A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo Basin strains of monkeypox virus

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Multiple monkeypox virus (MPXV) animal models have been discussed in previous studies, but no small animal models, nor most non-human primate models, demonstrated the protracted asymptomatic incubation phase seen in systemic human orthopoxvirus illness. Herein, we characterize a black-tailed prairie dog (PD) (Cynomys ludovicianus) model of infection, via intranasal and intradermal exposures, with the two MPXV clades. Daily observations of the animals were made (food consumption, general symptoms, disease presentation), while weights and virus evaluations (ocular, nasal, oropharyngeal, faeces, blood) were obtained/made every third day. Generalized rash became apparent 9–12 days post-infection for all animals. Individual animals demonstrated a range of symptoms consistent with human monkeypox disease. Measurable viraemias and excretas were similar for both clade-representative strains and persisted until at least day 21. Greater morbidity was observed in Congo Basin strain-challenged animals and mortality was observed only in the Congo Basin strain-challenged animals. The PD model is valuable for the study of strain-dependent differences in MPXV. Additionally, the model closely mimics human systemic orthopoxvirus disease and may serve as a valuable non-human surrogate for investigations of antivirals and next generation orthopoxvirus vaccines.

INTRODUCTION

Monkeypox is a zoonotic disease causing febrile rash illness in humans with a presentation similar to smallpox. Since eradication of variola virus (the causative agent of smallpox), monkeypox virus (MPXV) has emerged as the most significant public health threat in the genus Orthopoxvirus. As evidenced by the USA 2003 outbreak, MPXV is considered potentially problematic beyond its endemic African range. Variola virus was an exclusively human pathogen, the viral-specific determinants of host range are not understood, and the human disease lacks a representative surrogate animal model. The related orthopoxvirus MPXV is a zoonotic agent that causes febrile rash disease with clinical presentation similar to smallpox (Breman & Henderson, 2002; Jezek et al., 1987) and has been used for the study of systemic orthopoxvirus infections. In humans, MPXV and variola virus have similar asymptomatic incubation periods ranging from 7–17 days followed by development of generalized rash. However, smallpox had a much higher secondary attack rate (25–96 %) than monkeypox (5–11 %) (Arita & Gromyko, 1982; Fine et al., 1988). Previous studies defined two distinct MPXV clades, West African and Congo Basin, with unique disease manifestations (Chen et al., 2005; Likos et al., 2005). Human disease associated with West African MPXV infection is less severe and associated with less human-to-human transmission compared to Congo Basin infection (Breman et al., 1980; Foster et al., 1972). In the Democratic Republic of Congo (Congo Basin), monkeypox has a reported case fatality rate of ~10–13 %.

Supplementary figures are available with the online version of this paper. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention.
(Arita et al., 1985; Breman et al., 1980; Meyer et al., 2002), compared with up to 40% for smallpox (Breman & Henderson, 2002). Human monkeypox had never been reported outside of Africa until 2003, when an outbreak occurred in the USA resulting from human contact with infected prairie dogs (PDs) which were co-housed with imported African rodents (Hutson et al., 2007; Reed et al., 2004). This outbreak demonstrated the potential for MPXV importation and, together with observations of ongoing human monkeypox in Africa (Boumandouki et al., 2007; Hutin et al., 2001; Learned et al., 2005; Meyer et al., 2002; Mukinda et al., 1997; Mwanbal et al., 1997; Rimoin et al., 2007), further emphasized the importance of a more complete understanding of this serious human pathogen.

In vivo animal model comparisons of viruses from these two clades will provide valuable tools with which to evaluate host–pathogen factors responsible for pathogenic differences. Multiple animal species have been experimentally infected with MPXV (Marenikova & Seluhina, 1976; Shcheluhkina & Marenikova, 1975; Tesh et al., 2004; Zaucha et al., 2001); however, most of these models have little in common with the human disease progression, presentation or mortality from either monkeypox or smallpox. Based on observations of PDs infected during the USA outbreak (Guarner et al., 2004; Hutson et al., 2007), and previous experimentally infected PDs (Xiao et al., 2005), we believe these animals will serve as a relevant model for human monkeypox. In the Xiao et al. study, only West African MPXV was used and it was administered via an intranasal and an intraperitoneal route. Here, we show that PDs are a valuable model for study of strain-dependent differences in MPXV virulence, something that has not been previously studied in these animals. Additionally, we infected with an intranasal as well as an intradermal route, both of which are plausible natural routes of infection. Our described animal model mimics human monkeypox disease progression and presentation more closely than previous models, and thus will serve as a valuable surrogate for future investigations of human systemic orthopoxvirus diseases, including smallpox.

