

## **A tale of two clades: monkeypox viruses**

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Human monkeypox was first recognized outside Africa in 2003 during an outbreak in the USA that was traced to imported monkeypox virus (MPXV)-infected West African rodents. Unlike the smallpox-like disease described in the Democratic Republic of the Congo (DRC; a Congo Basin country), disease in the USA appeared milder. Here, analyses compared clinical, laboratory and epidemiological features of confirmed human monkeypox case-patients, using data from outbreaks in the USA and the Congo Basin, and the results suggested that human disease pathogenicity was associated with the viral strain. Genomic sequencing of USA, Western and Central African MPXV isolates confirmed the existence of two MPXV clades. A comparison of open reading frames between MPXV clades permitted prediction of viral proteins that could cause the observed differences in human pathogenicity between these two clades. Understanding the molecular pathogenesis and clinical and epidemiological properties of MPXV can improve monkeypox prevention and control.

## INTRODUCTION

Smallpox was a devastating disease caused by *Orthopoxvirus variola virus*. The naturally occurring disease was eradicated 25 years ago, enabling cessation of smallpox vaccination (with *Orthopoxvirus vaccinia virus*) worldwide. Prior to 1970, monkeypox, a disease caused by *Orthopoxvirus monkeypox virus* (MPXV), was recognized only in non-human host(s). Between 1970 and 1986, 10 cases of human monkeypox were reported from Western African countries (Sierra Leone, Nigeria, Liberia and Côte d'Ivoire) and 394 cases were reported from the Congo Basin countries of Cameroon, Central African Republic and Zaire [now Democratic Republic of the Congo (DRC)] (Jezek & Fenner, 1988). Disease reported in West Africa was less severe and demonstrated less human-to-human transmission than that from DRC (Ladnyj *et al.*, 1972; Foster *et al.*, 1972; Breman *et al.*, 1980), where the World Health Organization (WHO) focused their 1981–1986 surveillance efforts. These studies addressed clinical, epidemiological, ecological and biological properties of MPXV (Jezek & Fenner, 1988). Rash burden, hospitalization rates and illness severity (a global score incorporating degree of incapacitation, need for nursing care and rash burden) were used to define human disease morbidity. Monkeypox case-fatality rates in DRC were ~10 % in non-vaccinated individuals (Jezek & Fenner, 1988). Individuals vaccinated against smallpox were noted to have fewer lesions and generally less severe disease (Jezek & Fenner, 1988). Mathematical models implied that MPXV could not transmit indefinitely between humans in the absence of additional introductions from zoonotic host(s) (Fine *et al.*, 1988), making it unlikely that MPXV would fill the niche left by smallpox eradication. Subsequent to 1990, surveillance for monkeypox waned throughout Africa.

In 2003, an outbreak of febrile rash illness in the USA among humans and captive pet prairie dogs was attributed to MPXV-infected West African rodents imported from Ghana (CDC, 2003). Laboratory testing confirmed 37 human cases as of August 2003, all associated with ill prairie dogs (CDC, 2003; Damon, 2003); no human-to-human transmission was documented. Early observations suggested that the disease described during the USA outbreak was milder than had been previously described in DRC patients (Damon, 2003; Peters, 2003). Observations, which were based on single gene sequence phylogenies, indicated the monkeypox isolate identified in the USA belonged to a clade of MPXV distinct from that of the previously characterized DRC isolates (Reed *et al.*, 2004). Concurrent with the 2003 USA monkeypox outbreak, the Congo Basin nation Republic of Congo (RCG) reported 10 monkeypox cases and sustained human-to-human transmission (Learned *et al.*, 2005).

To discern potential pathogenic differences, we compared clinical and epidemiological features of confirmed human monkeypox disease, using retrospective datasets from outbreaks in the USA and DRC as well as laboratory detection of MPXV in blood samples from the concurrent USA and RCG outbreaks. We observed differences that, after controlling for population age and vaccination disparities, may be attributed to genetic differences in the geographically distinct viruses. Therefore, we sequenced and annotated five MPXV isolates to

identify genomic and proteomic differences, to enable more robust depiction of MPXV clades and to allow the development of hypotheses regarding differences in pathogenesis.

## METHODS

**Clinical and epidemiological comparisons.** To compare monkeypox infection in the USA, Western and Central Africa, we obtained a coded dataset generated from standardized data-collection forms at WHO, describing 404 African cases reported during 1970–1986, a time of enhanced surveillance for smallpox and, later, monkeypox. Because we were concerned that only severe cases of human monkeypox might be detected and reported during the early years, we limited our analyses to the 338 cases from 1981 to 1986 in DRC. During this time, public health authorities actively sought and reported all monkeypox cases in five regions of DRC (Jezek & Fenner, 1988), a situation more analogous to the likely complete reporting of USA human monkeypox cases. Although we initially intended to include only cases determined by direct evidence of MPXV infection (pock morphology on chorioallantoic membranes, electron microscopy visualization and/or tissue culture), comparison of serologically to non-serologically confirmed cases within DRC suggested that serologically confirmed cases may have been milder (perhaps due to less severe disease or rash burden, and therefore detected later in the disease progression). Serologically confirmed DRC cases were included to avoid potential biases. Eleven of 338 DRC case-patients died without laboratory testing and were excluded from the analysis. USA cases analysed were only those confirmed by PCR amplification specific for MPXV, viral isolation or immunohistochemistry with an appropriate epidemiological link. Information on USA cases was also collected using a standardized data-collection form.

Because age and prior vaccination for smallpox may affect the severity of human monkeypox illness, we stratified our analyses by these factors. We limited our analysis of DRC patients to the same age range as seen in the USA outbreak (6–48 years). This resulted in the exclusion of 200 cases (198 <6 years of age, two >48 years of age) from the 327 laboratory-confirmed DRC cases. Stratification by vaccination status, however, was confounded by age, and stratification by both vaccination status and age resulted in empty cells since no children in the USA were vaccinated against smallpox, and there were no adults in DRC that had not been vaccinated.

Both datasets included information on rash burden (lesion counts), hospitalizations, case outcomes and types of complications and/or sequelae. Additionally, the USA dataset included a variable explaining the reason for hospitalization (e.g. ‘for isolation’, or ‘severity of disease’), while the WHO dataset scored illness severity as one, two or three on the basis of the number of lesions, degree of incapacitation and need for nursing care. A score of three was given to individuals with  $\geq 100$  lesions and severe incapacitation requiring medical care (Jezek & Fenner, 1988), although individual scores for lesions and incapacitation occasionally differed (17 of 117 cases with known lesion counts were discordant). Therefore, for our comparisons, a severely ill individual from DRC was defined as anyone with  $\geq 100$  lesions who was hospitalized and had an illness severity score of three. A severely ill USA patient was similarly defined as having  $\geq 100$  lesions and was hospitalized and was noted to be hospitalized specifically for ‘severity of disease’.

**Statistical methods.** Two-sided Fisher's exact tests were used to compare infection characteristics between DRC and USA case-patients, and a non-parametric test (Wilcoxon rank sum) was used to compare ages between the two populations. For ease of evaluation, comparisons of infection characteristics between DRC and USA cases are presented as relative risk ratios (RR) with 95 % confidence intervals (CI).

**Strains.** The five sequenced MPXV strains were derived from isolates collected in Liberia, DRC, USA and RCG. MPXV-LIB-1970-184 (DQ011156; length, 200263 bp; G+C content, 33.09 mol%) was collected from a 3-year-old girl in Liberia in 1970. A crust sample was processed and passaged once in chorioallantoic membrane (CAM) and twice in African green monkey kidney cells (BSC-40) prior to DNA isolation for sequencing. Descriptive and initial laboratory information has been published (Foster *et al.*, 1972; Lourie *et al.*, 1972).

