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Virology 402 (2010) 72–82

Contents lists available at ScienceDirect

Virology

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

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ARTICLE INFO

Article history:
Received 14 September 2009
Returned to author for revision 12 November 2009
Accepted 4 March 2010
Available online 5 April 2010

Keywords:
Monkeypox
Orthopox
Pathogenesis

ABSTRACT

The prairie dog is valuable for the study of monkeypox virus (MPXV) virulence and closely resembles human systemic orthopoxvirus disease. Herein, we utilize a variable dose intranasal challenge with approximately 10^3, 10^4, 10^5, and 10^6 PFU for each clade to further characterize virulence differences between the two MPXV clades. A trend of increased morbidity and mortality as well as greater viral shedding was observed with increasing viral challenge dose. Additionally, there appeared to be a delay in onset of disease for animals challenged with lower dosages of virus. Mathematical calculations were used to determine LD_{50} values and based on these calculations, Congo Basin MPXV had approximately a hundred times lower LD_{50} value than the West African clade (5.9×10^3 and 1.29×10^5 respectively); reinforcing previous findings that Congo Basin MPXV is more virulent.

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Introduction

Orthopoxvirus monkeypox is a zoonotic agent that causes febrile rash disease in humans and a clinical presentation similar to discrete, ordinary smallpox (Breman et al., 1977; Jezek et al., 1987). Monkeypox virus (MPXV) is endemic to the rain forests of Central and Western Africa where it causes human disease believed to result from close contact between humans and infected rain forest dwelling animals or, in certain circumstances, via interhuman transmission (Di Giulio and Eckburg, 2004; Khodakevich et al., 1987a,b). Previous studies defined two distinct MPXV clades, West African and Congo Basin (Chen et al., 2005; Likos et al., 2005). Human disease associated with West African MPXV infection is less severe, is associated with <1% mortality, and is rarely associated with human to human transmission. Comparatively Congo Basin MPXV infection has a 10% case fatality rate in unvaccinated persons, and up to six sequential interhuman transmission events have been documented (Breman et al., 1980; Foster et al., 1972; Likos et al., 2005; Learned et al., 2005). Human MPXV was first reported outside of Africa in 2003, when an outbreak occurred in the United States resulting from human contact with infected prairie dogs (PDs) which had been co-housed with imported African rodents (Hutson et al., 2007; Reed et al., 2004; Reynolds et al., 2006). This outbreak together with observations of ongoing human MPXV in Africa (Hutin et al., 2001; Learned et al., 2005; Meyer et al., 2002), exemplifies the importance of a more complete understanding of this serious human pathogen.

Previous studies of the prairie dog MPXV model reported a disease that closely resembled human MPXV, and human systemic orthopoxvirus disease progression and presentation more closely than previously described models. Additionally, these studies (Hutson et al., 2009) have shown that PDs are a valuable model for the study of MPXV clade specific differences in pathogenicity. Comparisons of the disease caused by viruses from the two MPXV clades (West African and Congo Basin), in an animal model, can provide a system with which to evaluate host–pathogen factors responsible for virulence differences. In order to characterize the effect of challenge dose on disease manifestation we challenged animals with approximately 10^3–10^6 infectious virus particles of each of the two MPXV clades and evaluated virus shedding, clinical symptoms, and calculated LD_{50} mortality values associated with strains of each virus clade.

Results

Clinical findings

West African MPXV (Table 1)

With the exception of four animals in which inappetance was noted 1–3 days before lesion onset, no other symptoms were noted until the development of disseminated lesions on day 10 for most animals (range 9–27 days). One animal (PD6) in the 6×10^3 PFU West African dosage group first developed lesions on day 27 p.i. and was the only animal in this dosage group to lose weight and subsequently

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2010.03.012

die as a result of its infection (Fig. 1). For all other animals, lesions had completely resolved between days 24–27. Only one animal in the 6×10^2 dosage group developed lesions, compared to the other West African dosage groups in which all 4 animals per group presented lesions. Additionally, lesions did not develop on that animal until day 17 which was later than the majority of animals in the other dosage groups, and lesions were restricted to the genitalia area and inner extremities. Animals in the 6×10^3–6×10^5 PFU dosage groups tended to develop lesions centrifugally; lesions were first observed on the ventral surface of the legs or on the head followed lastly by the trunk and shaved area of the back. Lesions on the paws tended to be apparent later in disease progression and may have been due to secondary infection caused by the animal grooming lesions elsewhere on the body. Fig. 2 depicts the typical progression of lesions for one animal. On day 10, vesicles are first visible on inner extremities. By day 13 p.i., lesions are numerous (>25) on the inner extremities, abdomen and face. During days 17–20, lesions began drying and desquamation occurred. On day 24, hair loss and hypopigmentation were visible where lesions had been. There was some variation observed in the development of lesions on the back area that seemed to result from individual animal variability, and did not necessarily appear to be dosage dependent. Lesions observed on the inner surface of extremities and on the trunk and shaved back area typically developed macular, vesicular, and pustular evolution before drying and desquamation. In slight contrast, facial and paw lesions evolved from macular to vesicular, but did not seem to reach the pustular stage. There was a wide range in the number of counted lesions from 3 to >50. Although lesion count was greatest in one animal (PD6) in the 6×10^3 PFU challenge inoculum group, additional signs and symptoms (facial edema, breathing difficulties, and purulent discharge) were additionally prominent in the two higher challenge dose groups. Also, mortality occurred soon after lesion onset in the group challenged with the highest inoculum. A range of clinical symptoms was observed that generally increased in severity with increasing dosage of virus (Table 1). Additionally, lymphadenopathy was observed in two animals (6×10^3 and 6×10^4 PFU groups) both of which died during the study. Weights generally remained static in the 6×10^2 PFU and 6×10^3 PFU inoculum groups (mean % weight loss 6.65% and 6.14% respectively), with the exception of the one animal in the 6×10^3 PFU group which exhibited a delayed onset of obvious disease signs. In contrast, all animals in the 6×10^4 PFU and 6×10^5 PFU dosage groups lost weight, (mean % weight loss: 15.88% and 18.47% respectively)