**METHODS**

**Animal maintenance.** Wild-caught, juvenile black-tailed PDs (*Cynomys ludovicianus*) were obtained from Western Kansas. At time of infection, animals were approximately 2 years old. During experimental infections, animals were housed in large rat cages with aerosol filter tops. Cages were kept in a Duo-Flow biosafety cabinet in a BSL-3 animal room. Animals were cared for in accordance with CDC Institutional Animal Care and Use Committee (IACUC) under an approved protocol (1431REGPRAC-A1). Animal handling was performed using biosafety level 3 + personal protective equipment (PPE). In addition to PD chow and hay, animals were provided with monkey biscuits for added dietary enrichment.

**Viruses.** The West African MPXV strain, MPXV-2003-044, was isolated during the 2003 USA outbreak, and has previously been fully sequenced (Likos et al., 2005; Reed et al., 2004). The Congo Basin MPXV strain, MPXV-2003-358, was collected from a 2003 outbreak of monkeypox in the Republic of Congo (ROC) and has been fully sequenced (Likos et al., 2005). Both viruses had undergone two passages in African green monkey kidney cells (BSC-40) prior to seed pool production. The titres for both virus inocula were retitrated after appropriate dilutions were made for PD inoculation.

**Animal inoculation.** Infection of PDs with the two MPXV strains was done on separate occasions. The average starting weight for animals challenged with West African MPXV was 714.1 g (range 535–842 g), and the average for Congo Basin MPXV-challenged animals was 807.8 g (range 640–988 g). Animals were infected by either an intranasal (IN) or intradermal via scarification (ID-SC) route of inoculation while under anaesthesia. For both strains, groups of four animals were inoculated with $10^8$ plaque-forming units (p.f.u.) in a total volume of 10 μl IN (5 μl in each nostril). Similarly, for both strains, four animals were inoculated with $10^2$ p.f.u. in a total volume of 10 μl by ID-SC. For ID-SC animals, a small area on the right flank of the animals was shaved. Ten microlitres of virus solution was placed on this shaved area and a 28 gauge needle was used to gently probe to the superficial layers for ten replicates. Additionally for each strain, four animals were mock-infected with PBS.

**Observations and sampling.** Daily visual observations of the animals were made and recorded throughout the study. Lesion count, temperature, weight and tissue/excreta samples were collected every third day post-infection (p.i.) without anaesthesia. For both MPXV strain challenge studies, the relatively less fur-covered areas of the face, inner hind legs and genitalia were used to count lesions. Blood was collected from the saphenous vein in EDTA-coated tubes. Sterile individual Dacron swabs were used to collect excreta and were stored frozen without diluent. Swabs, faces and blood were processed and prepared for DNA analysis and virus isolation (see below).

** Necropsy and tissue specimen collection.** If death did not occur as a result of infection, at 35 days (West African MPXV) and 49 days (Congo Basin MPXV) animals were humanely euthanized and necropsied. Euthanasia dates were determined based on estimated time to cessation of viable virus [RT-PCR values greater than cycle threshold ($C_\text{t}$) =34] in excreta. Necropsies on all animals were performed according to IACUC standards in a BSL-3 laboratory and utilizing full BSL-3 PPE. Instruments were cleaned and decontaminated with 3% Amphyl and 10% Clorox bleach between collections of each tissue. Tissues were frozen at $-70^\circ$C prior to further processing. Oral and ocular swabs were collected with sterile individual Dacron swabs and stored frozen without diluent. Serum was separated from whole blood and processed for serology (see below). Tissues and swabs were subsequently processed and further prepared for DNA analysis and virus isolation (see below).