MPXV-ZAI-1979-005 (DQ011155; length, 196967 bp; G+C content, 33.09 mol%) was isolated from a severely ill, 1-year-old boy living in Zaire in 1978 (Breman *et al.*, 1980). The virus was isolated from a lesion scab, which was positive for poxvirus by electron microscopy and for monkeypox virus on CAM. DNA was derived from virus passaged five times on BSC-40 cells.

MPXV-USA-2003-044 (DQ011153; length, 198780 bp; G+C content, 33.08 mol%) and MPXV-USA-2003-039 (DQ011157; length, 198780 bp; G+C content, 33.08 mol%) were collected during the 2003 monkeypox outbreak in the USA from a prairie dog and human, respectively. The prairie dog isolate was from the lymph node of the animal associated with the initial (index) case recognized in Wisconsin. The human isolate was obtained from a skin vesicle of the exotic pet distributor who sold this prairie dog to the family that heralded the outbreak (Reed *et al.*, 2004). Both were passaged twice on BSC-40 cells prior to DNA isolation for sequencing. The MPXV-USA-2003-044 and MPXV-USA-2003-039 genomes have a single nucleotide difference at base 31 in the inverted terminal repeat region prior to the leftmost open reading frame (ORF).

MPXV-RCG-2003-358 (DQ011154; length, 197191 bp; G+C content, 33.09 mol%) was isolated from a 10-year-old girl infected in Impfondo, RCG, and admitted to the hospital on 9 June, 2003 (Learned *et al.*, 2005). A bloody rash-derived sample was processed and passaged twice in BSC-40 cells, followed by DNA isolation.

**Sequencing.** We sequenced the entire genome of five strains from USA and African MPXV isolates collected over 33 years and compared them to the published genome for MPXV-ZAI-1996-016 (Shchelkunov *et al.*, 2002) (NC\_003310). We compared genome nucleotide sequences to distinguish the clades, which we designated West African/USA and Congo Basin. Monkeypox virus DNA was prepared as previously described (Reed *et al.*, 2004). The genomic DNAs were used as templates for the production of 20 overlapping PCR amplicons designed to span the complete viral genome. Each amplicon (5–16 kb) was generated using the Expand High Fidelity PCR system (Roche Applied Science); for each DNA sequencing template, eight independent PCR reactions were pooled and treated with ExoSap-IT (USB). The templates

were sequenced by primer-walking both strands, using ABI Big-Dye 3.1 dye chemistry and ABI 3730XL automated DNA sequencers (both from PE Biosystems). Sequencing primers were synthesized by Integrated DNA Technologies. Approximately 2500 reads were obtained for each genome, resulting in a ninefold mean redundancy at each base position. Chromatogram data were assembled using Seqmerge (Wisconsin Package version 10.3; Accelrys), Phred/Phrap base-calling and assembly software (Ewing *et al.*, 1998; Ewing & Green, 1998) and Consed (Gordon *et al.*, 2001) for sequence editing.

**Analysis of genomic sequences.** Sequence annotation was done through a locally modified version of Poxvirus Orthologous Clusters software (Ehlers *et al.*, 2002). Genes were predicted using GeneMarkS (Besemer *et al.*, 2001) and Glimmer 2.02 (Delcher *et al.*, 1999) and then tested for the presence of regulatory elements and assigned an initial annotation by comparison to other poxvirus gene databases, using BLASTP (Altschul *et al.*, 1997). The remaining ORFs were then verified by manual inspection. Genome alignments were generated with Mavid (Bray & Pachter, 2004) with default parameters and then edited by hand to remove errors. The predicted protein sequence alignments in Fig. 5 and Supplementary Fig. S1 (available in JGV Online) were constructed using CLUSTALW (Higgins & Sharp, 1988). The diversity calculation,  $\pi$ , was calculated using a modification of the Nei & Miller method (1990) in the DNAsp software package (Rozas *et al.*, 2003). MrBayes (Ronquist & Huelsenbeck, 2003) was used to perform a maximum-likelihood Bayesian analysis of whole genomic nucleotide sequences from five MPXV isolates as well as from one isolate each of cowpox (CPXV-GRI; X94355) and vaccinia (VACV-COP; NC\_001559). The program settings were as follows: the maximum-likelihood model utilized six substitution types, base frequencies were set to the empirically observed values, and rate variation across sites was modelled using a gamma distribution with a proportion of sites being invariant. The Markov Chain Monte Carlo search was run with four chains for 5000000 generations. Trees were sampled every 1000 generations and the first 200 trees were discarded.

**Restriction fragment length polymorphism (RFLP) analysis.** Monkeypox virus DNA was prepared as previously described (Reed *et al.*, 2004). Amplification of the A-type inclusion (ATI) gene region followed standard protocol. The amplicon DNAs were used as template for RFLP analysis, using *Xba*I and *Bgl*II (data not shown) restriction endonuclease analysis to identify bands that co-migrate with MPXV DNA fragments (Meyer *et al.*, 1997).

**Analysis of clinical diagnostic samples.** A quantitative real-time PCR assay was used to assess the amount of viral DNA within clinical whole blood samples submitted from confirmed USA monkeypox cases, and from probable and confirmed RCG monkeypox cases to the Centers for Disease Control and Prevention (Atlanta, Georgia) during the summer of 2003. Each sample was confirmed as monkeypox, using a real-time PCR monkeypox-specific assay, and the DNA content quantified by triplicate runs, using an orthopox generic assay (Y. Li, V. Olson, T. Laue, M. Laker & I. Damon, unpublished data). Samples with MPXV DNA detected in

none or only one of the triplicate runs were considered negative, and those with DNA in two of three runs were considered equivocal. A sample was considered positive only if all three runs crossed the threshold. A dilution series of purified vaccinia virus was used to create a reference standard curve for the orthopoxvirus generic real-time PCR assay. Using this standard curve, the viral load in femtograms for each sample was calculated on the basis of the mean cycle at which fluorescence from the sample crossed the threshold. To determine the length of viraemia, viral load was plotted against the number of days after onset of rash or fever.

## RESULTS

### Comparison of clinical and epidemiological features of monkeypox in Africa and the USA

DRC patients were more likely than USA patients to have pronounced rash (rash count  $\geq 100$ ) (Table 1); this was true regardless of the laboratory method used to confirm DRC case-patient status (see Methods). The 53 DRC case-patients who were confirmed by serological tests alone were less likely to have pronounced rash (RR 0.7, 95 % CI 0.58–0.84 for  $\geq 100$  lesions) than were DRC case-patients who were confirmed by direct virus testing. Although hospitalization rates did not differ between DRC and USA patient populations, there were significantly more severely ill patients (see Methods) in the DRC than in the USA (Table 1). There were no significant differences between DRC and USA case-patients in complication rates or sequelae (data not shown). Monkeypox-related mortality and human-to-human transmission were features seen only in DRC (Tables 1 and 2).

Young age and unvaccinated status have both been associated with more severe disease and mortality (Jezek & Fenner, 1988). Case-patients in DRC were younger than those in the USA (median 11.5 years, range 6–44 vs 25.4 years, range 6–48, respectively,  $P < 0.001$ ). To control for this, we grouped case-patients by age ( $\geq 18$  or  $< 18$  years of age; Table 1). DRC case-patients in all age groups appeared to be at greater risk than USA patients for pronounced rash, but this difference was only significant in the population  $\geq 18$  years of age.

