even in an animal with few or no clinical signs. The sentinel animal, PD24, showed evidence of viral replication in the skin by BLI on day 9, despite lack of clinical signs. Experimentally infected animals began to show luminescence 3 dpi. If a similar time course occurs in natural infection, the sentinel was likely infected between 5 and 7 days after inoculation of the other animals. Nasal, oral and fecal titers were positive in inoculated animals on day 7 and in the cohoused sentinel on day 13. Our results are consistent with Hutson et al. (2011); sentinel prairie dogs cohoused with a single inoculated prairie dog had visible MPX lesions 13 dpi and 7–11 days after lesions were visible in the inoculated animal. Prairie dogs live in small family groups and display many

social bonding behaviors that allow ample opportunity for contact or aerosol transmission such as sleeping together, sharing food, and grooming (Hoogland 1995). Prairie dogs also fight occasionally and bite wounds are another possible route of infection, but no wounds or fights were observed during this experiment.

In several instances, there was disagreement between PCR and viral titration results. It was expected that some samples may be positive for viral DNA but not viable virus as reported by others (Hutson et al. 2007, 2011). One sample, the kidney of PD54 was positive by viral titration and not by Q-PCR. This sample was tested multiple times from new pieces of tissues. It is possible the virus occurs in a patchy, multifocal distribution in these tissues. This discreet multifocal distribution of pox-like lesions was evident by histopathology in skin and lung. For this reason, it is important to analyze tissues by multiple methods. Contamination is a second possibility, but tissues processed on the same day, before and after this sample were negative. Presence of PCR inhibitors could have also contributed to negative PCR results, and future studies should include an internal control.

In mice, infection of internal organs is well documented using in vivo imaging (Cook and Griffin 2003; Luker and Luker 2009, 2010; Ozkaya et al. 2012). However, in our study, no luminescence was evident in the liver, lung, brain, or kidney of infected prairie dogs, despite positive viral titers in postmortem tissues. Likewise, no luminescence was evident in spleen or intestines, although Q-PCR indicated viral presence at some time during the experiment. The large size of the prairie dogs and their dark hair probably reduced the sensitivity of this technique. A titer of 8.42×10^4 pfu/g tissue was found in the skin of PD24 at the time of death, but no luminescence was detected in this area on the last day of imaging. This titer is perhaps near the limit of detection by in vivo imaging. Bioluminescent light originating in internal organs must travel through the tissues to be detected by the CCD camera and is attenuated during this passage. The keratin and pigment in hair also attenuate the bioluminescent signal. For future work, hair can be shaved in specific regions and larger animals can be imaged in four views rather than two to maximize detection. Another potential addition to future studies is to image using several depths of focus, which may help to increase the sensitivity for organs that are far from the skin surface.

Despite limitations in other internal organs, bioluminescence accurately identified infection of the superficial cervical lymph nodes, potentially important sites of secondary MPXV replication. Lymphadenitis is an important clinical sign in humans and several animal models (Schultz et al. 2009; Damon 2011; Dyal et al. 2011). Studies of sheeppox virus infection in sheep and MPXV infection in cynomolgous monkeys have suggested that capripox virus and orthopox virus infections lead to cell-associated viremias that target macrophages (Zaucha et al. 2001; Bowden et al. 2008). Xiao et al. (2005) likewise demonstrated infection of prairie dog macrophages with MPXV by immunohistochemistry. It is believed that these poxviruses infect monocytic lineage cells at the primary site of infection and spread from there to lymphoid organs (lymph node, tonsil, thymus), skin, and lung, which are considered secondary sites of replication. Intestine, liver, kidney, spleen, ovary, and testis are considered tertiary sites of replication (Zaucha et al. 2001). The histologic changes in the prairie dogs in our study support a similar pathogenesis. However, viremia was not detectable in these animals until days 7 and 9. Whole blood was used for

analysis, which may have resulted in decreased sensitivity. A more sensitive sample for detection of viremia may have been buffy coat or peripheral blood mononuclear cells, as reported by others (Zaucha et al. 2001; Embury-Hyatt et al. 2012).

Enanthema of the oral cavity was a prominent feature of disease in this study, with vesicles noted on the tongue and oral mucosa. In previous studies using prairie dogs as a model of MPXV human infection, lesions were also noted on the tongue and oral mucosa (Guarner et al. 2004). Oral shedding of MPXV has been associated with these lesions as the vesicles in the mouth, like those in the skin, rupture and release copious amounts of virus. This is likely an important source of virus shedding and environmental contamination. In human cases of MPX, painful oral lesions are often described that limit the ability of patients to eat and drink (Huhn et al. 2005; Damon 2011). Experimentally infected macaques also displayed moderate to severe stomatitis (Goff et al. 2011). Hutson et al. (2010) described oral shedding of MPXV as early as 3 dpi, before the presence of lesions in the skin or mouth. Likewise, we identified oral shedding of virus in the secondarily exposed animal (PD24) in the absence of visible oral lesions. Furthermore, luminescence was evident in the area of lymph nodes of this animal via BLI before its oronasal region, and this was preceded by luminescence in its skin. Thus, the primary site of infection could have been skin in the secondarily exposed animal; alternatively the primary infection and viremia were below the level of detection of in vivo imaging and whole blood culture, and only secondary sites of replication were detected by BLI. In the animals experimentally infected by the IN route, luminescence was detected in the oronasal region before the local lymph node and may have been the route of lymph node infection via lymphatic drainage. In two animals (PD1 and PD24), viral shedding continued in the mouth after there was no evidence of luminescence in the lymph node. The other two animals were moribund and euthanized before resolution of lymph node infection.