**Sample preparation.** Sample processing was performed under BSL-3 conditions. For DNA analysis of blood, water was added if necessary to bring total volume to 200 μl. The sample was incubated at 55 °C for 1 h to inactivate virus. The EZ-1 DNA extraction robot (Qiagen) was used for genomic DNA extraction of all blood samples. For swabs collected during the West African MPXV challenge, 400 μl PBS was added to the swab. The swab extraction tube systems (SETS) (Roche) protocol was used to recover sample from a swab. Genomic DNA was prepared using either the AquaPure DNA isolation kit (Bio-Rad), or the BioRobot MDx workstation (Qiagen). For swabs collected during the Congo Basin MPXV challenge, 1 ml PBS was added to the swab and vortexed. Genomic DNA was then prepared using the AquaPure DNA isolation kit. For both virus strains, remaining swab lysate was used for virus isolation (see below). Tissue and fecal samples were placed in disposable bounce homogenizers. PBS (1 ml) was added to each weighed tissue sample in the 50 ml sterile tissue grinder and ground thoroughly to create a slurry. Genomic DNA was prepared...
from a slurry aliquot (100 μl) with BioRobot MDx workstation or AquaPure DNA isolation kit and the remaining samples were used for virus isolation (see below).

Real-time PCR analysis. Samples were tested by real-time PCR using forward and reverse primers and probe complementary to the conserved orthopoxvirus E9L (DNA polymerase) gene (Li et al., 2006). A representative sample from each animal was confirmed for MPXV DNA using forward and reverse primers and probe specific to the MPXV B6R gene (Li et al., 2006). MPXV DNA (10 fg–1 ng) was used as positive control for both tests.

Virus–tissue infectivity. Previous analyses demonstrated that real-time PCR detection of MPXV DNA was significantly more sensitive than detection of p.f.u. (Hutson et al., 2007). Therefore, specimens were first tested for the presence of orthopoxvirus DNA by PCR and, if positive, were subsequently evaluated for viable virus by tissue culture propagation. Each swab, faeces or tissue sample was titrated using tenfold dilutions of swab eluent or tissue slurry on BSC-40 cell monolayers, incubated at 36°C and 6% CO₂ for 72 h, and subsequently stained with crystal violet and formalin to reveal plaques.

Serological analysis. A modified version of the ELISA was used for analysis of anti-orthopoxvirus immunoglobulin types A and G (Hutson et al., 2007). Microtitre plates (Immulon II; Dynatech) were coated with 0.01 μg per well crude vaccinia virus (Dryvax grown in BSC-40 cells) in carbonate buffer on one half of the plate and an equal volume of BSC-40 cell lysate diluted in carbonate buffer on the other half and incubated overnight at 4°C. After inactivation with 10% formalin (buffered) for 10 min at room temperature, plates were blocked for 30 min at room temperature with assay diluent [PBS, 0.01 M, pH 7.4 (Gibco) + 0.05% Tween-20, 5% dried skim milk, 2% normal goat serum and 2% BSA] followed by three PBST (0.05% Tween-20) washes. PD sera (diluted 1:100 in assay diluent) was added to both halves of the plates and incubated for 1 h at 37°C. Plates were washed, and a 1:50,000 dilution (in assay diluent) of ImmunoPure A/G conjugate (Pierce) was added and incubated for 1 h at 37°C. Plates were washed, and peroxidase substrate (Kirkegaard & Perry Laboratories) was added, and absorbance was read on a spectrophotometer at 450 nm. Values reported represent the average of duplicate wells for each sample. Both positive and negative human anti-vaccinia sera were used as assay controls. The BSC-40 cell lysate half of each plate was used to generate a cut-off value (COV) for each plate by averaging all the values of the BSC-40 lysate virus half and adding two SD. Specimens were considered positive if the test sample's value was above the COV.

Statistical analyses. As data were not normally distributed, nonparametric statistical analyses were used (Lehmann, 1975). Levels of viral DNA, viable virus, anti-orthopoxvirus antibodies, and daily observations (weight, temperature, lesion counts) were compared between strains and routes of infection using the Wilcoxon rank-sum test. In order to evaluate differences in temperature, day zero temperature and the highest temperature recorded thereafter were used to determine the per cent increase in temperature for each PD. To evaluate weight differences, weights observed at day zero were used as the baseline and the lowest weight measured thereafter was used to determine per cent weight loss in each animal. Comparisons between the first and last day of DNA and viable virus were also evaluated between strains and routes using the Wilcoxon rank-sum test. ID-SC and IN groups were combined to increase numbers when comparing strains, and strains were combined to increase numbers when comparing route for DNA and viable virus. This was also done when comparing the number of lesions between strains. A P-value <0.05 was considered significant.