Although significantly more unvaccinated case-patients in the DRC than in the USA had pronounced rash (RR 5.6, 95 % CI 1.9–16.4; Table 2), they were not significantly more likely to be hospitalized. Overall, significantly more unvaccinated DRC than USA case-patients were severely ill; however, all unvaccinated, severely ill patients, regardless of their geographical origin, were  $< 18$  years old (Table 2). No smallpox-vaccinated patients in the USA had pronounced rash or were severely ill, despite an apparent longer time period between onset of illness and last vaccination ( $> 20$  years for the 2 of 10 USA patients who could recall their last vaccination vs  $9.4 \pm 4.3$  years, range 1–18, in the DRC). In sum, human disease was more transmissible and more severe among DRC than USA case-patients, and this finding was independent of patient age and vaccination status.

### Prolonged detection of viral nucleic acid

One hypothesis to explain the relative differences in disease severity and transmissibility would be a difference in the duration and magnitude of viraemia. We performed quantitative PCR for MPXV in those cases where whole blood samples were available from monkeypox patients in the USA and RCG (Fig. 1). Of 14 blood samples collected within 21 days of rash onset from 12 USA patients with confirmed infection, three were positive for MPXV DNA and two others were equivocal. All were negative past day 21, a time point likely exceeding the occurrence of viraemia in an otherwise healthy human host (Downie *et al.*, 1950). In contrast, two of three RCG samples obtained within 21 days of rash onset from three probable or

confirmed patients were positive for MPXV DNA; one of five samples collected after day 21 (day 33) was positive. These results suggested a longer presence of virus in the blood of RCG patients. No USA or RCG samples contained viable virus at the time of testing.

### **Single-gene phylogenies**

Previous comparative analysis of discrete genes suggests the existence of at least two monkeypox virus strains that are geographically separated (Reed *et al.*, 2004). PCR amplicon and RFLP analysis of isolates collected from the USA and Central and Western African outbreaks readily classified isolates into Central or Western African strains (Fig. 2). By this genotypic criterion, Gabon, Cameroon, RCG and DRC isolates fell within the Central African clade, perhaps more appropriately called the Congo Basin clade, while Nigerian, Liberian and USA (ex-Ghana) isolates grouped into the West African clade (Fig. 2).

### **Whole genome phylogenetic analysis**

To establish definitively the existence of two clades and understand proteomic differences, we sequenced genomes of five geographically and/or temporally distinct strains (Supplementary Tables S1–S4 and Methods). Genomic sequences of individual West African-derived (Liberia and USA) and Congo Basin monkeypox strains showed an overall nucleotide identity of ~99 % within geographical regions and only ~95 % nucleotide identity across geographical groupings (Table 3). Of note, there was only a single nucleotide difference between MPXV-USA-2003-044 (prairie dog) and MPXV-USA-2003-039 (human) over one or two cycles of viral transmission, likely from a common source (Reed *et al.*, 2004).  $\pi$  (Nei & Miller, 1990), a measure of ORF nucleotide diversity across all strains, was greater among predicted immunomodulatory/host-range protein encoding ORFs within the left and right genome termini than among essential virus replication/transcription ORFs encoded within the central region (Fig. 3). Most ORFs with  $\pi$  values  $>6$  were fragments of orthopoxvirus homologues. Phylogenetic analyses, using maximum-likelihood and parsimony methodologies (Ronquist & Huelsenbeck, 2003; Swofford, 2002), were conducted using the four geographically distinct MPXV genomes, the previously described MPXV-ZAI-1996-016 (Shchelkunov *et al.*, 2002), and were rooted with cowpox virus strain Grishak (CPXV-GRI) and vaccinia virus strain Copenhagen (VACV-COP). All analyses resulted in identical topologies depicting two distinct MPXV clades: West African/USA and Congo Basin (Fig. 4).

### **Predicted protein sequence comparisons**

To analyse proteins that discriminate clades and that may cause the observed differences in human monkeypox disease pathogenesis, we focused on the previous finding that a small number of amino acid changes significantly alter protein function in other orthopoxviruses (Rosengard *et al.*, 2002). Using a predicted change of  $\geq 5$  aa as an indication of

potentially significant altered function, we identified nine proteins with differences conserved across the two clades (Fig. 5 and Supplementary Fig. S1). Four of these nine are involved with various aspects of the viral life cycle (including replication and transcription) and include the monkeypox orthologues of VACV-COP or vaccinia virus strain Western Reserve (VACV-WR) late transcription factor H5R (VLTF-4) (Kovacs & Moss, 1996; Beaud & Beaud, 1997; Black *et al.*, 1998; Murcia-Nicolas *et al.*, 1999; Brown *et al.*, 2000) (Supplementary Fig. S1a), essential morphogenesis factor A9L (Yeh *et al.*, 2000) (Supplementary Fig. S1b), non-essential, early expressed DNA ligase A50R (Kerr & Smith, 1989; Smith *et al.*, 1989a; Colinas *et al.*, 1990) (Supplementary Fig. S1c) and actin tail nucleation protein A36R (Parkinson & Smith, 1994; Sanderson *et al.*, 1998; Wolffe *et al.*, 1998; Frischknecht *et al.*, 1999; Ward *et al.*, 2003) (Supplementary Fig. S1d).

The remaining five proteins that consistently demonstrated geographically associated differences have been experimentally demonstrated to be involved with either immune evasion or host range determination in other poxviruses. These include the interleukin-1 $\beta$  (IL1 $\beta$ ) receptor orthologue (affects the febrile response and virulence) (Smith & Chan, 1991; Spriggs *et al.*, 1992; Alcamì & Smith, 1992, 1996), the SPI-1 orthologue (apoptosis regulation, host range) (Smith *et al.*, 1989b; Kotwal & Moss, 1989; Senkevich *et al.*, 1993; Thompson *et al.*, 1993; Ali *et al.*, 1994; Kettle *et al.*, 1995; Brooks *et al.*, 1995; Macen *et al.*, 1996; Shisler *et al.*, 1999; Moon *et al.*, 1999; Legrand *et al.*, 2004), the vaccinia C7L orthologue (a host range factor) (Perkus *et al.*, 1990; Oguiura *et al.*, 1993), the myxoma M-T4 orthologue (apoptosis regulation) (Barry *et al.*, 1997; Shchelkunov *et al.*, 1998; Hnatiuk *et al.*, 1999; Price *et al.*, 2002) and the orthologue of the complement control protein (CCP) (inhibits the classical and alternate complement pathways) (Isaacs *et al.*, 1992; Miller *et al.*, 1997; Smith *et al.*, 2000; Rosengard *et al.*, 2002; Isaacs *et al.*, 2003).

Fragmentation of the West African/USA MPXV IL1 $\beta$  receptor orthologue was observed. Either a single nucleotide deletion results in truncation after the signal peptide (Fig. 5a) or, if the second methionine is used to initiate translation, only the first two immunoglobulin domains (Smith & Chan, 1991) are translated. Within the Congo Basin MPXV clade, the MPXV-ZAI-1979-005 orthologue also lacked the third immunoglobulin domain, while MPXV-ZAI-1996-016 and MPXV-RCG-2003-358 orthologues had full-length sequences (Fig. 5a).