The consistent presence of necrotizing dermatitis with MPXV antigen detected by immunohistochemistry suggests that the skin is a target organ for this virus in infected prairie dogs, consistent with orthopoxviruses in other species (Chapman et al. 2010). Lymphoplasmacytic pneumonia was present in all animals and, although mild in the sentinel, it was accompanied by pulmonary necrosis and syncytia formation in the IN inoculated prairie dogs and was considered severe enough to have contributed to death. Severe viral pneumonia due to MPXV infection has been described in mice (Osorio et al. 2009) and nonhuman primates (Dyall et al. 2011). The severity of lymphoid tissue necrosis in affected prairie dogs suggested that this was another target organ for MPXV. Although this conceivably might have led to disseminated intravascular coagulopathy, compelling histologic evidence of this was not found. The clinical relevance of the gastritis and endocarditis is unknown.

In summary, this study provides further support for prairie dogs as animal models for MPXV infection, informing our knowledge of how MPXV might establish routes of shedding in humans and rodents. We also demonstrated that BLI can be used to detect real time spread of virus within individual animals and potential routes of shedding and transmission. Use of BLI reduces the number of animals used and allows investigators to follow individual animals through the disease course, especially important in wild animals.

Use of BLI is also appropriate for monitoring MPXV infection in animal species that display few or no clinical signs, and these subclinical infections are likely to occur in the natural maintenance hosts. Future BLI studies will be used to investigate which rodent species play critical roles in the epidemiology of MPX.

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Figure 1. Dorsal in vivo bioluminescent imaging (BLI) of black-tailed prairie dogs (*Cynomys ludovicianus*) experimentally infected with a recombinant luminescent *Monkeypox virus* shows that viral replication at the site of intranasal infection is evident by day 3 postinoculation. By day 7, BLI reveals viral replication at distant sites.

Figure 2.

Ventral views of black-tailed prairie dogs (*Cynomys ludovicianus*) infected with *Monkeypox virus* expressing luciferase show viral replication (indicated by luminescence) in the oronasal area on day 3 postinoculation, which spreads to the superficial cervical lymph nodes by day 5. Abdominal distension, one of the main clinical signs, is also evident in these ventral images.

Figure 3.

Quantification of luminescence of black-tailed prairie dogs (*Cynomys ludovicianus*) infected with luminescent *Monkeypox virus* and a cohoused sentinel prairie dog (PD24). The sentinel animal begins to show an increase in luminescence on day 7, likely the day he was infected. Luminescence in the sentinel peaked on day 18 and dropped to baseline levels by day 25.

Figure 4.

Dorsal (top row) and ventral (bottom row) bioluminescent images of a sentinel prairie dog (PD24) cohoused with experimentally infected prairie dogs indicates viral replication, as shown by luminescence, in the absence of clinical signs of monkeypox infection. Luminescence was evident 9 days postinoculation of the cohoused prairie dogs, although quantification of luminescence indicates that luminescence was increasing as early as day 7 (Fig. 3). Viral replication was detected in the dorsal skin and then the superficial cervical lymph node and the mouth. All are likely secondary sites of replication. Viral replication was below detectable levels using bioluminescent imaging by day 25.

Figure 5.

Tissues from adult black-tailed prairie dogs (*Cynomys ludovicianus*) inoculated intranasally with *Monkeypox virus* with firefly luciferase gene (MPXV-USA-2003/LUC). (A) Hematoxylin and eosin (H&E) stained section of skin from prairie dog PD1, with syncytial cells (arrowheads), intraepidermal neutrophils (thick arrow), and intense inflammation at the dermal–epidermal interface (thin arrow). (B) H&E section of lymph node from PD54. Note syncytial cells (arrowhead), acute necrosis and edema (thick arrow), and necrotic cellular debris in the subcapsular region (thin arrow). (C) H&E section of bronchiolar region of lung from PD1 with syncytia (arrowhead) and necrotic inflammatory debris along the basement membrane of bronchiolar epithelium. (D) Same section of lung as C with immunohistochemical stain using vaccinia mouse hyperimmune serum and horseradish peroxidase as a detection label for *Monkeypox virus* antigen. Note brown viral antigen staining in the bronchiolar epithelium (thin arrow) and necrotic debris along the basement membrane of the bronchiolar epithelium (thick arrow).

Figure 6.