RESULTS

Clinical observations (Table 1)

General observations. For all animals in all viral-infected groups, disseminated or generalized lesions emerged in a centrifugal pattern; lesions were first noted on the head or ventral surfaces of extremities, followed by lesion development on the trunk. Lesions observed on ventral surface of extremities and on the trunk developed through the typical macular, vesicular, pustular evolution before drying and desquamation. In slight contrast, facial lesions evolved from macular to vesicular, but did not reach the pustular stage. The Congo Basin strain-infected PDs had a significantly higher per cent increase in temperature compared to the West African strain-infected PDs (P=0.01; per cent mean increase West African: 2.88%; per cent mean increase Congo Basin: 5.98%). There was also a greater trend in weight loss in the Congo Basin strain-infected PDs (mean percentage: Congo Basin 10.53%; West African 6.57%); however, this trend was not considered significant (P=0.1089)

West African MPXV challenge. The animals inoculated with the West African strain of MPXV via IN and ID-SC routes had similar disease presentations with generalized lesions appearing between days 9 and 12 (Supplementary Fig. S1, available in JGV Online). All four animals inoculated via the ID-SC route developed a scarification site (SS) lesion between days 6 and 9. Until the development of this primary lesion for the ID-SC group, or the disseminated lesions for the IN group, no observable symptoms were noted. Lesion count was slightly lower for the IN inoculated animals compared with the ID-SC animals, and in contrast to what was observed with the ID-SC inoculation, only one IN animal developed more than one lesion on the inner leg area or genitalia. Similarly seen in both groups was the development of a slightly haemorrhagic lesion on the ventral surface of the legs on two of the ID-SC animals and one IN animal. Clinical symptoms were comparable between the two West African-inoculated groups. All of the West African MPXV-inoculated animals recovered from disease with secondary lesions beginning to resolve between days 18 and 28. At necropsy (day 35), two ID-SC PDs had what appeared to be secondary bacterial infections at the primary site of inoculation, while the other two PDs’ primary lesion sites remained scabbed.

Congo Basin MPXV challenge. As was seen in the West African MPXV-infected animals, the animals inoculated with the Congo Basin strain of MPXV had similar disease presentations with generalized lesions appearing between days 9 and 12 (Supplementary Fig. S2, available in JGV Online). All four animals inoculated via the ID-SC route developed a SS lesion on day 6, also comparable to the
West African ID-SC animals. Until the development of this primary lesion for the ID-SC group, or the disseminated lesions for the IN group, no observable symptoms were noted. Similarly to West African animals, lesion count was slightly less for the Congo Basin IN animals compared with the ID-SC animals, with the exception of one ID-SC animal which, unlike the West African animals, developed a SS lesion, but died on day 11 before disseminated lesions occurred. Another ID-SC animal was found dead on day 12 and one IN animal was found dead on day 13. Additionally, unlike the West African IN animals, all Congo Basin MPXV IN-infected animals developed lesions on the face and inner legs. Clinical symptoms were comparable between the two Congo Basin-inoculated groups. All of the surviving Congo Basin MPXV infected animals’ secondary lesions began to resolve between days 18 and 28, similar to West African infected animals. One ID-SC animal developed an apparent secondary bacterial infection at the SS (~ day 15), lasting until approximately day 35 and crustng over by day 42. The other animal that survived ID-SC inoculation never developed a bacterial infection at the site of scarification. For this animal, the primary lesion that developed began to resolve around day 15, with the scab completely falling off by day 30.

**Molecular and virologic findings (Table 1)**

**West African MPXV challenge.** MPXV DNA was detectable in all samples (ocular, oral, nasal, faecal, blood) at days 9–15 from the West African MPXV ID-SC inoculated group as well as the IN-inoculated group (with the exception of faeces). Viral DNA was initially detected in blood, followed by oral and nasal excreta, then in ocular and faecal excreta (Fig. 1a) in the ID-SC-inoculated animals. Similar results were seen for IN-inoculated animals, with the exception of DNA initially being detected in oral secretions (Fig. 1b). Viable virologic analyses provided similar kinetics for each group, but virus isolation was not attempted from blood (Fig. 1c and d). Peak MPXV DNA was observed from day 6 to 15, with oral samples yielding the highest levels for both groups (Fig. 2a). For both inoculation routes, viable virus was detectable beginning on day 6 for oral and nasal swabs and the count was highest for oral swabs (Fig. 1a). Viable virus was detectable on day 9 for faecal and day 12 for ocular swabs. Viral shedding continued from these sites until days 15–21 (Fig. 1c and d). Peak MPXV viable virus was observed from day 9 to 18, with the oral sample having the highest load for both groups (Fig. 2b). Blood was collected from all West African MPXV-injected animals on day 35, and all had anti-orthopoxvirus antibodies (Fig. 3a). West African ID-SC-infected animals had significantly higher anti-orthopoxvirus antibodies than West African IN animals ($P = 0.03$). At time of necropsy, all four animals in the ID-SC group had MPXV DNA detected in the lesion sample. In addition, three eyelid samples, one skin sample, one oral swab, one lymph node and one gonad were MPXV DNA-positive. The four IN-inoculated