Insertion/deletion events in the upstream regions of the West African/USA MPXV orthologues of vaccinia SPI-1 (C12L) and VAC-COP-C7L were predicted to affect these expressed proteins, in addition to amino acid changes. A 4 nt deletion in the variable repeat region upstream of the West African/USA MPXV vaccinia SPI-1 (C12L) orthologue may produce, dependent on promoter usage, an in-frame fusion resulting in a variable length string of amino-terminal ILY repeats (Supplementary Figs S1e, S1f, S2 and Online Supplementary Results and Discussion). Upstream of the West African/USA MPXV VAC-COP-C7L orthologue, a single nucleotide deletion was predicted to result in an amino-terminal fusion of 17 aa to the protein, depending upon which predicted promoter is used (Fig. 5b, Supplementary Fig. S3 and Online Supplementary Results and Discussion). A BIMAS-predicted 9 mer epitope of this gene product, conserved between vaccinia and variola, is protective as a single peptide vaccine

against a lethal intranasal vaccinia mouse challenge model in HLA-A2.1 transgenic mice (Snyder *et al.*, 2004). In all monkeypox isolates sequenced, this epitope was perturbed (Fig. 5b), such that the BIMAS-predicted dissociation half-life decreased three to fourfold (to 107 or 89 min). The West African/USA strain predicted protein sequences contained a unique 9 mer epitope with a predicted dissociation half-life even greater than that of the vaccinia/variola epitope (437 min vs 365, respectively).

Two immunomodulatory proteins of the Congo Basin clade viruses were fragmented or absent in the West African/USA MPXV clade. The West African/USA MPXV orthologue of the myxoma virus apoptotic regulator (M-T4) was predicted to be severely truncated (Fig. 3, box E), similar to the non-functional vaccinia B9R gene product (Price *et al.*, 2002). Although there was minimal overall amino acid conservation (~24 %), Congo Basin orthologues retained the conserved cysteine residues and, thus, may exhibit anti-apoptotic function and permit infection of lymphocytes (Fig. 5c) (Barry *et al.*, 1997). Lastly, the MPXV orthologue of the vaccinia CCP and smallpox virus inhibitor of complement enzymes was completely absent in West African/USA isolates (Fig. 3, box B). The orthopoxvirus orthologues interfere with the classical and alternative pathways of complement activation and prevent complement-mediated virus neutralization. The Congo Basin strains are predicted to express a CCP, albeit with a truncated fourth short consensus repeat (Fig. 5d). This MPXV protein is reported to inhibit the classical complement pathway (Smith *et al.*, 2000).

## DISCUSSION

We demonstrated significant differences in epidemiological and clinical features of human monkeypox disease caused by Congo Basin and West African-derived (USA) monkeypox viruses. More pronounced morbidity, mortality, human-to-human transmission and viraemia were (and are currently) seen in Congo Basin human monkeypox disease. Hospitalization rates, which are potentially biased due to differences in access to care and cultural/medical practices, did not differ significantly. More impartial measures of morbidity, such as global scores of illness severity or pronounced rash, did show significant differences between populations. Our analysis suggested these clinical findings were independent of age and/or smallpox vaccination status. Although these diverse disease presentations could reflect genetic, immunological and nutritional differences in host population or route of infection, we demonstrated significant and conserved differences in the viral genomes sequenced from case-isolates from these two regions.

Whole genome analysis confirmed the existence of two monkeypox clades, which previously was suggested by single-gene phylogenies (Reed *et al.*, 2004) and whole-genome RFLP analysis (Mackett & Archard, 1979; Esposito & Knight, 1985), and provided clues to understanding the differences in human monkeypox disease pathology. More nucleotide divergence was seen at the right and left termini of the genome, a finding consistent with previous observations of general orthopoxvirus genome structure (Esposito & Knight, 1985; Shchelkunov *et al.*, 2002). Although the effect of various selective pressures has not been extensively evaluated, our observations from the USA outbreak suggested MPXV was stably replicated because only a single nucleotide substitution existed between human and prairie dog MPXV isolates. Although the *in vitro* and *in vivo* significances of proteins predicted to differ between the two clades remain to be validated, it is noteworthy that these changes have been reproducibly maintained for over 30 years among MPXV isolates obtained from the two regions.

A number of proteins were identified as candidates that might affect the observed different human disease manifestations. Their orthologues in other orthopoxviruses have been shown to promote viral persistence, and/or to evade immune recognition and clearance. The clade-specific orthologues may modulate viral pathogenesis or host response, perhaps playing a role in the observed differential clearance of virus from the blood of individuals infected with these strains.

ORF comparisons clearly predicted loss of function for the West African/USA MPXV CCP. Both vaccinia and variola orthologues interfere with the classical and alternate complement pathways (Rosengard *et al.*, 2002). If Congo Basin MPXV CCP is effective in blocking complement-enhanced viral neutralization, as observed in vaccinia (Isaacs *et al.*, 1992), one would expect prolongation of viraemia; this may partly explain the ability to detect MPXV nucleic acid by PCR in blood specimens collected at longer intervals following onset of rash in individuals infected with the Congo Basin MPXV clade. The haemorrhagic manifestation of skin lesions observed in the USA outbreak (Reed *et al.*, 2004) may correlate with a lack of complement inhibition (Miller *et al.*, 1997).

A novel prediction, based on previous studies with vaccinia and on sequence comparative analysis with the BIMAS program, was the potential for a peptide of the West African/USA clade to contain a unique 9 aa sequence. This new epitope, not present in the Congo Basin clade and derived from the C7L-orthologue predicted amino-terminal fusion, is expected to facilitate efficient immune recognition and clearance of West African/USA MPXV-infected host cells.

VAC-WR IL1 $\beta$  receptor inhibits IL1-induced murine T- and B-lymphocyte proliferation *in vitro* (Spriggs *et al.*, 1992), which is anticipated to diminish host immune recognition and viral clearance. The West African/USA isolates lack a predicted IL1 $\beta$  receptor orthologue. *In vivo* models of systemic disease suggest functional IL1 $\beta$  receptor decreases the pathogenic febrile (cytokine-induced) response (Alcami & Smith, 1996); febrile response has not been systematically evaluated in either human disease population and may be of interest for further study.

The myxoma virus protein M-T4 is important for host range and lymphocyte infection (Barry *et al.*, 1997). Absence of M-T4 attenuates disease in both intranasal and intradermal rabbit models and enhances the inflammatory response (Barry *et al.*, 1997). Therefore, absence of this protein in West African/USA strains may also contribute to our observed decrease in viraemia and ultimately effect a milder disease presentation.

Combined, these observations suggest that the effect of changes among a relatively small number of ORFs could account for the differences in viral clearance and pathogenesis of human infections with West African/USA and Congo Basin MPXVs. If transmission correlates with duration and magnitude of viral presence and shedding, the observations reported here may explain why no human-to-human transmissions have been observed in the West African/USA case series.

It is possible that the clade-specific orthologues may also influence disease in reservoir or susceptible species. Host species-specific effects of the monkeypox virally encoded IL1 $\beta$  receptor are suggested by studies demonstrating the cowpox virus orthologue has different affinities for mouse and human IL1 (Alcami & Smith, 1996). Similar adaptations of the Congo Basin MPXV orthologue could affect disease presentation in different host species, and the absence of a West African/USA orthologue could differentially affect presentation of the virus in different hosts, influencing viral host range.

On the basis of comparisons of the entire genome of five different MPXV isolates, we have verified and elaborated on the preliminary observation of the existence of two well-developed, geographically distinct clades of MPXV, suggesting significant divergence in their evolutionary histories. Evolutionary divergence may also correlate with subtle differences in the natural histories of reservoir host species or subspecies as has been observed with other viruses (Plyusnin & Morzunov, 2001; Gonzalez, 1996), and this possibility will be the subject of future studies.

In an era of heightened awareness to the potential of nefarious bioterrorist events, the emergence of MPXV in the USA serves as a timely reminder that orthopoxviruses continue to naturally exploit novel ecological and geographical niches. A better appreciation for the steps

involved in the evolution of zoonotic orthopoxviruses, including MPXV, may well be relevant to understanding the events that led to the evolution of variola virus, a pathogen with severe human pathogenicity, efficient transmission and highly specialized (human) host range.

