Virology 475 (2015) 129–138

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Attenuation of monkeypox virus by deletion of genomic regions

Juan G. Lopera [a,*], Elizabeth A. Falendysz [b], Tonie E. Rocke [b], Jorge E. Osorio [a,**]

[a] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison, WI, USA
[b] National Wildlife Health Center, U.S. Geological Survey, Madison, WI, USA

ARTICLE INFO

Article history:
Received 11 September 2014
Returned to author for revisions
20 October 2014
Accepted 3 November 2014
Available online 1 December 2014

Keywords:
Monkeypox virus
Variola virus
Vaccinia virus
Monkeypox virus pathogenesis
Virulence factors
Viral replication
In vivo bioluminescence imaging

ABSTRACT

Monkeypox virus (MPXV) is an emerging pathogen from Africa that causes disease similar to smallpox. Two clades with different geographic distributions and virulence have been described. Here, we utilized bioinformatic tools to identify genomic regions in MPXV containing multiple virulence genes and explored their roles in pathogenicity; two selected regions were then deleted singularly or in combination. *In vitro* and *in vivo* studies indicated that these regions play a significant role in MPXV replication, tissue spread, and mortality in mice. Interestingly, while deletion of either region led to decreased virulence in mice, one region had no effect on *in vitro* replication. Deletion of both regions simultaneously also reduced cell culture replication and significantly increased the attenuation *in vivo* over either single deletion. Attenuated MPXV with genomic deletions present a safe and efficacious tool in the study of MPX pathogenesis and in the identification of genetic factors associated with virulence.

Introduction

Monkeypox virus (MPXV; *Poxviridae*, *Orthopoxvirus*) causes human monkeypox (MPX), a disease with a clinical presentation that resembles human smallpox (Cann et al., 2013; Huhn et al., 2005). Human MPX is endemic in equatorial Africa, and no specific wild animal reservoir has been identified. Recent epidemiologic studies have shown an increase in human MPX cases in the last 30 years in the central region of Africa (Reynolds and Damon, 2012; Rimoin et al., 2010). In 2003, the virus was introduced to the United States through the importation of infected rodents from West Africa (Likos et al., 2005; Reynolds and Damon, 2012). In 2005, human MPX cases were identified in southern Sudan (now South Sudan), and are thought to be associated with translocations of infected humans or animals from Central Africa (Nakazawa et al., 2013).

The emergence of MPXV, as well as its potential threat as a bioterrorism weapon, warrants the study of its mechanisms of infection, virulence factors, and pathogenesis (Breman and Henderson, 1998; Reynolds and Damon, 2012; Rimoin et al., 2010). Genomic comparisons of MPXV human isolates have revealed the existence of two highly diverse clades of MPXV with different geographic distribution and pathogenicity. Infections with MPXVs isolated from Central Africa (MPXV/Congo) cause more severe disease in humans and rodents compared to viruses in the West African clade (MPXV/USA) (Chen et al., 2005; Hutson and Damon, 2010; Likos et al., 2005). Comparisons between these MPXV clades and related orthopoxvirus (OPXV) genomes have shown that although these are closely related viruses, they contain several highly variable genomic regions (termed here regions [R]) that are characterized by high mutation rates, insertions, deletions, and gene truncations (Chen et al., 2005; Hendrickson et al., 2010; Shchelkunov et al., 2001). In MPXV and other OPXVs these regions contain several known host range and immunomodulatory (IMM) genes (Seet et al., 2003). These genes have evolved specifically to simultaneously inhibit diverse processes such as pattern-recognition receptor signaling, apoptosis, chemokine and cytokine function, and lymphocyte and antibody activity (Fernandez de Marco Mdel et al., 2010; Hammarlund et al., 2008; Kindrachuk et al., 2012; Weaver and Isaacs, 2008). Whereas previous evaluations of MPXV virulence factors have predominantly focused on the function and characterization of individual viral genes, these studies demonstrated that individual genes were not solely responsible for the observed differences in pathogenicity between MPXV clades (Estep et al., 2011; Hudson et al., 2012). In contrast, we evaluated the effect of deletions of large genomic regions in MPXV that contain several host range and IMM genes to facilitate selection of candidate virulence factors involved in pathogenesis. Specifically, we quantified and compared the effect of deletion of two genomic regions in a Central African MPXV-Congo strain. The impacts of these deletions on replication