<table>
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<tr>
<th>MPXV strain:</th>
<th>West African</th>
<th>Congo Basin</th>
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<tbody>
<tr>
<td>Inoculation route:</td>
<td>ID-SC</td>
<td>IN</td>
</tr>
<tr>
<td>Localized lesion/SS lesion onset</td>
<td>Days 6–9</td>
<td>NA</td>
</tr>
<tr>
<td>Generalized lesion onset</td>
<td>Days 9–12</td>
<td>7–17 (avg 11.5)</td>
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<tr>
<td>Objective and subjective symptoms</td>
<td>Lethargy, inappetence, confusion, crusty nose, crusty lips, bloody oral swab, hypopigmentation, secondary infection at SS</td>
<td>Inappetence, crusty nose, nasal discharge, bloody oral swab, respiratory distress, hypopigmentation</td>
</tr>
<tr>
<td>Mortality</td>
<td>0/4</td>
<td>0/4</td>
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<tr>
<td>MPX viable virus in swabs and faeces (range)</td>
<td>Days 6–21</td>
<td>Days 3–21</td>
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<td>Peak MPX viable virus</td>
<td>Days 9–18</td>
<td>All anti-OPXV Ab positive (significantly higher titres than West IN)</td>
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<td>Anti-OPXV antibodies (Ab)</td>
<td>Days 12 and 18</td>
<td>All surviving animals anti-OPXV Ab positive</td>
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**Table 1. Comparison of disease presentation and molecular findings in prairie dogs infected with MPXV**

Animals were challenged with either the West African or Congo Basin strain of MPXV. For each virus strain, animals were inoculated by either an IN ($n=4$) or ID-SC ($n=4$) route of infection. Samples were taken every third day p.i. and were tested for DNA and viable virus. Blood was taken upon death or, if death did not occur, on day 35 for animals infected with West African MPXV and day 49 for Congo Basin MPXV. OPXV, Orthopoxvirus.
animals had two MPXV DNA-positive eyelid samples, as well as one skin and one lesion. None of these MPXV DNA-positive samples yielded viable virus.

Congo Basin MPXV challenge. The Congo Basin MPXV-inoculated PDs were sampled 2 weeks longer than the West African MPXV-infected animals. This was done in order to observe whether these animals might shed virus longer (suggested by real-time PCR data) than the West African MPXV-challenged animals. In addition, a swab of the inner leg area (secondary lesions) was taken for both groups, and a swab of the SS was taken from the ID-SC-inoculated group. Overall, animals inoculated with Congo Basin MPXV resulted in similar onset, kinetics and cessation of detectable DNA and viable virus as that seen in the West African MPXV-infected animals (Figs 4 and 5). Congo Basin MPXV infected via ID-SC manifested DNA in blood earlier than other tissues, compared with those infected via the IN route (Fig. 4). However, all samples, with the exception of the ID-SC blood samples, yielded positive MPXV DNA beginning on days 6–9, comparable to what was seen in the West African MPXV samples. Also similar to the West African-inoculated animals, MPXV DNA levels peaked on days 9–21 (Fig. 6a). An interesting difference seen in the Congo Basin-challenged animals was that faecal samples yielded the highest MPXV DNA loads for both inoculation routes; however, comparable to the West African-infected group, the oral samples yielded the highest viable virus loads (Fig. 6). Also comparable to what was seen in the West African MPXV-inoculated animals, viable virus was detectable beginning on day 6 for samples from ID-SC and IN animals (Fig. 5). Viral shedding occurred for both groups until day 18–21 for all samples except the IN ocular sample, which had viable virus only until day 12 (Fig. 5). Peak MPXV viable virus was observed from days 12 to 18 (Fig. 6b), again similar to the peak observed during the West African MPXV infection. Blood was collected post-mortem on the three animals that

Fig. 1. Quantification of West African MPXV within infected PDs. Animals were challenged with West African MPXV by either an ID-SC (n=4; a, c) or IN (n=4; b, d) route of infection. Samples were taken every third day p.i. and were tested for DNA (a, b) and viable virus as p.f.u. (c, d). Average values were plotted on a log scale. For (a) and (b), a value of 1 was added to all samples with less than 1 fg of DNA for visual representation on a log scale. Error bars, SD.