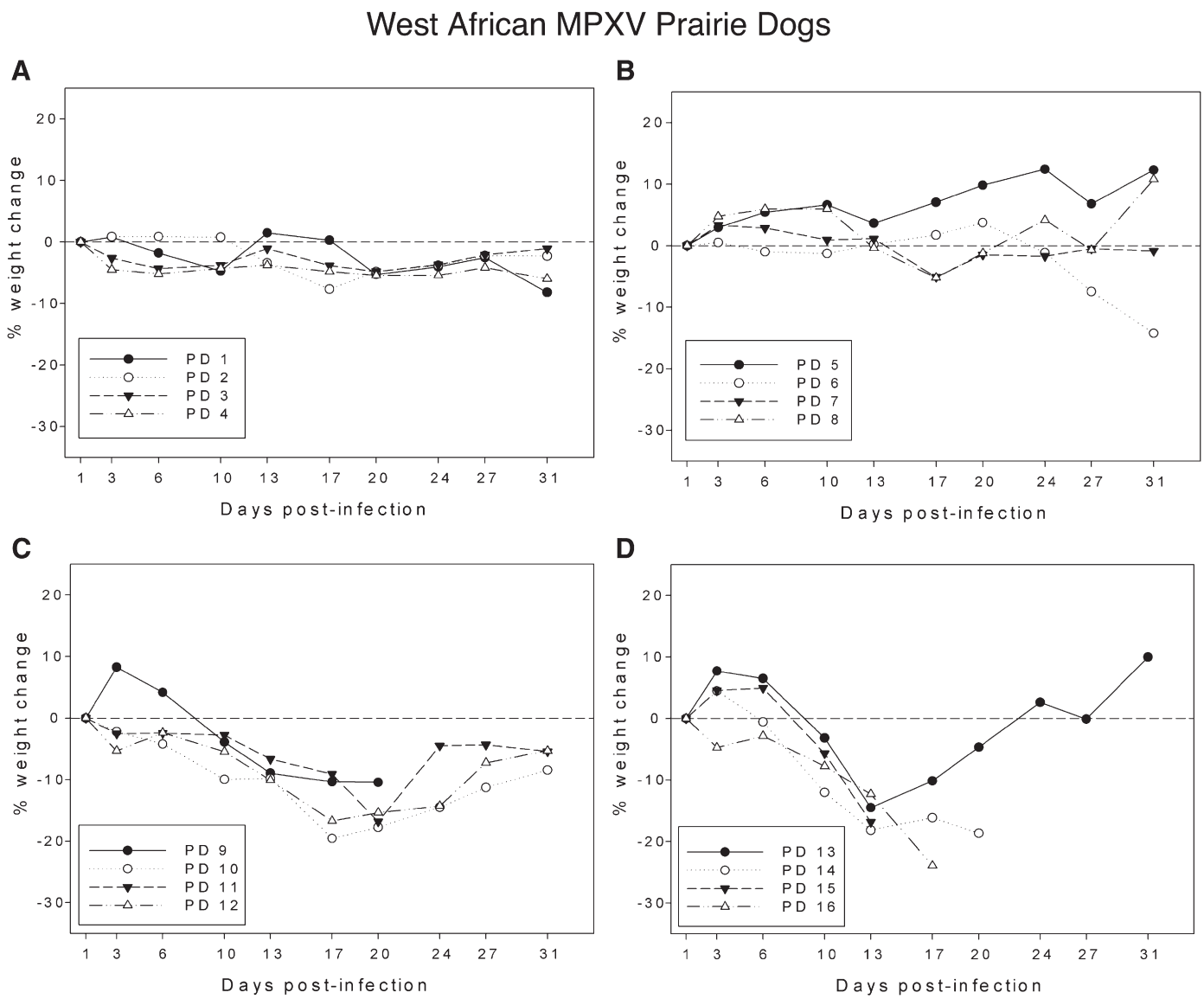

Fig. 1. West African MPXV: individual animal percent weight change. Groups of prairie dogs ($n=4$) were inoculated with 6×10^2 (A), 6×10^3 (B), 6×10^4 (C), or 6×10^5 (D) PFU of West African MPXV via an IN route.

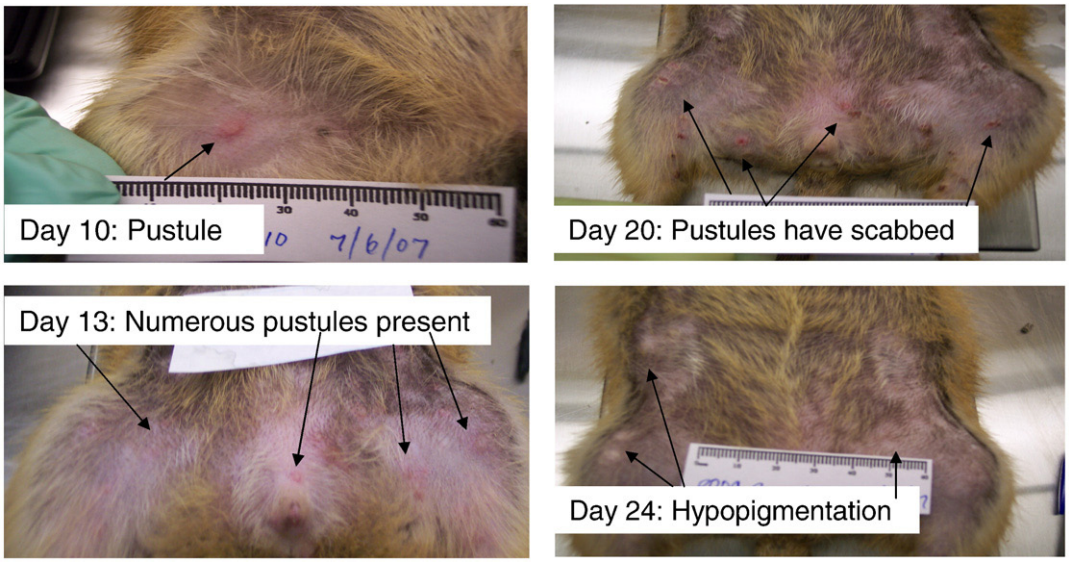

Fig. 2. Groups of prairie dogs ($n=4$) were inoculated with 6×10^2, 6×10^3, 6×10^4, or 6×10^5 PFU of West African MPXV via an IN route. The lesion progression for one animal in the West African 6×10^3 PFU group is shown beginning on day 10 p.i.

(Fig. 1). Mortality correlated with increasing viral challenge dose of virus for the West African inoculum groups (Table 1). Using the Reed–Muench equation, we were able to determine that the LD_{50} for West African MPXV is 1.29×10^5 PFU. A similar LD_{50} of 3.6×10^5 was calculated utilizing Probit analysis.

Congo Basin MPXV (Table 2)

Similar to what was observed with West African MPXV, with the exception of three animals in which inappetance was noted 1–3 days before lesion onset, no other symptoms were noted until the development of disseminated lesions usually on days 9–13 (most often on day 10), with lesions typically resolving between days 24 and 27. As seen with West African MPXV, only one of the 8×10^2 PFU dose animals developed lesions, compared to all 4 animals in each of the other dosage groups. Additionally, the animal in the 8×10^2 PFU dose group that developed lesions did not do so until day 13 which was slightly later than all other Congo Basin dosage groups, comparable to that seen with the West African 6×10^2 dosage group animal. The Congo Basin and West African animals had similar lesion presentation as described in the West African MPXV clinical findings and shown in Fig. 2. The number and overall range (3 to >50) of counted lesions were not dissimilar between the West African and Congo Basin infected animals, but animals challenged with the 8×10^4 and 8×10^5 doses of Congo Basin MPXV had up to two fold higher maximal lesion counts compared to those challenged with 6×10^4 and 6×10^5 PFU doses of West African MPXV. A range of clinical signs and symptoms were observed that increased in severity between 8×10^2 PFU and the higher challenge Congo Basin MPXV doses of virus (Table 2),

Table 1
Comparison of disease presentation and molecular findings for prairie dogs infected with different dosages of West African MPXV. Groups of prairie dogs ($n=4$) were inoculated with 6×10^2, 6×10^3, 6×10^4, or 6×10^5 PFU of West African MPXV via an IN route. Oral swabs were taken twice a week and blood was taken upon death or euthanasia.

Dosage[a]	6×10^2	6×10^3	6×10^4	6×10^5
Animals that developed lesions	1/4	4/4	4/4	4/4
Generalized lesion onset	Day 17 (PD2)	Days 10 (PDs 5, 7 and 8), and 27[b] (PD6)	Days 9 (PD9), 10 (PDs 10 and 12), and 13 (PD11)	Days 9 (PD16), and 10 (PDs 13, 14 and 15)
Counted lesions	5	3 to >50	4 to >20	10 to >25
Objective and subjective symptoms for individual animals (other than lesions)	(PD2) Inappetance (PDs 1, 3, and 4); no clinical symptoms observed	(PDs 5 and 8) Nothing other than lesions (PD6)[c] swollen LNs, facial edema, inappetance (PD7) lethargic	(PD9)[c] Inappetance, facial edema, labored breathing, nasal pus, lethargic (PD10) Inappetance, crusty nose, nasal blood/pus, bloody oral swab (PD11) crusty nose, swollen paws–lesions (PD12) Inappetance, swollen paws–lesions, crusty face	(PD13) Inappetance, nasal congestion, lethargy, facial/nasal edema, swollen paws–lesions (PD14)[c] Inappetance, facial/nasal edema, nasal congestion, disorientation, lethargy, labored breathing (PD15)[c] Inappetance, swollen LNs, nasal blood/pus, labored breathing, lethargy (PD16)[c] Inappetance, lethargy, facial/nasal edema, nasal pus, swollen paws–lesions, labored breathing
Mortality #	0/4	1/4 (PD6)	1/4 (PD9)	3/4 (PDs 14, 15 and 16)
Mortality (day post infection)	(NA)	33[b]	15	13, 14 and 17
Oral swab MPXV DNA (range)	Days 13–24	Days 6–24 (20–31[b])	Days 6–24	Days 3–24
Oral swab MPXV VV (range)	Days 13–17	Days 6–20 (24–31[b])	Days 6–20	Days 3–17
Highest viral load in oral swabs	5.4×10^4	2.4×10^6	1.4×10^7	8.6×10^7
Peak mean viral load in oral swabs	2.7×10^4	7.2×10^5	4.6×10^6	2.5×10^7
Anti-OPXV antibodies in all animals	1/4	4/4	4/4	3/4
Anti-OPXV antibodies in survivors	1/4	3/3	3/3	1/1

VV: viable virus (PFU/ml).
[a] All dosages were titrated but necessitated serial dilution to achieve countable plaque numbers.
[b] Animal had a delayed onset in infection.
[c] Animal died or was euthanized.