Further efforts to understand the contribution of these distinct MPXV clades to human disease will continue to influence and contribute to informed decision-making relevant to interruption of monkeypox transmission to humans, possible future outbreak responses, diagnostic test deployment and even possible outbreak-related decisions regarding vaccination and therapeutics.

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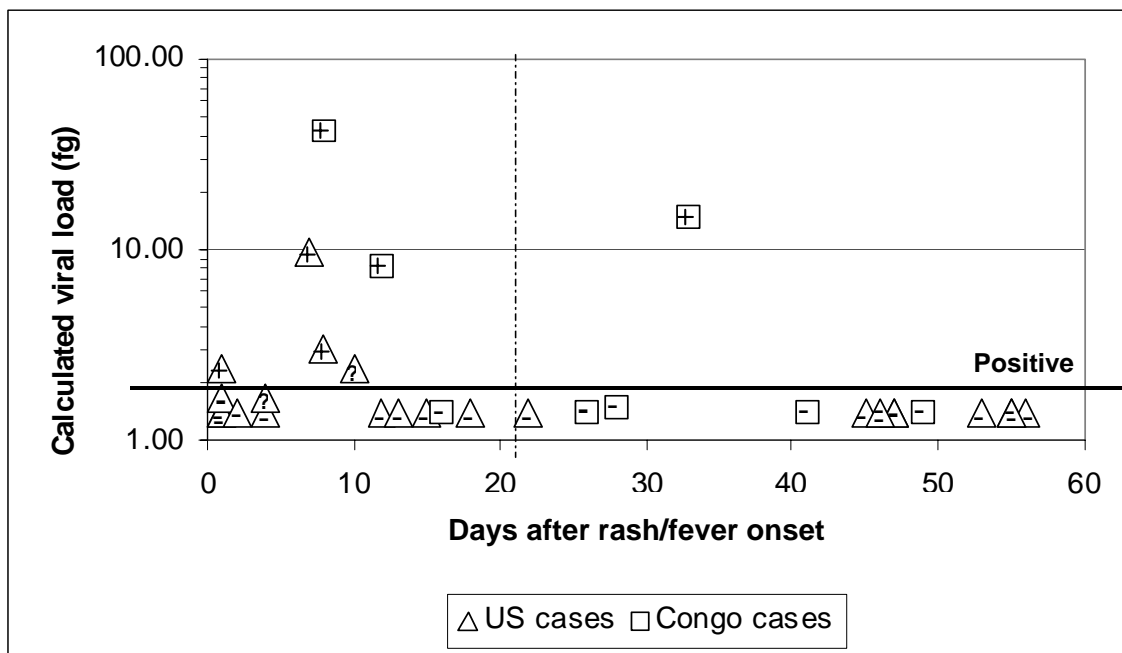
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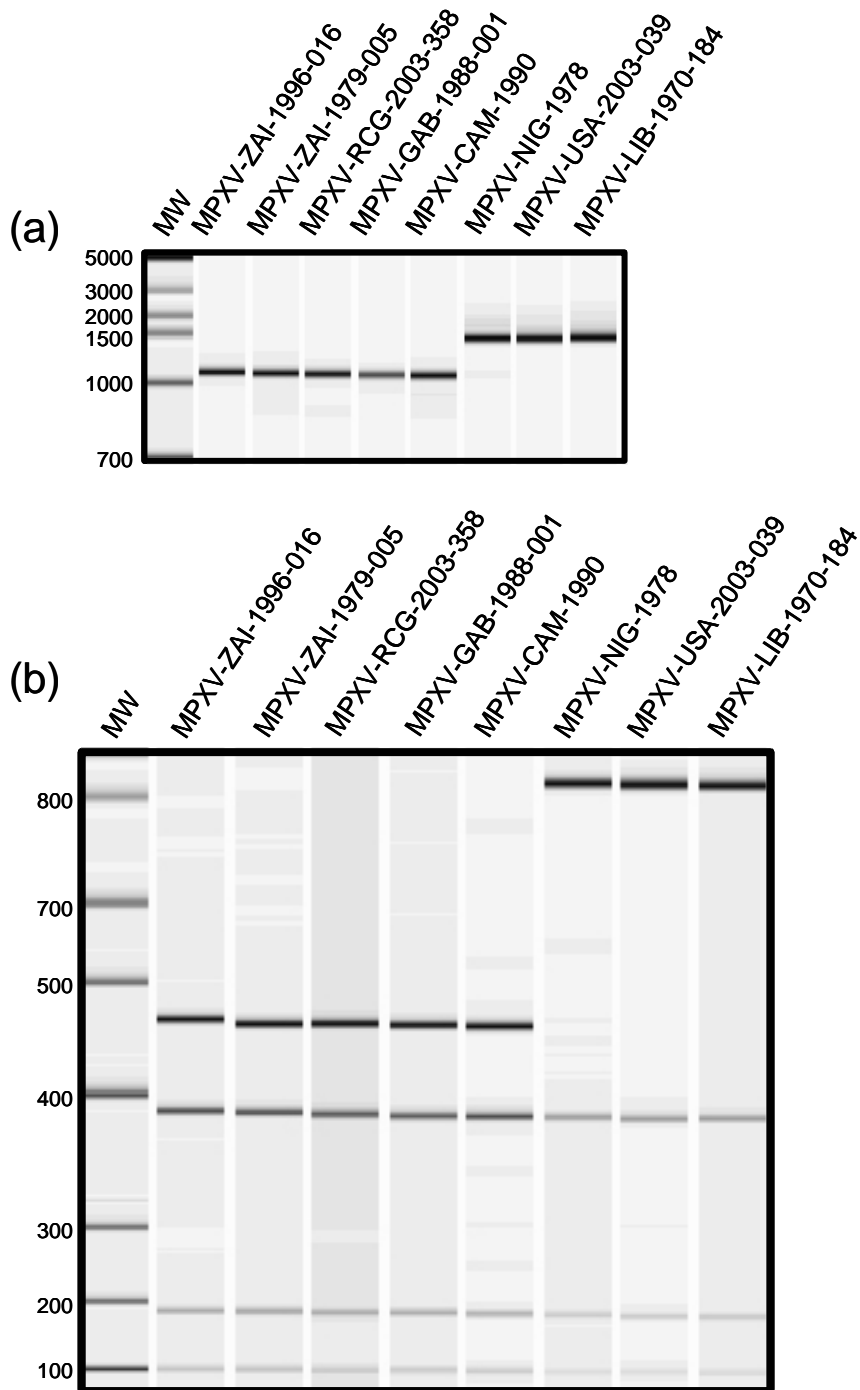
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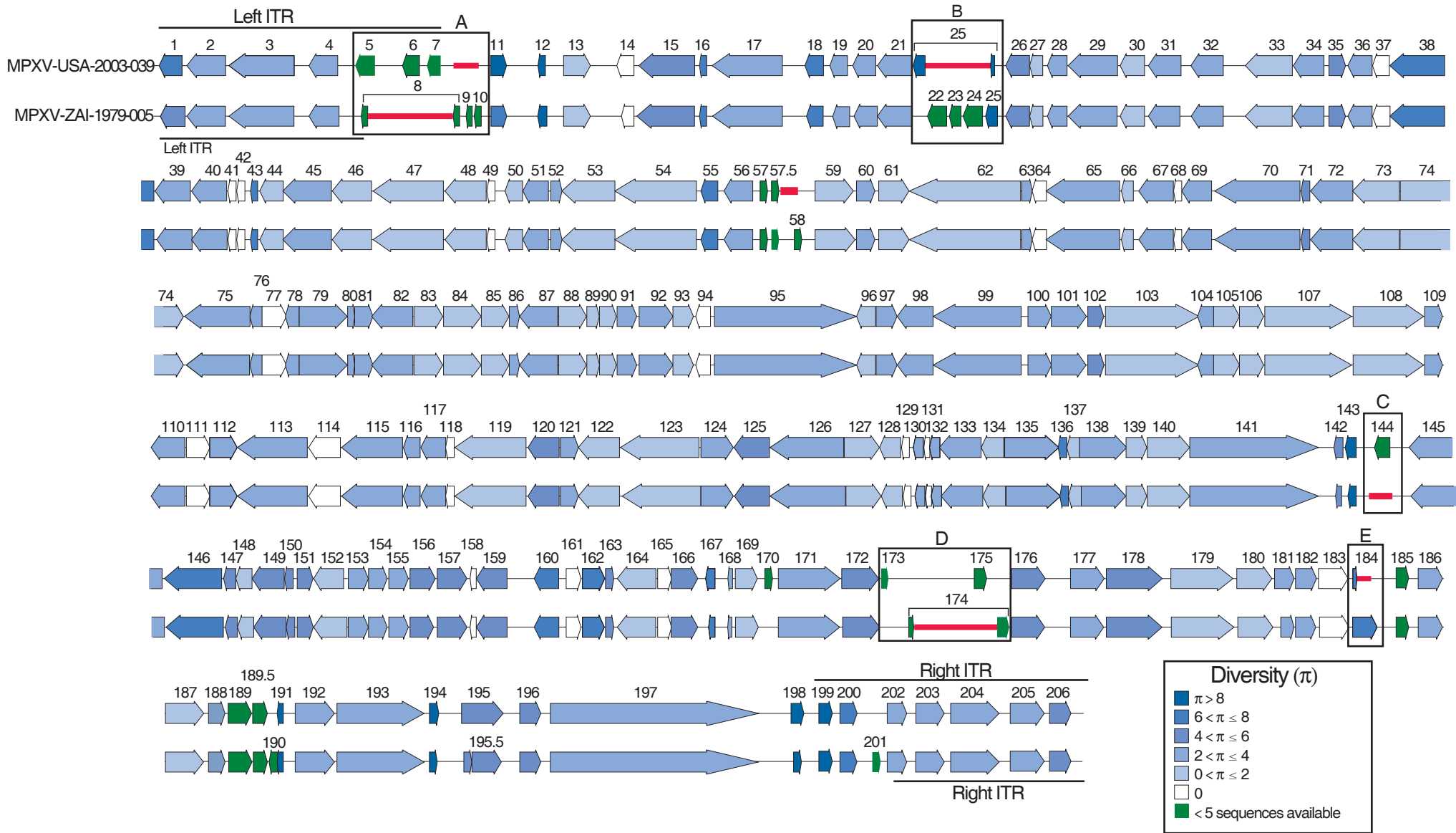
**Fig. 1.** PCR analysis of whole blood diagnostic samples. PCR testing of whole blood received as diagnostic specimens from confirmed USA (triangles) or probable and confirmed RCG (squares) patients in 2003 by CDC. Each sample was run in triplicate, using a real-time PCR monkeypox-specific assay for confirmation and an orthopox generic assay for quantification (Y. Li, V. Olson, T. Laue, M. Laker & I. Damon, unpublished data). Positive (+) and equivocal (?) indicate that fluorescence was above the threshold in 3/3 and 2/3 assays, respectively. Negative (–) indicates that fluorescence was above the threshold cycle in only 1/3 or no assays. The viral load, in femtograms (fg), calculated from a reference standard curve, is plotted against days after rash/fever onset. The dashed line indicates 21 days after rash onset. Occasionally, multiple specimens were collected on the same day after rash/fever onset and were found to have the same viral load; these are indicated by more than one symbol (+ or –) contained within the square or triangle.