Fig. 2. Quantification of peak values seen in West African MPXV-infected PDs. Animals were challenged with West African MPXV by either an IN (n=4) or ID-SC (n=4) route of infection. Samples were taken every third day p.i. and were tested for DNA (a) and viable virus (b). The average peak value was calculated by taking the maximum average value for each sample from each animal group. The numbers on the bars correspond to the day that the maximum average value was seen.
**Fig. 3.** Serological analysis of PDs infected with MPXV. Animals were challenged with West African MPXV by either an IN \((n=4)\) or ID-SC \((n=4)\) route of infection. Blood was taken upon death, or if death did not occur, on day 35 for animals infected with West African MPXV (a) and day 49 for Congo Basin MPXV (b). A modified version of the ELISA was used for analysis of anti-orthopoxvirus immunoglobulin types A and G. Values reported represent the average of duplicate wells for each sample. An asterisk (*) indicates an animal that died during the study.

**Fig. 4.** Quantification of Congo Basin MPXV DNA within infected PDs. Animals were challenged with Congo Basin MPXV by either an ID-SC \((n=4); a\) or IN \((n=4); b\) route of infection. Samples were taken every third day p.i. and were tested for DNA. Average values were plotted on a log scale. A value of 1 was added to all samples with less than 1 fg of DNA for visual representation on a log scale. Error bars, SD.
died spontaneously, and on day 49 for the remaining animals. The blood from the animals that died during the study was negative for orthopoxvirus (OPXV) antibodies. The other five convalescent Congo Basin-inoculated animals had anti-OPXV antibodies detected at time of necropsy with similar levels as were observed in the serum collected from the West African-challenged animals (Fig. 3b). Unlike the West African-challenged animals, there was no significant difference seen between the antibody levels. At time of euthanasia, the five animals that survived had several MPXV DNA-positive samples which were negative for viable virus. This included two lesion samples and one skin sample from the ID-SC animals as well as one blood and one heart sample from the IN animals. The three animals that died during the course of the observations had high levels of both viral DNA and viable virus in the majority of samples tested (Fig. 7).

None of the uninfected animals, housed under the same conditions, showed any evidence of MPXV infection during the duration of the study. Furthermore, all samples taken from these control animals were negative for MPXV DNA when tested with real-time PCR (data not shown) and were negative for OPXV antibodies (Fig. 3).

**DISCUSSION**

Consistent with human MPXV infections (Huhn et al., 2005; Jezek et al., 1987; Reynolds et al., 2004), MPXV infection in PDs was asymptomatic during a 6–12 day incubation period, after which obvious skin lesions developed and subsequently resolved approximately 28 days p.i. for surviving animals. Prior to development of generalized lesions, viral shedding could be detected in oropharyngeal secretions, consistent with descriptions of the oral enanthem of human systemic orthopoxvirus disease. Generalized lesions developed and evolved at a similar rate in a centrifugal pattern, similar to disease progression for human monkeypox (Arita et al., 1985; Jezek et al., 1987).