Titers of *Monkeypox virus* (plaque-forming units per gram [pfu/g] of tissue) in tissues of three experimentally infected and one sentinel (PD24) black-tailed prairie dogs (*Cynomys ludovicianus*). Viral titer for each tissue was calculated using the Reed and Muench method of calculating viral titer from 50% tissue culture infectious dose plate assays in a 96 well format, with Vero cells.

Figure 7.

Viral titers of swabs and blood from four black-tailed prairie dogs (*Cynomys ludovicianus*) infected with *Monkeypox virus* show increasing titers in experimentally infected animals up until death. Titers peaked on days 13–18 and then decreased in the uninoculated sentinel animal (PD24).

Table 1

Tissues from experimentally inoculated and sentinel black-tailed prairie dogs (*Cynomys ludovicianus*) with *Monkeypox virus* (MPXV) expressing firefly luciferase gene (MPXV-USA-2003/LUC) were examined histologically. Numbers are prairie dog identification numbers examined and those that were found with lesions, by tissue type.

Tissue	PD ^a examined	PD with lesions ^b
Brain	1, 54	1
Brown fat	1, 19, 24, 54	-
Choroid plexus	1, 54	-
Esophagus	19, 54	-
Eye	1	-
Heart	1, 19, 24, 54	19
Intestine	1, 19, 24, 54	NS (1, 24)
Kidney	1, 19, 24, 54	-
Liver	1, 19, 24, 54	NS (1, 24, 54)
Lung	1, 19, 24, 54	1, 19, 24, 54
Lymph node	1, 19, 24, 54	19, 54
Pancreas	19, 54	-
Pituitary gland	1	-
Salivary gland	1, 19, 54	-
Skin	1, 19, 24, 54	1, 19, 24
Spleen	1, 19, 24, 54	NS (19, 24)
Stomach	1, 19, 54	54
Testis	1, 24, 54	-
Thymus	1	1
Thyroid gland	1, 19, 24, 54	-
Tongue	19	-
Tonsil	1, 54	1, 54
Trachea	1, 19, 24, 54	19, 24

^aPD = prairie dog identification number

^bNS = nonspecific lesions (mild lesions of unknown etiology but unlikely to be associated with MPXV); dashes indicate no lesions found in any prairie dogs.

Table 2

Detection of *Monkeypox virus* or viral DNA in prairie dog (*Cynomys ludovicianus*) tissues by viral plaque assay and real time PCR. Tissues from infected prairie dogs and one sentinel prairie dog (PD24) were assayed for monkeypox virus using an E9L orthopoxvirus real time PCR assay and 50% tissue culture infectious dose (TCID50) plaque assay in Vero cells. Results are reported as TCID50/PCR. Incongruous results between the two assays are italic, most often positive by PCR but not by cell culture; the only exception is indicated by bold face. Pos = positive; Neg = negative; NC = not collected; NT = not tested.

Tissue	Prairie dog identification number			
	PD1	PD19	PD24	PD54
Bladder	NC	Neg/NT	Neg/NT	NC
Brain	<i>Neg/Pos</i>	Pos/Pos	Neg/Neg	<i>Neg/Pos</i>
Duodenum	NC	NC	NC	Neg/NT
Esophagus	NC	Pos/Pos	NC	Pos/Pos
Heart	Neg/Neg	<i>Neg/Pos</i>	Neg/Neg	<i>Neg/Pos</i>
Intestine	<i>Neg/Pos</i>	<i>Neg/Pos</i>	<i>Neg/Pos</i>	Neg/Neg
Kidney	Neg/Neg	<i>Neg/Pos</i>	<i>Neg/Pos</i>	Pos/Neg
Liver	<i>Neg/Pos</i>	Pos/Pos	Neg/Neg	<i>Neg/Pos</i>
Lung	Pos/Pos	Pos/Pos	<i>Neg/Pos</i>	Pos/Pos
Pancreas	NC	NC	Neg/NT	NC
SC Lymph Node*	<i>Neg/Pos</i>	Pos/Pos	<i>Neg/Pos</i>	Pos/Pos
Salivary Gland	Neg/Neg	NC	Neg/Neg	Pos/Pos
Skin	NC	Pos/Pos	Pos/Pos	Pos/Pos
Spleen	Neg/Neg	<i>Neg/Pos</i>	Neg/Neg	<i>Neg/Pos</i>
Stomach	<i>Neg/Pos</i>	Pos/Pos	<i>Neg/Pos</i>	NC
Thyroid	NC	<i>Neg/Pos</i>	NC	NC
Testis/Ovary	<i>Neg/Pos</i>	NC	NC	NC
Thymus	Neg/Neg	NC	Neg/Neg	Pos/Pos
Tongue	Pos/Pos	Pos/Pos	Pos/Pos	Pos/Pos
Tonsil	<i>Neg/Pos</i>	NC	NC	NC
Trachea	<i>Neg/Pos</i>	Pos/Pos	Neg/Neg	Pos/Pos

* Superficial cervical lymph node