Table 2
Comparison of disease presentation and molecular findings for prairie dogs infected with different dosages of Congo Basin MPXV. Groups of prairie dogs ($n=4$) were inoculated with 8×10^2, 8×10^3, 8×10^4, or 8×10^5 PFU of Congo Basin MPXV via an IN route. Oral swabs were taken twice a week and blood was taken upon death or euthanasia.

Dosage[a]	8×10^2	8×10^3	8×10^4	8×10^5
Animals that developed lesions	1/4	4/4	4/4	4/4
Generalized lesion onset	Day 13 (PD21)	Day 10 (PDs 23, 24 and 25), Day 9 (PD26)	Day 10 (PDs 27, 28, 29 and 30)	Day 10 (PDs 31, 32, 33 and 34)
Counted lesions	3	3 to >25	8 to >50	10 to >50
Objective and subjective symptoms for individual animals (other than lesions)	(PD21) slightly swollen paws–lesions, nasal pus (PD19)[b] Inappetance (PDs 20 and 22) No clinical symptoms observed	(PD23)[b] Inappetance, nasal pus/crusty (PD24)[b] Inappetance, facial edema, ruffled coat, lethargy, nasal pus/congestion, oral/nasal blood (PD25)[b] Inappetance, swollen LNs, wheezing/labored breathing, nasal pus/congestion, facial edema, bloated, lethargy (PD26)[b] Inappetance, neck/face edema, oral/nasal blood	(PD27) Inappetance, nasal blood/pus, nasal edema (PD28) Inappetance, lethargy, nose/face edema, swollen paws–lesions, nasal congestion (PD29)[b] Inappetance, nasal/oral pus/blood, labored breathing, facial edema, lethargy, matted/swollen eye (PD30)[b] Inappetance, facial edema, nasal pus, swollen LNs, lethargy	(PD31) Inappetance, nasal pus, front paws swollen-lesions, lethargy (PD32)[b] Inappetance, facial edema, lethargy, labored breathing, mouth/nasal pus/blood (PD33) Inappetance, facial edema, nasal pus/blood, oral blood, lethargy, paw lesions (PD34)[b] Inappetance, labored breathing, facial edema, nasal pus, lethargy
Mortality #	1/4 (PD19)	4/4 (PDs 23, 24, 25 and 26)	2/4 (PDs 29 and 30)	2/4 (PDs 32 and 34)
Mortality (day post infection)	11	11, 15, 17 (2)	11, 17	11, 13
Oral swab MPXV DNA (range)	Days 6–27	Days 3–17	Days 3–24	Days 3–31
Oral swab MPXV VV (range)	Days 6–10	Days 3–17	Days 3–20	Days 3–20
Highest viral load in oral swabs	1.0×10^6	3.0×10^8	3.7×10^8	1.6×10^8
Peak mean viral load in oral swabs	2.5×10^5	1.3×10^8	1.1×10^8	5.3×10^7
Anti-OPXV antibodies in all animals	1/4	3/4	3/4	2/3[c]
Anti-OPXV antibodies in survivors	1/3	0/0	2/2	2/2

VV: viable virus (PFU/ml).
[a] All dosages were titrated but necessitated serial dilution to achieve countable plaque numbers.
[b] Animal died or was euthanized.
[c] One animal's blood was not able to be collected post mortem.

these signs and symptoms appeared also more numerous in a greater number of animals compared to the 6×10^3–6×10^5 PFU West African challenged animals. Weight loss remained static (3.26%) only in animals in the lowest dosage group. The three groups of animals challenged with 8×10^3–8×10^5 PFU virus groups all lost weight (16.27%, 14.19%, and 17.86% respectively) (Fig. 3). Lymphadenopathy was also observed in two animals (8×10^3 and 8×10^4 PFU groups), which were the similar dosage groups in which lymphadenopathy was observed in the West African infected animals, and as observed in West African challenged animals, both of these Congo Basin animals died during the study. Mortality did not correlate linearly with the dose of Congo Basin MPXV (Table 2). Using the Reed–Muench equation, which accounts for variability in small group response (in this case to mortality associated with viral dose), we calculated the LD_{50} for Congo Basin MPXV as 5.9×10^3 PFU, approximately 100 times lower than West African MPXV. Probit analysis was inappropriate for the Congo Basin data due to an inadequate fit.

Molecular and virologic findings

West African MPXV (Table 1)

Although only 1 animal in the 6×10^2 PFU dosage group developed disseminated lesions, three animals in this group had detectable levels of MPXV DNA in their oral swabs for at least one day p.i. beginning on days 13–17 (data not shown); the animal that developed lesions was also the only one of these that was assay-positive for viable virus (PD2) (Fig. 4). All 4 animals in each of the 6×10^3–6×10^5 PFU dosage groups were positive for MPXV DNA beginning as early as day 6 for 6×10^3 and 6×10^4 PFU dose groups and as early as day 3 for the 6×10^5 PFU dose group. Levels were detectable until at least day 24 for animals that survived infection in all three groups (Table 1). Viable virus kinetics were similar as that observed for DNA; the animal in the 6×10^2 PFU group that subsequently developed lesions and shed viable virus, was virus-positive beginning on day 13, slightly later than other groups. Animals in the 6×10^5 PFU dose group had detectable virus levels slightly earlier on days 3–6 (Table 1, Fig. 4). Viable virus shedding continued until days 17–20 for those animals that survived infection. In general, levels of viable virus in oral swabs increased with each subsequent dosage of virus; with the 6×10^5 PFU dosage group having the highest level at 8.6×10^7 PFU/ml (Figs. 4 and 5). Except for the 6×10^2 PFU dose group, in which only one animal had detectable viral DNA in necropsy tissues (the same animal that had highest levels of DNA and the only animal with detectable viable virus in oral swabs), all other animals that survived infection had low levels of detectable DNA in at least one necropsy tissue (data not shown). Viable virus was only detected in necropsy tissues from those animals that died or were euthanized during the study (Fig. 6). In the 6×10^3–6×10^5 PFU dosage groups of animals, almost all animals had developed OPXV antibodies at time of death or by the end of the study if death did not occur. The exception was one of the 6×10^5 PFU dose animals which died before the development of detectable antibodies (Fig. 7). Only one of the 6×10^2 PFU dose animals had OPXV antibodies when tested, the same animal that developed disseminated lesions as well as the only animal in this inoculum group with detectable viable virus in oral swabs as well as viral DNA at necropsy.