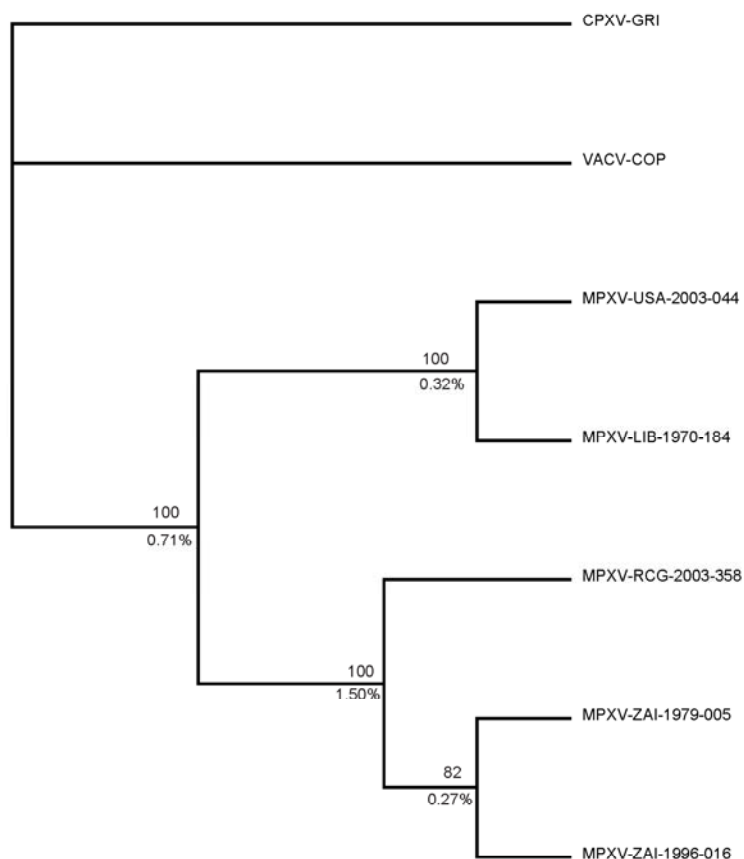
**Fig. 2.** Single-gene PCR and RFLP analysis of MPXV isolates from selected regions of Africa. (a) Single-gene (ATI) PCR products from various MPXV isolates. (b) RFLP using restriction endonuclease *Xba*I of the ATI-PCR product.



**Fig. 3.** Genome comparison of MPXV-USA-2003-039 and MPXV-ZAI-1979-005. Genome alignment of MPXV-ZAI-1979-005 and MPXV-USA-2003-039 with ORFs superimposed over the alignment. ORFs encoded on top and bottom strands are indicated by arrows. Alignment gaps >500 bases are indicated by red bars. ORF nucleotide diversity ( $\pi$ ) (Nei & Miller, 1990) among five MPXV strains is shown by shading of the arrowheads and is not reported (green arrowheads) if <5 strains are available. Box A left: 2.3 kb insertion in MPXV-USA-2003-039 containing three ORFs within left inverted terminal repeat (ITR). These ORFs are duplicated within the right ITR; thus, MPXV-USA-2003-039 contains two copies and MPXV-ZAI-1979-005 only one. Box A right: 684 bp insertion in MPXV-ZAI-1979-005 containing three small ORFs that are fragments of CPXV-GRI C3L and C4L. Box B: 1.9 kb insertion in MPXV-ZAI-1979-005 containing three small ORFs that are fragments of a 512 aa kelch-like protein of VACCOP C2L. ORF 22 is 216 aa and similar to a secreted complement-binding protein (Rosengard *et al.*, 2002; Smith *et al.*, 2000). Box C: 500 bp insertion in MPXV-USA-2003-039 containing a 110 aa ORF similar to a 1284 aa A-type inclusion protein in cowpoxvirus strain Brighton. Box D: 2.2 kb insert in MPXV-USA-2003-039 containing a 112 aa ORF with kelch-like properties. Box E: 500 bp deletion in MPXV-USA-2003-039 that causes an ORF truncation (homologue of myxoma M-T4) (Barry *et al.*, 1997).