* Corresponding author. Tel.: +1 608 890 0480; fax: +1 608 262 7420.
** Corresponding author. Tel.: +1 608 890 0252.
E-mail addresses: loperapena@wisc.edu (J.G. Lopera), osorio@svm.vetmed.wisc.edu (J.E. Osorio).

http://dx.doi.org/10.1016/j.virol.2014.11.009
0042-6822/

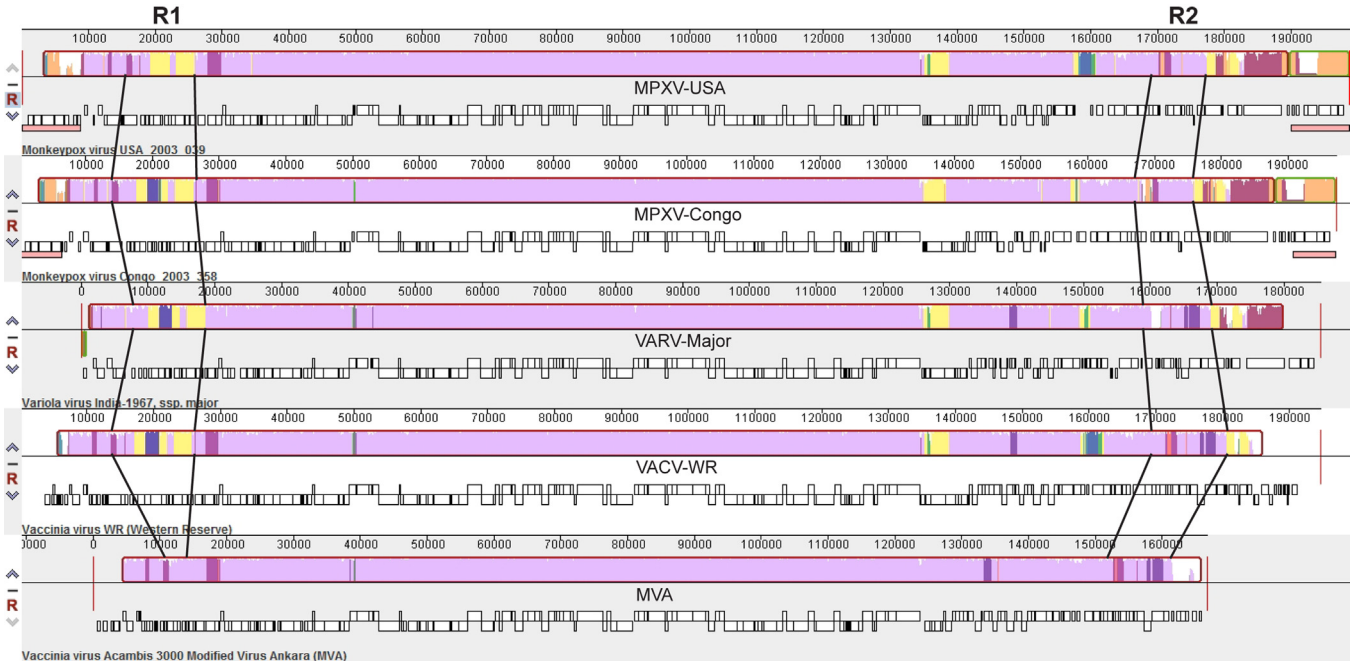

Fig. 1. Sequence comparisons and identification of target genomic regions. The colored bars inside the genomic blocks represent the level of sequence similarities between viruses. Regions with high sequence similarity are shown in light purple. Regions with mutations, insertions, and deletions of genes are represented in colored bars. White bars represent genomic sequences present in only one viral genome. Boxes surround predicted genomic regions in MPXV clades. Lines link orthologous genomic regions in MPXV, variola virus (VARV), vaccinia virus (VACV) and modified vaccinia ankara (MVA) genomes. Genomic regions were selected based upon sequence analysis utilizing MAUVE and DAMBE software. Regions characterized in this study are indicated by R1 and R2.

kinetics, host immune response, host tissue tropism and virulence were analyzed using cell culture, immunoassays and *in vivo* imaging. The research described herein identified regions in the MPXV genome that can inform the selection of candidate genes for future study to more fully dissect the molecular determinants of virulence in MPXV.