Congo Basin MPXV (Table 2)

Similar to the findings from the West African MPXV challenge, although only one 8×10^2 PFU dose animal developed disseminated lesions, all 4 animals in this group had low levels of viral DNA detected in oral swabs (data not shown). In contrast to that observed with the West African challenged animals, viral DNA was generally detectable earlier from oral swabs (day 3) in the three higher dosage groups. For animals that survived infection, DNA was detectable until days 27–31 (Table 2), slightly longer than that observed with the West African MPXV strain. Within the 8×10^2 PFU dose group, only two animals had detectable viable virus in the oral swabs (Fig. 8). One of these animals

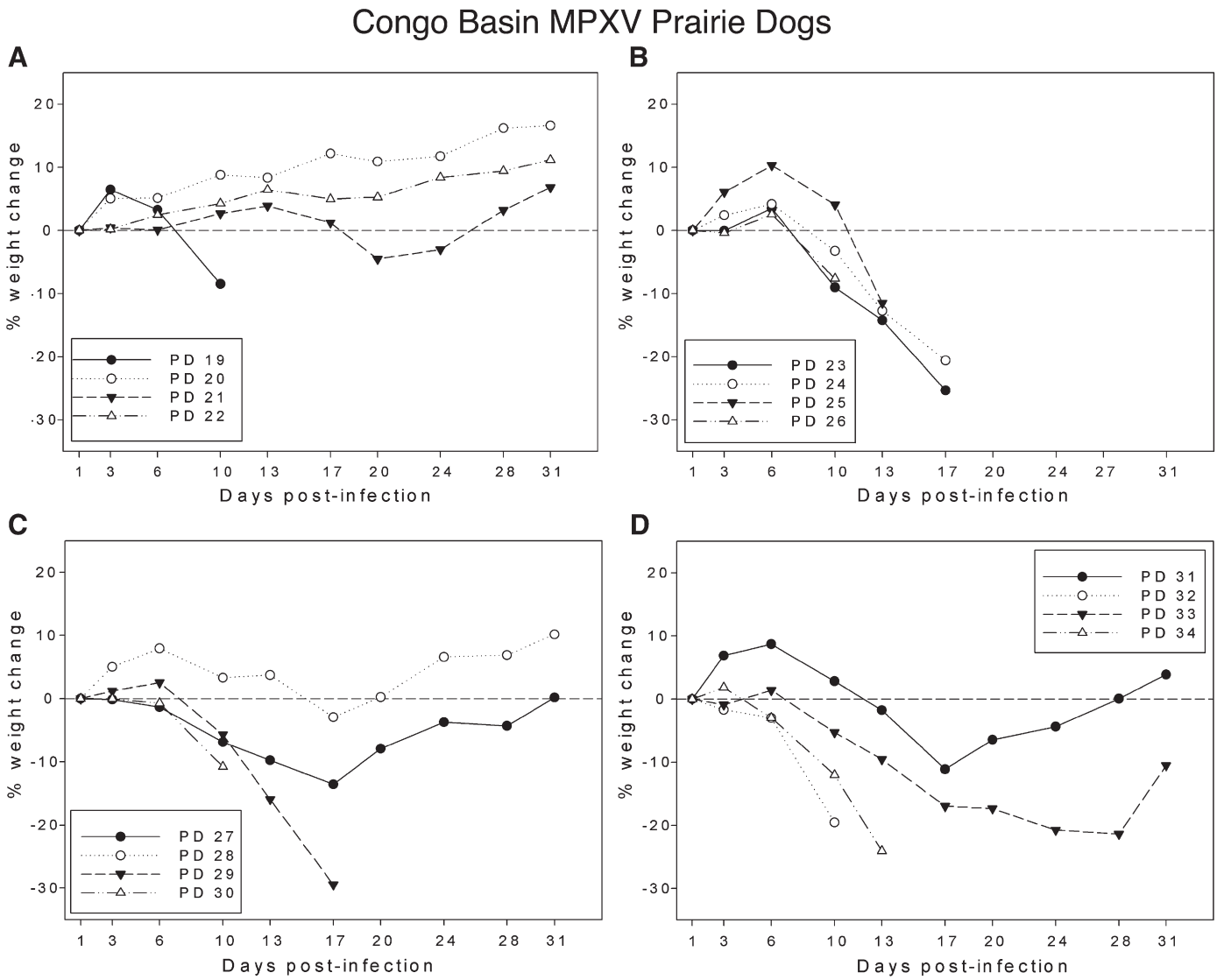

Fig. 3. Congo Basin MPXV: individual animal percent weight change. Groups of prairie dogs ($n=4$) were inoculated with 8×10^2 (A), 8×10^3 (B), 8×10^4 (C), or 8×10^5 (D)PFU of Congo Basin MPXV via an IN route.

began shedding virus on day 6 and was the only animal to perish in this dosage group, the other was the only animal in the 8×10^2 PFU inoculum group that developed lesions. For the three other dosage groups, viable virus kinetics correlated with DNA results. Viable virus was detectable in oral swabs beginning on day 3 for all animals in the 8×10^3–8×10^5 dosage groups and continued from those animals that survived infection until days 17–20 (Fig. 8). The 8×10^2 PFU dose animals had the lowest levels of viable virus, the 8×10^3–8×10^5 PFU dosage animals had similar levels (Figs. 8 and 5), with peak viral loads approximately 1–2 logs higher than that seen with similar dose West African MPXV challenged animals. Consistent with the West African MPXV challenge, animals that survived infection had low levels of viral DNA at time of necropsy but no viable virus (data not shown); animals that died or were euthanized due to MPXV infection had high levels of viable virus throughout tissues tested (Fig. 9). The exception to this was the only 8×10^2 PFU dose animal that developed disseminated lesions and OPXV antibodies (PD21), and which unlike other animals that survived infection, still had viable virus in a crusted lesion sample taken from the back at study end (Fig. 9). All of the animals in the three highest dosage groups that survived infection had developed an immune response by study end (Fig. 7). 4 animals died before developing OPXV antibodies, one animal was unable to be tested (PD34) due to inadequate blood sample post mortem, and two animals that were infected with 8×10^2 PFU survived infection, but did not develop an immune response.

Control animals

None of the mock-infected animals, housed under the same conditions, showed any evidence of MPXV infection during the duration of the study, such as lesions or weight loss. Furthermore, all samples taken from these control animals were negative for MPXV DNA when tested with PCR and were negative for OPXV antibodies (Fig. 7).

Statistical comparisons

Although weight loss occurred in more animals infected with Congo Basin MPXV (Figs. 1 and 3), when comparing the mean % weight losses between MPXV strains in the top three dosages, the values were not considered statistically significant (P values: 0.16, 0.68, and 0.68 respectively). When comparing weight loss between

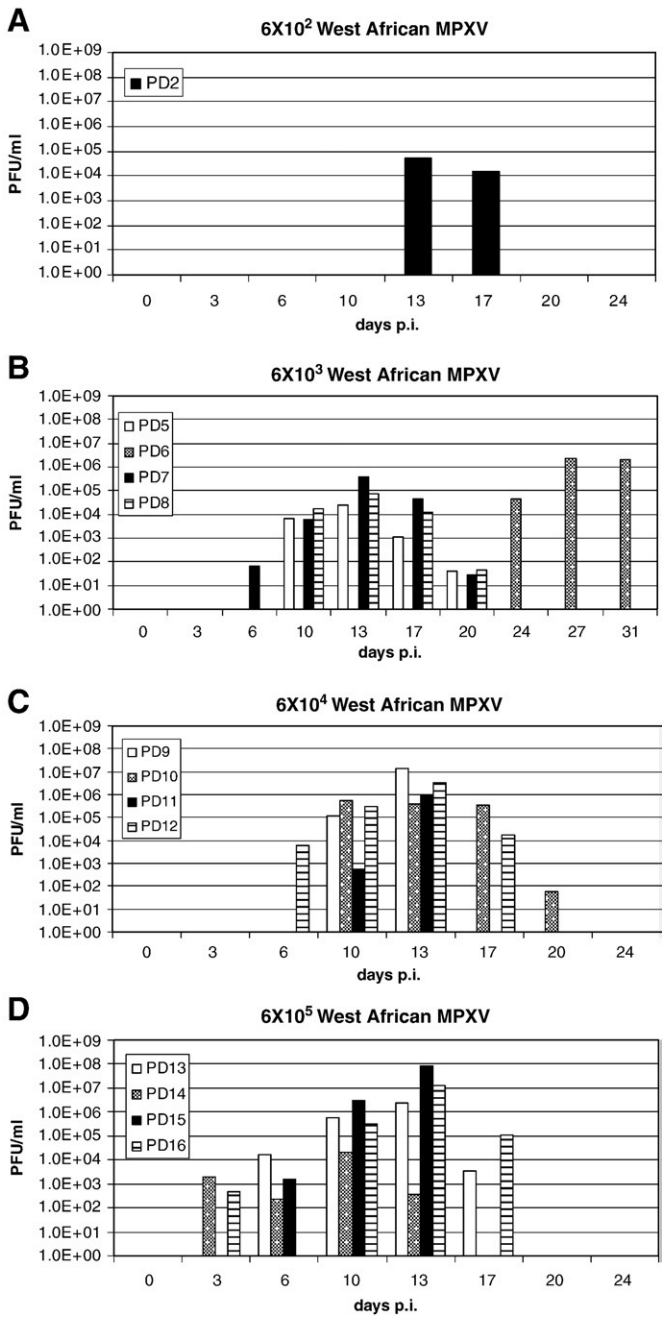

Fig. 4. West African MPXV viable virus results for oral swabs. Groups of prairie dogs ($n=4$) were inoculated with 6×10^2 (A), 6×10^3 (B), 6×10^4 (C), or 6×10^5 (D) PFU of West African MPXV via an IN route. Oral swabs were taken twice a week and subsequently tested for infectious virus. Values are shown on a log scale.