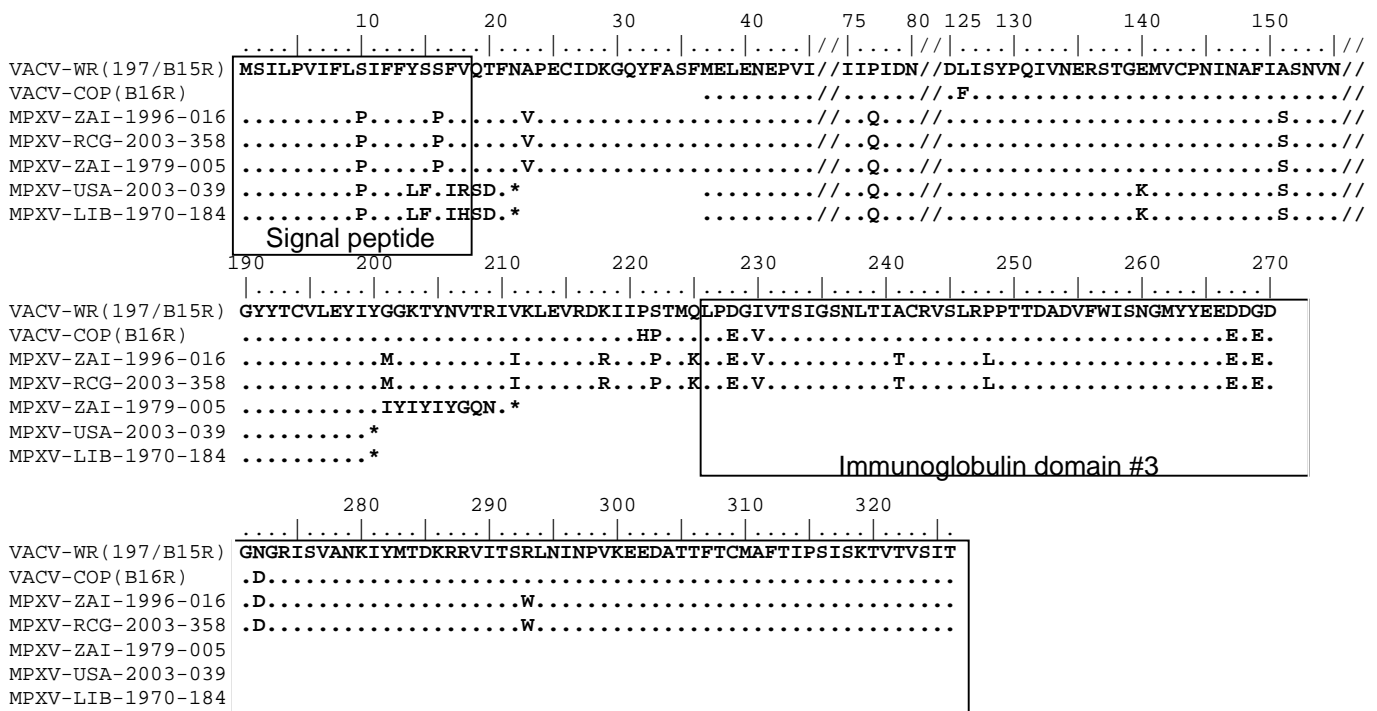


**Fig. 4.** Phylogeny of monkeypox viruses. Phylogeny based on the whole genomic alignments of five MPXV genomes, rooted with cowpox virus strain Grishak-90 (CPXV-GRI; X94355), and vaccinia virus strain Copenhagen (VACV-COP; M35027), using both parsimony and maximum-likelihood analyses (Ronquist & Huelsenbeck, 2003; Swofford, 2002). All topologies were identical. This figure represents the maximum-parsimony cladogram with bootstrap values (above) and absolute genetic distances (uncorrected p, below) superimposed to indicate nodal support.

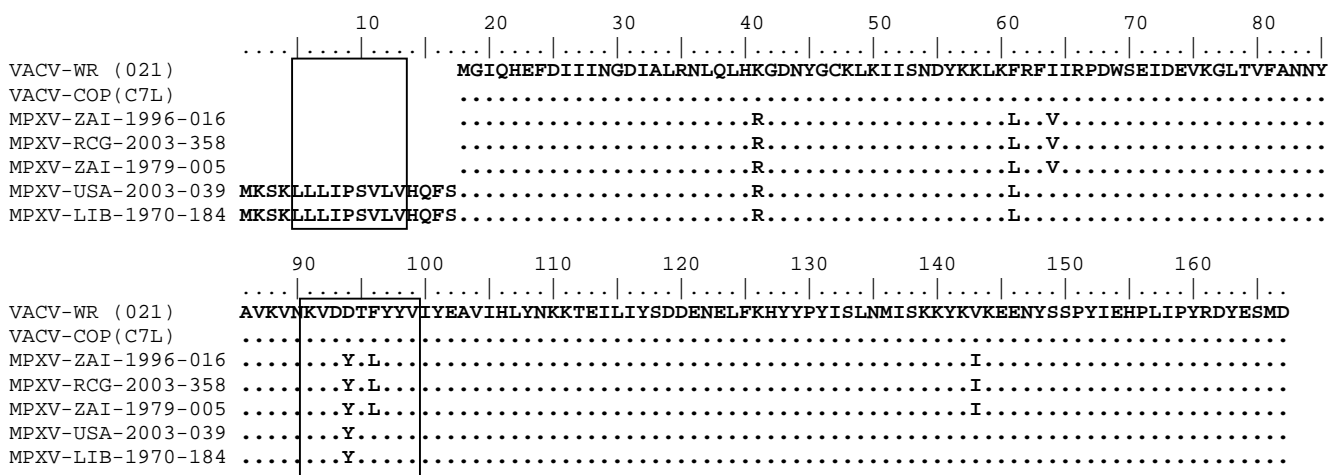


**Fig. 5.** Predicted protein sequences that discriminate MPXV clades. Predicted amino acid sequences demonstrating differences between MPXV clades are aligned with orthopoxvirus orthologues (reference proteins are in parentheses). Areas of complete homology are deleted from the alignment (//). Identical amino acid (.), gaps within the coding region (-) and early ORF terminations (\*) are indicated. Boxes mark regions of known/predicted function or interest: (a) signal peptide and immunoglobulin domain #3 (Smith & Chan, 1991); (b) putative antigenic peptides (Snyder *et al.*, 2004); (c) conserved cysteines (C) (Barry *et al.*, 1997); (d) short consensus repeat #4 (Smith *et al.*, 2000). Abbreviations: vaccinia virus strain Copenhagen (VACV-COP), vaccinia virus strain Western Reserve (VACV-WR), variola virus strain Bangladesh (VARV-BSH), cowpox virus strain Grishak-90 (CPXV-GRI), myxoma virus strain Lausanne (MYXV-LAU), vaccinia virus complement control protein (VCP), smallpox inhibitor of complement enzyme (SPICE).