In addition to disseminated vesiculo-pustular lesions, several other disease symptoms in the PD MPXV model were similar to those seen in human cases. One potentially painful symptom of human monkeypox cases is oral lesion development (Huhn et al., 2005; Jezek et al., 1987; Reynolds et al., 2006). Although we could not photograph lesions in the oral cavities of animals since animals were not anaesthetized during sampling, we were able to observe lesions on/inside the mouths of several animals. Furthermore, bloody oral swabs collected from some animals were probably caused by oral lesions. A complication in human disease is development of secondary bacterial skin infections at lesion sites (Jezek et al., 1987). Although no disseminated lesions developed secondary bacterial infections, several of the SSs did. Diarrhoea is seen in a small percentage of human cases (Jezek et al., 1987), and similarly, was observed in one infected animal. Bronchopneumonia is another rare, late sequela of human
could be indicative of upper airway oedema causing stridor. Crusty noses and nasal discharge from several of the animals suggests upper respiratory involvement; human disease in the USA and Africa has documented upper airway symptoms of sore throat and choryza (Huhn et al., 2004, 2005; Reynolds et al., 2006). Finally, inappetence and lethargy observed in PDs are commonly observed in humans (Jezek et al., 1987). A primary difference between the clinical presentations of human smallpox and monkeypox is the occurrence of lymphadenopathy in MPXV-infected individuals. Interestingly, we did not observe these sequelae in the PDs. However, we isolated viable virus from lymph node tissue in the three animals that succumbed to Congo Basin MPXV, indicating that the lymphatic system was involved during the course of infection, even though swelling was not noted. Either the condition did not occur, or was not substantial enough to be observed. Although disease manifestations are not identical in these two species, there are many similarities in disease progression, clinical manifestations and mortality rates between that seen in humans and in PDs infected with MPXV. Our model used a relatively low level of infectious dose as well as two plausible natural routes of infection, indicating that the PD is an ideal model for human MPXV infection.

Viral DNA was detected in the blood of most animals between days 6 and 15. Although we did not attempt viral isolation on blood samples, the presence of detectable MPXV DNA early in infection is evidence of viraemia during this time period. Viable virus was isolated from oral, ocular and nasal swabs, as well as faeces, during the study, demonstrating the shedding potential of MPXV via multiple excreta. Oral swabs were especially rich in viable virus, with average peak levels greater than $10^4$ p.f.u. for the West African- and $10^5$ p.f.u. for Congo Basin MPXV-infected animals. Interestingly, the faecal samples from the Congo Basin MPXV-infected animals had significantly more DNA (days 6–28) and viable virus (day 18) than the West African MPXV-infected animals ($P$-values between $3 \times 10^{-4}$ and 0.046). This suggests, at least in this

Fig. 6. Quantification of peak values seen in Congo Basin MPXV-infected PDs. Animals were challenged with Congo Basin MPXV by either an IN ($n=4$) or ID-SC ($n=4$) route of infection. Samples were taken every third day p.i. and were tested for DNA (a) and viable virus (b). The average peak value was calculated by taking the maximum average value for each sample from each animal group. The numbers on the bars correspond to the day that the maximum average value was seen.

Fig. 7. Quantification of virus within three PDs that died due to infection with the Congo Basin strain of MPXV. Animals were challenged with Congo Basin MPXV by either an IN ($n=4$) or ID-SC ($n=4$) route of infection. Two ID-SC animals (nos 13 and 16) died on day 12 and 11 p.i. One IN animal (no. 19) died on day 13 p.i. Animals were necropsied and samples were tested by tissue culture.
experimental animal model, there are potential strain-dependent differences in transmission. Viable virus levels peaked at similar times for both MPXV strains, days 9–18 for West African and 12–18 for Congo Basin. For both strains, infected animals continued shedding viable virus from certain samples until day 21 p.i., information lacking for 2003 USA monkeypox outbreak animals and which might be potentially useful for formulating future outbreak control measures. This period of viable MPXV shedding from PDs was similar to human monkeypox outbreaks, with isolation of viable virus from samples taken up to 18 days after the development of rash (Arita et al., 1985). The serology results confirmed that all animals, except those that died during the study, had developed immune responses to orthopoxviruses. This assay was designed to capture all Ig antibodies, but may not adequately capture IgM antibodies. The animals that perished most likely died before developing IgG responses which would have been detected with this ELISA. At time of necropsy, some animals that survived the challenge had low levels of viral DNA in a few tissues, but no viable virus. However, the three animals that died during the Congo Basin MPXV challenge had high levels of viral DNA and viable virus throughout the majority of tissues tested, suggesting that MPXV has a wide range of tissue tropism. In one or more of these three animals, samples including the oral swab, eyelid, tongue, lung, liver, spleen and scent glands had levels of viable virus at or above $10^7$ p.f.u. ml$^{-1}$.