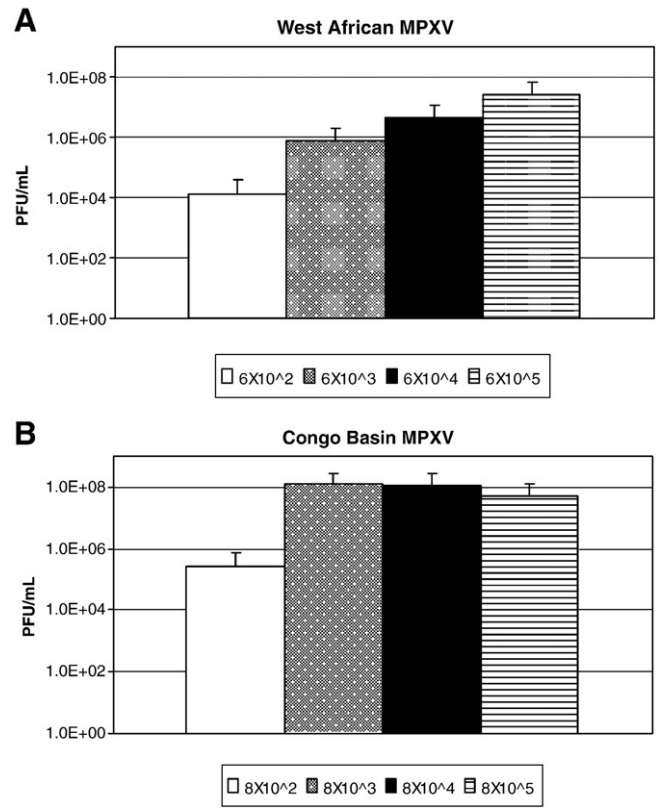

Fig. 5. Peak mean viral load for oral swabs. Groups of prairie dogs ($n=4$) were inoculated with one of four dosages of either West African MPXV (A) or Congo Basin MPXV (B) via an IN route. Oral swabs were taken twice a week and subsequently tested for infectious virus. Values are shown on a log scale. Error bars, SD.

dosages in each of the two strains, P values were significant between the two lower dosages of the West African strain compared to the two higher dosages ($P=0.0059$). Furthermore, a weak positive correlation between percent weight loss and increased viral load in oral swabs was found for all animals infected with either strain of MPXV (Pearson $r=0.423$; $P<0.0001$). As oral swab viral levels increased, animals were more likely to have a decrease in weight. Comparison of mean viral loads in oropharyngeal swabs between individual dosages and strains demonstrated a trend of increasing viral titers with increasing viral inoculum dose, however did not yield statistically significant differences. When all mean values were combined for each MPXV strain, Congo Basin animals had statistically higher levels of virus compared to West African MPXV infected animals (7.31×10^7 vs. 7.66×10^6; $P=0.021$) and viral loads in tissues taken from necropsy tended to be higher in those animals that succumbed to the Congo Basin MPXV (Figs. 6 and 9). However, because these animals died on different days, precise temporal comparisons are not possible.

Discussion

In this study, we were able to reproduce our previous findings, validating the utility of this model, and we were able to characterize additional aspects of disease progression and clade specific disease differences, by looking at a range of challenge doses. We were able to observe lymphadenopathy in some animals infected with each strain; an important observation in human MPX cases which distinguishes the disease presentation from that of smallpox. Consistent with descriptions of the oral enanthem of human systemic orthopoxvirus disease preceding the skin rash, viral shedding was detected in oropharyngeal secretions prior to development of generalized lesions on the prairie dog skin.

Animals infected with each strain of MPXV had a range of documented lesions that were not all consistently dose-dependent beyond the lower threshold dose (6×10^2 and 8×10^2) for both MPXV strains; this was especially true for the Congo Basin clade challenged animals. Animals challenged with the three higher doses of Congo Basin clade virus, for instance, all had maximal levels of virus in oropharyngeal samples; in West African challenged animals a clearer dose-shedding correlation is evident. This may be a sign of the greater virulence of the Congo Basin virus clade. The number of systemic signs and symptoms appeared dose-dependent in MPXV challenges of PDs with both virus clades, with a greater number of disease signs and

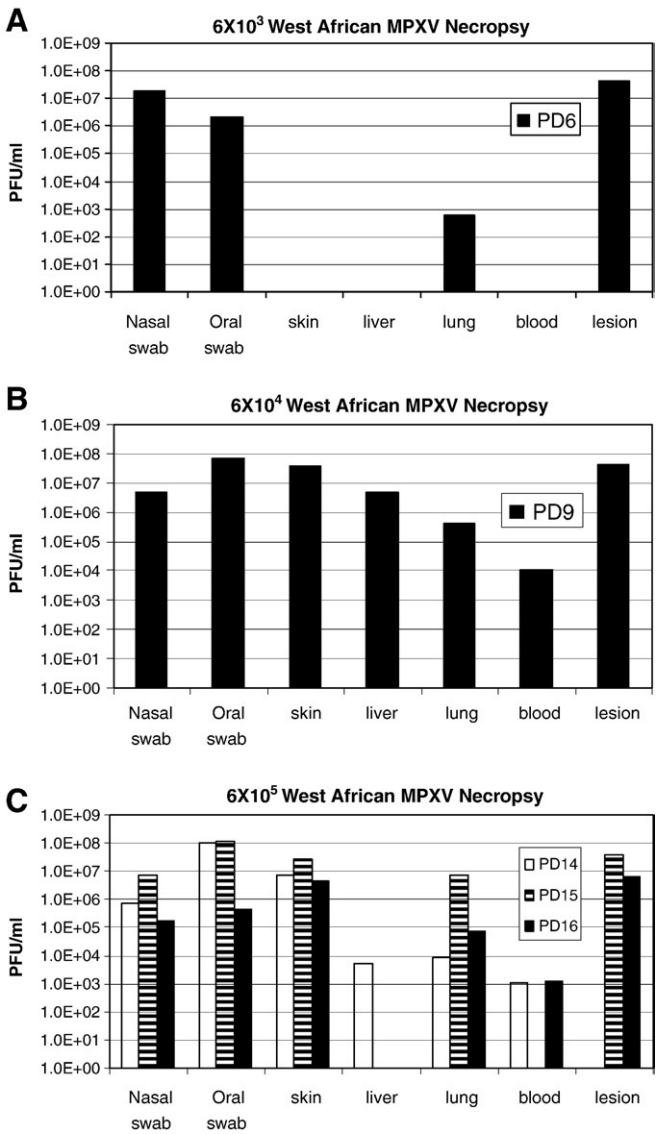

Fig. 6. West African MPXV viable virus results for necropsy samples. Groups of prairie dogs ($n=4$) were inoculated with 6×10^2, 6×10^3 (A), 6×10^4 (B), or 6×10^5 (C) PFU of West African MPXV via an IN route. If death did not occur, animals were euthanized 31–34 days p.i. and necropsies performed. Results are shown on a log scale for those animals which yielded infectious viral samples.