Results

Identification of genomic regions and generation of recombinant viruses

Genomic alignments and sequence comparisons between MPXV clades and other related OPXV helped to identify two target genomic regions (R) located at the 5′ and 3′ ends of the genomes (Fig. 1). Sequences of MPXVs and related OPXVs were obtained from Genbank (GB: DQ011157, DQ011154, NC001611, NC006998 and AY603355) and analyzed using bioinformatic tools. Genomic regions were selected based on mutation rates and presence of large-scale evolutionary events such as genomic rearrangement, inversion, truncation, insertion, and deletion. Additionally, each genomic region was selected by its degree of divergence between related viruses and its density of known virulence genes (Fig. 1 and Table 1). Through this analysis, two genomic regions were selected for in-depth analysis (Fig. 1). Three recombinant viruses (MPXV-ΔR1, MPXV-ΔR2, and MPXV-ΔR1/R2) containing deletions of individual or combined genomic regions were constructed (Fig. 2). Recombinant viruses were plaque purified, titered and assayed by cell culture and *in vivo* imaging.

Deletion of genomic regions in MPXV affected the replication kinetics and plaque size in cell culture

One-step growth curves and plaque size measurements were used to evaluate whether the deletion of genomic regions in MPXV affected *in vitro* growth and other phenotypic characteristics of gene-deleted viruses. Deletion of target genomic regions in MPXV reduced the *in vitro* growth characteristics of two of the recombinant viruses. MPXV-ΔR1 and MPXV-ΔR1/R2 viruses showed significant reduction in viral replication and total yield, as well as cell-to-cell spread. At 24, 48, and 72 h post-infection (pi), MPXV-ΔR1 and MPXV-ΔR1/R2 replicated to significantly lower peak titers ($p < 0.001$) and formed significantly smaller mean plaque sizes ($p < 0.01$) as compared to parental MPXV-Congo/Luc+ (Fig. 3A and B respectively). Interestingly, for MPXV-ΔR2 virus, the growth kinetics in cell culture appeared normal and comparable to parental MPXV-Congo/Luc+. The lag and rise periods of exponential growth curves were of similar duration along with peak titers (Fig. 3A). In addition, MPXV-ΔR2 virus did not differ in plaque size when compared to the parental MPXV-Congo/Luc+ (Fig. 3B). One-step growth curves in A549 cells showed similar results to Vero cells; however no differences in plaque phenotype were observed between parental and gene-deleted MPXVs (data not shown).

In vivo imaging demonstrated MPXV attenuation by deletion of genomic regions

Biophotonic *in vivo* imaging of CAST/EiJ mice was used to determine the pathogenicity of MPXV-ΔR1, MPXV-ΔR2, and MPXV-ΔR1/R2. At day 4 pi, viral spread and increased viral replication was seen in MPXV-Congo/Luc+ infected mice as compared to all of the gene-deleted viruses (Figs. 4 and 5A). Viruses containing deletions did not show signals of viral spread at day 4 pi. Between day 6 and 8 pi, morbidity, high viral replication, and generalized viral dissemination were observed in the control MPXV-Congo/Luc+ group (Figs. 4 and 5). All MPXV-Congo/Luc+ infected mice died or were euthanized by 8 days pi (Fig. 4). Only animals infected with parental MPXV-Congo/Luc+ exhibited significant weight loss ($p < 0.01$) (Fig. 5B). Weight change was not significantly different between groups of mice infected with MPXV