**(a) B16R of VACV-COP homologues (IL1 $\beta$ -receptor)**



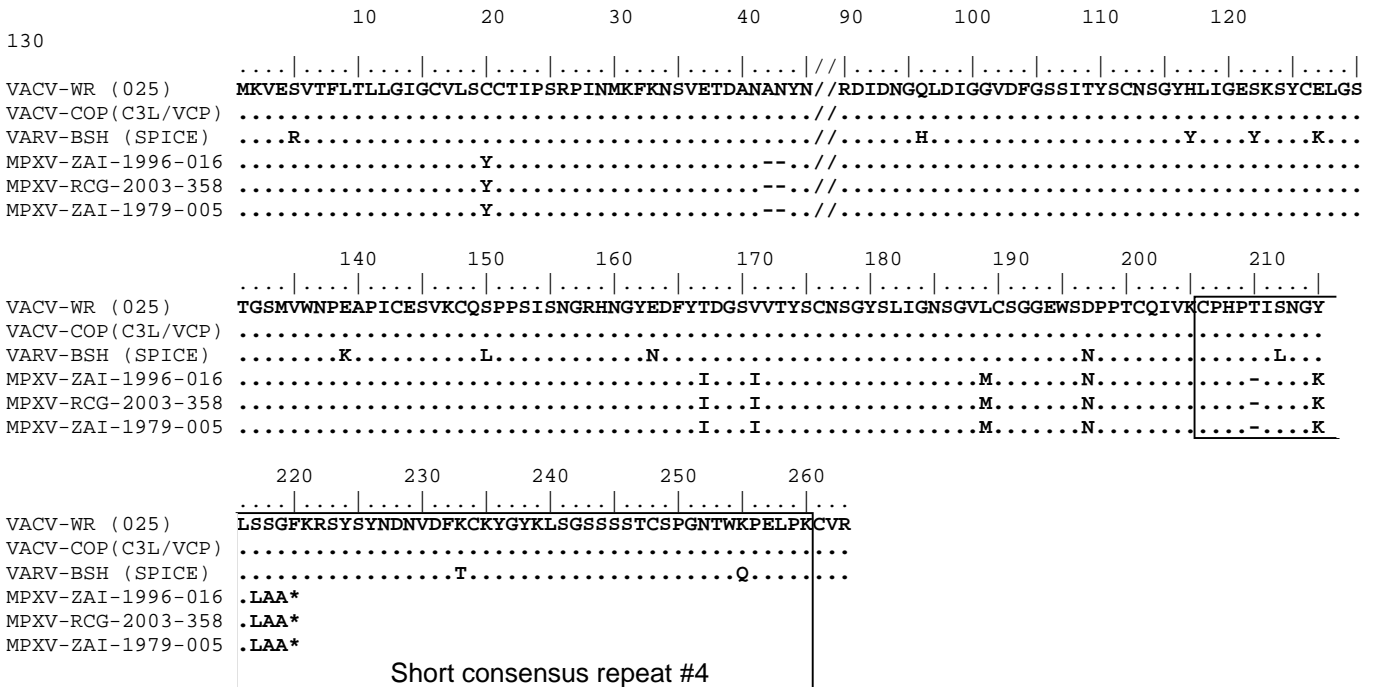
**(b) C7L of VACV-COP homologues (host range factor)**



**(c) B9R of VACV-COP homologues (M-T4-like apoptosis regulator)**



**(d) C3L of VACV-COP homologues (CCP)**





**Table 1.** Clinical and epidemiological characteristics of DRC (1981–1986) and USA (2003) monkeypox cases by age

Analysis restricted to only those DRC cases within the same age range as USA cases, aged 6–48 years.

Characteristic	All					≥18 years					<18 years				
	DRC (n=127)		USA (n=37)		RR (95 % CI)	DRC (n=21)		USA (n=29)		RR (95 % CI)	DRC (n=106)		USA (n=8)		RR (95 % CI)
	n	%	n	%		n	%	n	%		n	%	n	%	
Case fatality rate	3	2.4	0	0.0		0	0.0	0	0.0		3	2.7	0	0.0	
Cases due to secondary transmission	40	31.5	0	0.0		14	66.7	0	0.0		26	24.5	0	0.0	
No. of lesions															
<100	39	33.3	24	88.9	Referent*	11	61.1	20	95.2	Referent	28	28.3	4	66.7	Referent
≥100	78	66.7	3	11.1	6.0 (2.0–17.6)	7	38.9	1	4.8	8.2 (1.1–60.2)	71	71.7	2	33.3	2.2 (0.69–6.7)
Unknown	10	–	10	–		3	–	8	–		7	–	2	–	
Hospitalized	51	40.2	14	37.8	1.1 (0.7–1.7)	3	14.3	10	34.5	0.41 (0.13–1.3)	48	45.3	4	50.0	0.9 (0.44–1.9)
Severely ill†	37	29.1	1	2.7	10.8 (1.5–75.9)	3	14.3	0	0.0	Undefined	34	32.1	1	12.5	2.6 (0.4–16.4)

\*Relative risk for ≥100 rash lesions among DRC and USA monkeypox cases using cases with <100 rash lesions as comparison group; referent data are included for completeness.

†‘Severely ill’ patients include those DRC cases with ≥100 rash lesions that were hospitalized and had an illness severity score of ‘three’. USA patients considered severely ill had ≥100 rash lesions that were hospitalized and were specifically noted to be seriously ill (see Methods).

**Table 2.** Clinical and epidemiological characteristics of DRC (1981–1986) and USA (2003) monkeypox cases, aged 6–48 years, by vaccination status

Characteristic	Unvaccinated					Vaccinated				
	DRC* (n=80)		USA (n=26)		RR (95 % CI)	DRC (n=44)		USA (n=10)		RR (95 % CI)
	n	%	n	%		n	%	n	%	
Case fatality rate	2	2.5	0	0.0		1	2.3	0	0.0	
Cases due to secondary transmission	20	25	0	0.0		19	43.2	0	0.0	
No. of lesions										
<100	20	26.3	20	87.0	Referent	18	47.4	4	100	Referent
≥100	56	73.7	3	13.0	5.6 (1.9–16.4)	20	52.6	0	0.0	Undefined
Unknown	4	–	3	–		6	–	6	–	
Hospitalized	34	57.5	10	38.5	1.1 (0.64–1.9)	15	34.1	4	2.5	0.85 (0.36–2.0)
Severely ill†	26	32.5	1	3.8	8.5 (1.2–59.2)	9	0	0		Undefined

\*No DRC cases older than 14 years were unvaccinated.

†The mean age of unvaccinated DRC cases with severe disease was 7.4 years, range 6–14 years. The single severely ill USA case was <18 years of age.

**Table 3.** Pairwise comparisons of monkeypox sequences

Right quadrant: percentage nucleotide identity (calculated as no. of matches/total length of alignment). Left quadrant (upper number): no. of single nucleotide polymorphisms (SNPs). Left quadrant (lower number): no. of insertions/deletions (indels, each indel counting as one event).

Strain	Strain					
	MPXV-RCG- 2003-358	MPXV-ZAI- 1979-005	MPXV-ZAI- 1996-016	MPXV-USA-2003- 039 (human)	MPXV-USA-2003- 044 (prairie dog)	MPXV-LIB- 1970-184
MPXV-RCG-2003-358	–	99.7	99.5	94.9	94.9	94.7
MPXV-ZAI-1979-005	55 28	–	99.5	94.8	94.8	94.5
MPXV-ZAI-1996-016	172 55	168 58	–	94.7	94.7	94.4
MPXV-USA-2003-039 (human)	788 158	786 160	876 175	–	100.0*	98.9
MPXV-USA-2003-044 (prairie dog)	789 158	786 160	877 175	1 0	–	98.9
MPXV-LIB-1970-184	775 164	759 172	860 181	176 50	177 50	–

\*Contains one SNP on the left end of the genome (before the first ORF).