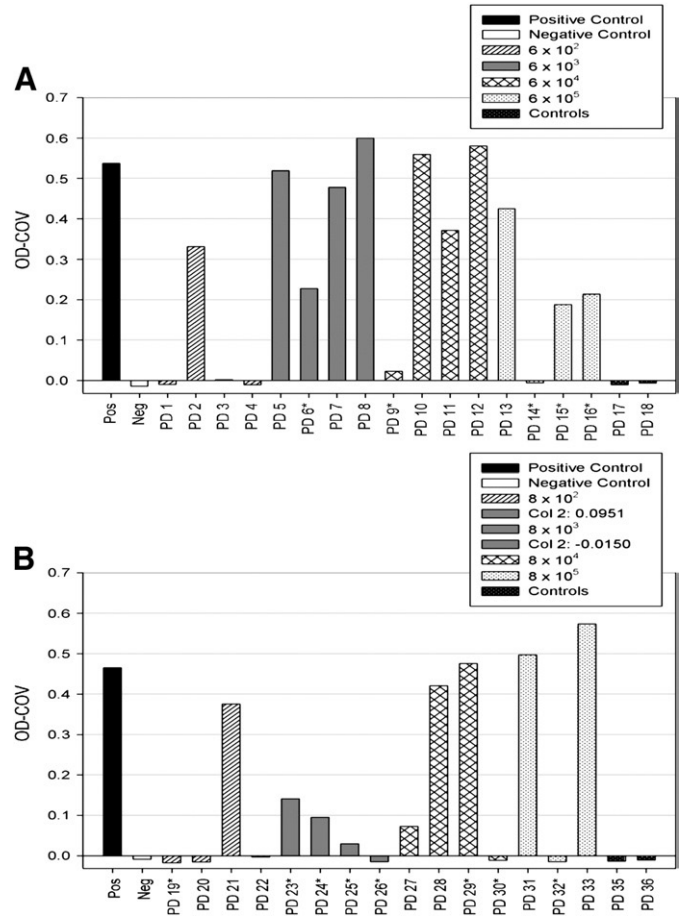

Fig. 7. Serology results for MPXV infected prairie dogs. Groups of prairie dogs were inoculated with either West African MPXV (A) or Congo Basin MPXV (B) via an IN route. Serum samples were taken upon death or at time of necropsy (except PD34 which could not have blood collected post mortem). OD values for OPXV antibodies are shown for each dosage group. Asterisks next to an animal number indicate an animal that perished or was euthanized during the study.

symptoms evident in Congo Basin challenged animals. Mortality observed for both the Congo Basin MPXV group and the West African MPXV group was slightly greater than in our previous studies (previous study deaths: $n=2$, $n=0$ respectively), although, there was variability within the Congo Basin MPXV dosage groups. Because these were wild caught animals, and not inbred, there is a greater probability that variations between individual hosts affected specific disease progression and mortality. All four animals in the Congo Basin 8×10^3 dosage group had very high loads of virus in oral swabs, which could have contributed to the 100% mortality in this group. Although animals were quarantined and screened for initial health status, it is possible that subtle variability in their health influenced the disease course. Additionally, as described in the Materials and Methods section, the investigators followed a strict euthanasia procedure as mandated by our Animal Care and Use Committee. Some animals that had to be euthanized following these criteria may well have survived infection if allowed to recover. Therefore, it is possible that the euthanasia of animals affected our LD50 calculations.

Although study group sizes are small, there is a trend towards later onset of clinical disease symptoms, and observations of delayed onset of viral shedding in oropharyngeal secretions in the animals challenged with the lower viral inocula. This trend is more evident in the West African challenged animals than in the Congo Basin challenged animals. In the West African group challenged with 6×10^2 infectious virions, virus was not seen in these secretions until day 13 or after. For those animals challenged with the two higher doses, viable virus in oropharyngeal samples occurred on day 6 or earlier. The most extreme example was the animal (PD6) in the 6×10^3 PFU West African MPXV dosage group; MPXV DNA was not detectable until day 20 p.i. and lesions were not present until day 27 p.i. The 27 day timeframe until lesion onset was similar to the animal in the 6×10^2 West African dosage group in which lesions were not apparent until day 17 p.i. PD6 was the only animal that lost weight in the 6×10^3 PFU dosage group and was found dead on day 33 p.i. We considered the possibility that PD6 was actually infected sometime after day 0 or re-infected during the course of the study, but felt this was not the likely explanation for the delayed disease onset. All animals were individually housed and none of the 4 control animals became infected during the study thus the delayed disease presentation in the animals challenged with lower viral loads may be

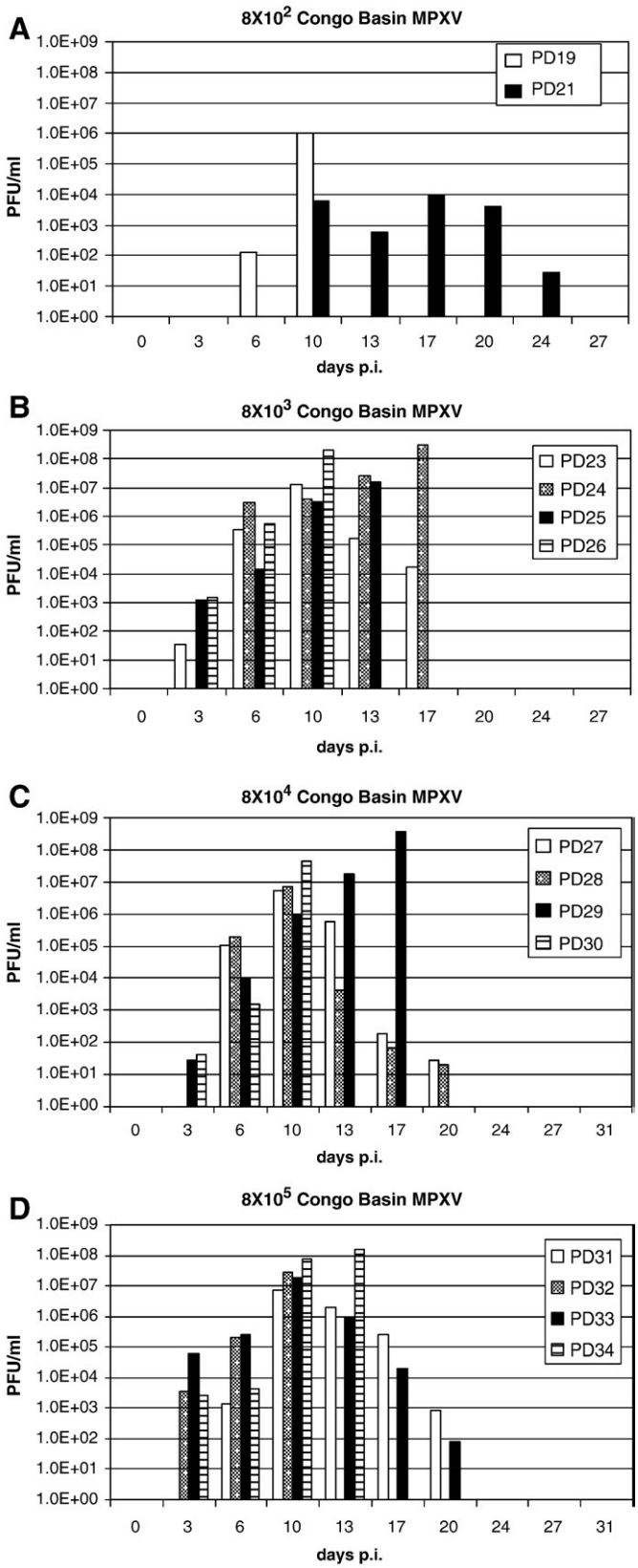

Fig. 8. Congo Basin MPXV viable virus results for oral swabs. Groups of prairie dogs ($n=4$) were inoculated with 8×10^2 (A), 8×10^3 (B), 8×10^4 (C), or 8×10^5 (D) PFU of Congo Basin MPXV via an IN route. Oral swabs were taken twice a week and subsequently tested for infectious virus. Values are shown on a log scale for animals which yielded infectious viral samples.