Similar to human infection (Breman et al., 1980; Foster et al., 1972; Jezek et al., 1987; Likos et al., 2005; Reynolds et al., 2006), the Congo Basin MPXV strain was more pathogenic than the West African MPXV in the PD model. Morbidity was higher in Congo Basin MPXV-infected animals as measured by lesion count. A greater trend in weight loss and a significantly higher per cent increase in temperature in Congo Basin MPXV-challenged animals were also noted. Furthermore, mortality rates of 50% (ID-SC) and 25% (IN) were seen in the Congo Basin MPXV-infected animals, compared with 0% in West African MPXV-infected animals. This is concordant with observations of human disease, where Congo Basin MPXV causes 10–13% mortality compared with 0% with West African MPXV. The ability to discern differences in virulence for MPXV from the two clades in this animal model should benefit future studies. Having such an animal model will hopefully allow better understanding/identification of MPXV virulence factors.

It is noteworthy that the observed mortality rate was lower than that observed in a previously published PD challenge study (Xiao et al., 2005) in which West African MPXV challenge administered IN caused 60% mortality and administered intraperitoneally caused 100% mortality. There are several possible explanations for this difference. Xiao et al. used a slightly higher dosage of virus ($10^7$ p.f.u.) and practices such as orbital bleeding and daily anaesthetizing for sampling, compared with our current study administering $10^{4.5}$ p.f.u., bleeding from the saphenous vein and animal sampling every third day without anaesthesia. Also, throughout our study, animals were provided with highly palatable ‘monkey biscuits’, potentially a key dietary factor when anorexia occurred. Additionally, during the Xiao et al. study, the intraperitoneal animals did not develop disseminated lesions, suggesting that this inoculation does not mimic natural disease progression, unlike animals infected in our study.

The incubation period before onset of secondary lesions (12 days versus 9–12 days), as well as the time it took for lesions to resolve, were similar for IN and ID-SC West African MPXV-infected animals in our study. Interestingly, this observation is somewhat different than that seen in human cases during the USA outbreak, in which humans who received bites or scratches (the transmission route that the ID-SC model most closely mimics) had a shorter incubation period compared with infection via the respiratory route (Reynolds et al., 2006). Additionally, in the USA human monkeypox study, a significant difference in rash burden between the two routes of exposure was not observed. However, we did see significantly higher numbers of lesions in the ID-SC-infected animals compared with the IN-infected animals ($P=0.02$). We consistently observed the development of a primary lesion at the site of scarification (days 6–9) before the onset of disseminated lesions (days 9–12), which is similar to the disease progression of inoculation smallpox versus respiratory route-acquired smallpox (Fenner, 1948, 1990). Similarly, the incubation period of the probable respiratory route of human monkeypox was observed to be longer than inoculation/complex exposure to MPXV (Reynolds et al., 2006). The differences seen in these previous human studies, compared with our PD model, might be attributed to differences between a retrospective (USA investigation) versus a prospective (our study) study design, or different host–pathogen interactions in our experimental animal model compared with human infection. Also, the infection dose for our PD study may well have been higher than in naturally acquired human monkeypox cases.

There were several study limitations. Since we routinely collected swabs instead of whole tissue, samples cannot be directly compared to evidence of virus per gram of tissue, but instead must be related to internally consistent collection methods. For the faecal samples, not surprisingly, there were some bacterial and/or fungal contaminations that may have inhibited viral propagation in cells. Also, we had some sample preparation variation because the swabs from the Congo Basin MPXV challenge were extracted in a larger amount of PBS compared with West African MPXV challenge samples. Although this was accounted for when calculating MPXV yield, the slightly higher dilution may have lowered the amount of detectable virus in marginally positive samples. Also, because it is challenging to do large scale studies with large rodents, our relatively small sample size limited statistical analysis. Finally, because PDs are wild-caught animals, not inbred laboratory-acquired animals; there is individual animal
variability which may affect the consistency of disease progression.

Our results are concordant with previous data (Xiao et al., 2005; Hutson et al., 2007; Guarner et al., 2004) that showed PDs are highly susceptible to MPXV. We found both inoculation routes gave similar infection results, and either route would be a good mode of inoculating animals in future experiments that may mimic natural transmission. Furthermore, because our model has a prolonged incubation period, disseminated lesions, as well as clear differences in virulence of the two MPXV strains, the PD MPXV model has the potential to be a valuable model for disseminated human orthopoxvirus disease, including both monkeypox as well as smallpox. The PD MPXV model will be valuable in the study of virulence factors within the two clades, orthopoxvirus therapeutics, transmission/shedding of MPXV, as well as the study of other human orthopoxviruses.

REFERENCES