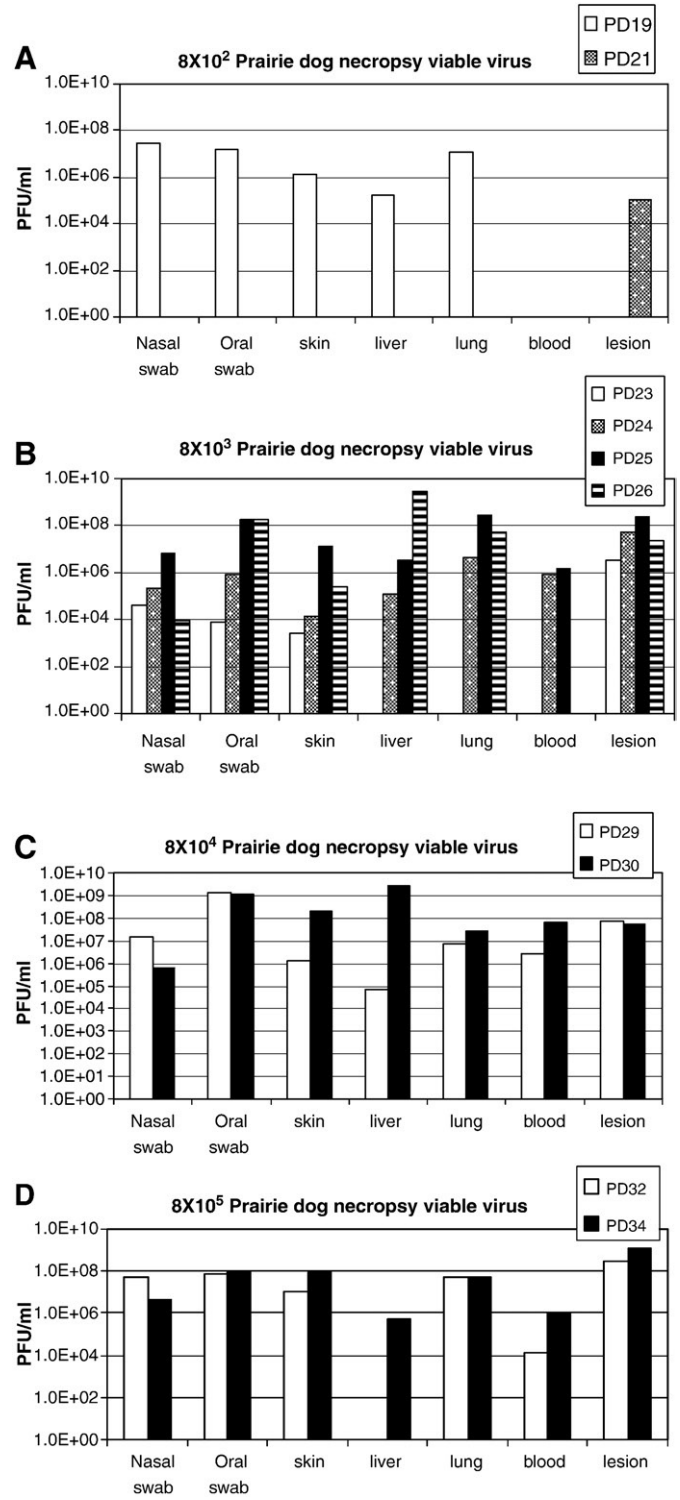

Fig. 9. Congo Basin viable virus results for necropsy tissues. Groups of prairie dogs ($n=4$) were inoculated with 8×10^2 (A), 8×10^3 (B), 8×10^4 (C), or 8×10^5 (D) PFU of Congo Basin MPXV via an IN route. If death did not occur, animals were euthanized 31–34 days p.i. and necropsies performed. Results are shown for those animals which yielded infectious viral samples on a log scale.

related to the lower viral inoculum; this hypothesis may better be tested with a larger sample size.

Although comparison of mean viral loads in oropharyngeal swabs between individual dosages and strains did not yield statistically significant differences, a trend of increasing viral titers with increasing

viral inoculum dose was apparent and when all mean values were combined for each MPXV strain, Congo Basin animals had statistically higher levels of virus. Additionally, the duration of MPXV DNA and infectious virus shedding in the oropharynx tended to occur earlier, attain higher levels, and persist longer for Congo Basin MPXV infected animals. Similar to human infections, the Congo Basin MPXV strain was more virulent in the prairie dogs based on morbidity and mortality. In general higher viral titers were seen in tissues of the Congo Basin MPXV infected animals, but more animals in this group died from the infection and were then necropsied during peak viral loads as compared to the West African group where more necropsies occurred at the end of the study when viral infections had cleared or were likely waning. Because of this, it is unclear whether the greater mortality seen in Congo Basin MPXV animals is attributable to higher viral tissue loads. A future serial sacrifice study would allow us to compare viral loads on set days to determine if there were statistical differences. PD mortality was higher in those animals infected with the Congo Basin MPXV strain, but was also less linearly dose correlated than that observed with the West African MPXV infected animals. Because the lowest dosage utilized was close to the LD_{50} value for the Congo Basin strain, one would not expect to see a linear relationship between the top three dosages. If lower dilutions had been used, we likely would have observed a more linear dose response. Utilizing the Reed–Muench formula (Reed and Muench, 1938), we were able to calculate the LD_{50} for the Congo Basin MPXV as 5.9×10^3 and the LD_{50} for West African MPXV as 1.29×10^5. This mathematical equation is ideal to use with small numbers of animals and takes into account "accidental" survivals or deaths by in effect canceling each other since equal number of dilutions is taken on each side of the endpoint (Reed and Muench, 1938). The calculated LD_{50} of approximately two logs difference between MPXV strains is consistent with previous studies and is consistent with observed pathogenic variations in humans infected with the two clades of MPXV.

The lack of detectable antibodies seen in some of the animals that perished during the study may have been due to limitations in the ELISA assay utilized (which does not detect IgM antibodies), but may also indicate that higher challenge doses were able to more efficiently circumvent host defenses, and caused death prior to the onset of an adaptive immune response. The serologic results confirmed that all surviving animals in the three highest inoculum groups for each strain developed immune responses to orthopoxviruses. For the animals challenged with the lowest inoculum of each MPXV strain, some surviving animals did not develop an immune response. This could suggest that the innate immune responses in these animals were able to clear the lower levels of challenge virus, without acquisition of adaptive immunity and/or that no viable virus reached susceptible cells to initiate productive infections which would result in anti-OPXV antibody production. This could also explain the delayed disease onset in those animals challenged with the lower viral doses; where the innate immune system could temporarily dampen viral replication for a finite period until viral loads were sufficient to overcome this and manifest as late onset symptomatic illness. Future studies evaluating virus loads in internal organs over the course of disease, with varying challenge doses may better assess this hypothesis.

Several other species have been considered as models for the comparison of MPXV strains including non-human primates (Chen et al., 2005; Saijo et al., 2009), ground squirrels (Sbrana et al., 2007), and inbred mice (Osorio et al., 2009; Hutson et al., 2010). Although these studies conclude as we do that the Congo Basin strain of MPXV is more virulent, they each have limitations in the modeling of human MPX disease. Unlike that seen in human outbreaks, the ground squirrel model was 100% lethal with both strains of MPXV. Additionally this model does not have the characteristic incubation period or development of disseminated lesions as is seen in human MPX. When comparing the MPXV strains in inbred mice, animals were resistant to disease. However, when immunodeficient mice were used, 100% mortality was seen with both MPXV strains and none of the inbred mice developed the lesions that characterize human infection. Non-human primate (nhp) disease models more closely mimic human rash illness disease progression than the two previous described models, but current nhp models lack an asymptomatic incubation period characteristic of human systemic orthopoxvirus disease.

The prairie dog MPXV model has many similar features as human MPX disease. The differences in MPXV clade LD_{50} values determined here are useful benchmarks for additional applications of this model to understand potential interventions for human MPX. Future antiviral efficacy studies can utilize the determined LD_{50} values to guide appropriate challenge dosages. If death as an endpoint is important for a particular study, challenge dosages above the LD_{50} should be utilized. Since development of disseminated lesions is an important observation of human MPX, the lowest dosage for each of the strains (6×10^2 and 8×10^2 PFU) should not be used for such a study. If lesion presentation with minimal mortality is desired for a particular efficacy study, staying above the lowest dilution, but below the determined LD_{50} for a particular strain would be optimal.

This study confirms previous observations (Hutson et al., 2009; Xiao et al., 2005) that showed PDs are an excellent model for studying human monkeypox and refines the MPXV experimental infection model by generating LD_{50} values in PDs for both MPXV clades. Future studies with the model system, including serial sacrifice studies, may allow us to better assess the interplay of host and pathogen in disease progression. This information will be critical for future MPXV studies, including investigations which will define virulence factors within the two clades, evaluate orthopoxvirus therapeutics, and study possible modes of virus transmission.

Materials and Methods

Animals

Wild-caught, juvenile black-tailed PDs (*Cynomys ludovicianus*) were obtained from western Kansas. At time of infection, animals were approximately 3 years old and had been prescreened for absence of anti-orthopoxvirus antibodies. The average starting weight for animals challenged with West African MPXV was 995 g (range 728–1267; 9 males, 7 females), and the average for Congo Basin MPXV challenged animals was 970 g (range 727–1366; 11 males, 5 females). During experimental infections animals were housed individually in large (12.13″ × 23.38″ × 209.00″) rat cages with aerosol filter tops. Cages were kept in a Duo-Flow biosafety cabinet in an animal Biological Safety Level-3 (ABSL-3) animal room. Animals were cared for in accordance with CDC Institutional Animal Care and Use Committee (IACUC) guidelines under an approved protocol (1431REGPRAC-A1). In addition to PD chow and hay, animals were provided with monkey biscuits for added dietary enrichment.

Viruses

The West African MPXV strain, MPXV-USA-2003-044, was isolated during the 2003 U.S. outbreak (Likos et al., 2005; Reed et al., 2004) and the Congo Basin MPXV strain, MPXV-ROC-2003-358, was collected from a 2003 outbreak of MPXV in the Republic of Congo (ROC) (Likos et al., 2005). Both viruses have been fully sequenced and underwent two passages in African green monkey kidney cells (BSC-40) prior to seed pool production; purified preparations of virus were used for animal challenges.

Animal inoculation

Infection of PDs with one of the two MPXV strains was done on separate occasions to reduce the potential for cross-contamination and to facilitate the logistics of monitoring animals. Inocula dosages

were calculated based on the mortality rates observed in the author's previous study (Hutson et al., 2009). Both virus strain stocks were serially diluted in phosphate-buffered saline (PBS) tenfold so that the intended infectious doses ranged from 10^3 to 10^6 PFU. Inocula titers were immediately re-confirmed by standard plaque assay (as described below) post infection and were found to be 8×10^2 to 8×10^5 PFU (Congo Basin) and 6×10^2 to 6×10^5 PFU (West African). In order to assure we had accurately inferred the titer of all challenge inoculums, virus dilutions were made using the identical methodology as used during the study, on two separate occasions, and titrated on BSC40 cells. The inocula inferred as used in the study were accurately calculated within ±21 PFU.

Animals were infected by an intranasal (IN) route of inoculation while under complete anesthesia using 5% isoflurane administered through a veterinary vaporizer. For each virus strain, groups of four animals were inoculated with one of the virus dilutions in a total volume of 10 µl IN (5 µl in each nostril). Additionally, four animals were mock-infected with PBS.

Observations and sampling

Daily visual observations of the animals were made (food consumption, mobility, general symptoms, and disease progression) and recorded throughout the study. Strict euthanasia criteria were applied throughout the study as follows: any animal that became unresponsive to touch, lost 25% or more starting body weight, or accrued a total score of 10 on the following scale was humanely euthanized; decreased activity (2 points); lethargy, unsteady gait and inappetance (3 points each); labored breathing and recumbency (5 points each). Blood was collected from the femoral vein into EDTA coated tubes prior to study onset as well as when the animal expired or was euthanized. Lesion count, weights, and oropharyngeal swabs were collected twice a week for 31 days post infection (p.i.), with the exception of the animals in the West African 6×10^3 dosage group which were not euthanized until day 34 due to one animal having delayed illness onset. It became apparent on day 27 that this animal was becoming moribund and in order to observe a complete picture and to have greater denominators, the animals in this group were followed until day 34. 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. Additionally, an area on the back of the animal was shaved prior to the start of the study in order to better visualize the development of lesions on the trunk. Sterile individual Dacron swabs were used to collect oropharyngeal samples and were stored frozen without diluent. Swabs, feces, and blood were processed and prepared for DNA analysis and virus isolation (see below).

Necropsy and tissue specimen collection

If death or euthanasia of animals did not occur as a result of infection, animals were humanely euthanized and necropsied at 31 or 34 days p.i. Necropsies on all animals were performed according to IACUC standards in an ABSL-3 laboratory and utilizing full ABSL-3 PPE. Samples taken during necropsy included: oral swab, lesion (skin if lesion was not present), lung, liver, whole blood and skin. Instruments were cleaned and decontaminated with 3% Amphyl and 10% Clorox bleach between collections of each tissue. Tissues were frozen at −70 °C prior to further processing. Oral and nasal 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-2 conditions with BSL-3 work practices. For DNA analysis of blood, 100 µl of water was added to 100 µl of blood in order to bring to a total volume of 200 µl. Samples were incubated at 55 °C for an hour to inactivate viable virus particles. The BioRobot EZ-1 Workstation (Qiagen) was used for genomic DNA extraction of all blood samples. For each swab collected, 400 µl of PBS was added. The swab extraction tube systems (SETS) (Roche) protocol was used to recover sample from the swab. DNA was extracted from 100 µl of the swab lysate using the EZ-1 DNA extraction robot (Qiagen). The remaining swab eluate was used for virus isolation (see below). Tissue samples were placed in disposable dounce homogenizers. PBS (1 ml) was added to each tissue sample in the 50 ml sterile tissue grinder and ground thoroughly to create a slurry. Genomic DNA was extracted from a slurry aliquot (100 µl) with EZ-1 DNA extraction robot (Qiagen) 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 probes complimentary to the conserved orthopoxvirus (OPXV) 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 probes specific to the MPXV B6R gene (Li et al., 2006). MPXV DNA (10 fg–1 ng) was used as positive controls for both tests. A positive sample produced CT values (in duplicate) of 37 or below. A weakly positive sample displayed CT values of 38–39 (duplicates).

Virus–tissue infectivity

Previous analyses demonstrated that real-time PCR detection of MPXV DNA is an assay for detecting trace amounts of MPXV DNA (Hutson et al., 2007). Therefore, specimens were first tested for presence of OPXV DNA by PCR and, if positive, were subsequently evaluated for viable virus by tissue culture propagation. Each swab or tissue sample was titrated in duplicate using 10 fold dilutions of swab eluate or tissue slurry on BSC-40 cell monolayers, incubated at 35.5 °C and 6% CO_2 for 72 h, and subsequently stained with crystal violet and formalin to visualize plaques. Titers were expressed as PFU per milliliter of tissue homogenate, blood or swab eluate.

Serologic analysis

A modified ELISA was used for analysis of anti-OPXV immunoglobulin types A and G as previously described in detail (Hutson et al., 2009).

Data analyses

The highest percentage weight loss was calculated for each animal. The baseline day zero weight and the lowest weight recorded thereafter were used to determine the percent weight loss for each animal. If an animal did not lose weight during the course of the study, it was given a value of zero for percent weight loss. Percent weight loss and highest oropharyngeal swab titers were compared; between Congo Basin and West African infected animals controlling for the dose, between doses controlling for MPXV strain, and between MPXV strains regardless of dose of virus. The two sample *t*-test and the Wilcoxon rank sum test were utilized and a *P*-value of ≤0.05 was considered statistically significant. The correlation coefficient was calculated between percent weight loss and oropharyngeal swab titer using Pearson correlation analysis.

For calculation of LD_{50} values for each strain, Probit analysis and the Reed–Muench method (which accounts for small sample size and non-linear observations) were utilized (Reed and Muench, 1938).

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