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Genome sequence variation among isolates of monkey B virus (*Macacine alphaherpesvirus* 1) from captive macaques

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ABSTRACT

Complete genome sequences of 19 strains of monkey B virus (*Macacine alphaherpesvirus* 1; BV) isolated from several macaque species were determined. A low level of sequence variation was present among BV isolates from rhesus macaques. Most variation among BV strains isolated from rhesus macaques was located in regions of repetitive or quasi-repetitive sequence. Variation in coding sequences (polypeptides and miRNAs) was minor compared to regions of non-coding sequences. Non-coding sequences in the long and short repeat regions of the genome did however exhibit islands of conserved sequence. Oral and genital isolates from a single monkey were identical in sequence and varied only in the number of iterations of repeat units in several areas of repeats. Sequence variation between BV isolates from different macaque species (different BV genotypes) was much greater and was spread across the entire genome, confirming the existence of different genotypes of BV in different macaque species.

1. Introduction

The genus *Macaca* is the most ecologically successful and widely distributed nonhuman primate (NHP) taxon, reaching its zenith in Asia where 18 of the 19 species of macaques are found. Like other NHP, macaques are naturally infected with their own complement of herpesviruses analogous to their human counterparts. (Estep et al., 2010) The macaque alpha-herpesvirus (*Macacine alphaherpesvirus* 1; monkey B virus, BV) is closely related to the human herpes simplex viruses HSV1 and HSV2. In macaques BV infection can be oral and/or genital, is usually asymptomatic, the virus establishes latency in sensory ganglia, and various forms of stress can result in reactivation of BV from latency and shedding of infectious virus in bodily secretions. (Huff et al., 2003; Huff and Barry, 2003; Keeble, 1960; Weigler, 1992) A few studies have shown that free-ranging macaques in habitat countries are seropositive for BV, but the genetic diversity of BV in naturally occurring macaque populations has not been investigated. (Engel et al., 2002, 2008; Jones-Engel et al., 2006; Lee et al., 2015).

While BV rarely causes serious disease in healthy adult macaques, when transmitted to humans or other primate species BV can be neuropathogenic. Only about 50 cases of zoonotic BV infection, all of which have occurred in the context of exposure to laboratory or captive animals, have been documented since the virus was first recognized in 1932, but most (~75%) have been lethal. (Davidson and Hummeler, 1960; Huff and Barry, 2003; Palmer, 1987; Weigler, 1992) The high mortality associated with zoonotic BV infection makes this virus the single most serious zoonotic concern for animal care and research personnel working with or around macaque monkeys.

In the 1960s a strain of BV isolated from a laboratory rhesus macaque (*M. mulatta*) was adapted to replicate in primary rabbit kidney cells and used to prepare a formalin-inactivated vaccine. (Hull, 1971; Hull and Nash, 1960; Hull et al., 1962) However, given the high pathogenicity of BV in humans and the low incidence of zoonotic BV infections, this vaccine has not been used beyond initial clinical testing. This BV strain (designated E2490) has since served as the reference strain of BV and its genome sequence has been determined. (Ohsawa et al., 2002a, 2003; Perelygina et al., 2003).

The high lethality associated with zoonotic BV infections in the laboratory led to BV being considered extremely neuropathogenic in humans. Consistent with this assumption, asymptomatic zoonotic BV infections have not been reported, although several cases are suspected to be the result of reactivation of latent BV in patients lacking a history of overt BV infection. (Fierer et al., 1973; Palmer, 1987; Weigler, 1992) Inconsistent with this assumption of extreme neuropathogenicity are

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the hundreds of thousands of exposure incidents that occur outside of the laboratory setting. Millions of free-ranging, urban, pet and temple macaques are distributed throughout the natural range of these highly commensal animals and contact with humans is ubiquitous and often results in bites, scratches and mucosal splashes. (Engel et al., 2002; Fuentes, 2006) However, there has never been a documented case of pathogenic zoonotic BV transmission following exposure to macaques in Asia or Gibraltar. (Craig et al., 2015; Engel et al., 2002; Jones-Engel et al., 2006, 2008) Obviously, factors such BV strain variation the amount of virus transmitted likely play a part in determining whether a clinically apparent zoonotic infection results.

For decades rhesus macaques have been the most widely used macaque species in biomedical research. As far as is known all zoonotic BV patients had contact with rhesus monkeys, although some had contact with other macaque species as well. Based on DNA sequence of very small regions of the BV genome (~1.3 kbp) it has been suggested that perhaps BV isolates from rhesus macaques were somehow different from BV of other macaque species, having greater pathogenicity to humans. Several studies have examined BV isolates from different macaque species housed in zoos or research centers in the US and found that BV isolates can be classified into distinct 'genotypes' based on DNA sequence differences. (Ohsawa et al., 2002b; Smith et al., 1998; Thompson et al., 2000) Since the number of macaque species examined is small and all BV isolates were from captive bred as opposed to wild monkeys, the veracity of BV genotypes remains to be seen. If distinct BV genotypes related to the macaque species do exist, the question of their relative neurovirulence or zoonotic potential is obviously of importance. Aside from one study in mice, comparative neurovirulence of different BV strains or genotypes has not been reported. The one study that tested several rhesus BV (BVrh) isolates in mice using i.m. inoculation did observe wide differences in neurovirulence, with the 50% infectious dose ranging from $10^{1.5}$ to $>10^{6}$ PFU. (Ritchey et al., 2005).

Aside from the reference BVrh strain E2490, the only other published BV genome sequence is that of an isolate (E90-136) from a young cynomolgus macaque (*M. fascicularis*) that died of a generalized infection. (Ohsawa et al., 2014; Simon et al., 1993) Since E2490 and the cynomolgus BV isolate viruses are different BV genotypes (BVrh & BVcy, resp.), comparison of these genome sequences provides little insight regarding the degree of sequence variation within or between BV genotypes. Here we report the sequence of several BVrh isolates as well as some isolates of different BV genotypes to begin to address the extent of genetic variation among BV isolates among captive macaques.

2. Materials and methods

2.1. Viruses

BV strains sequenced and what is known regarding their origin are listed in Table 1. The reference strain of BV (E2490) was isolated from a rhesus macaque (*M. mulatta*; BVrh) in the early 1950s when most monkeys used in research were still imported from the wild, so this isolate may have been from a wild-caught monkey. Since there are a number of differences between published E2490 sequences from different laboratories (Ohsawa et al., 2002a, 2003; Perelygina et al., 2003), the E2490 genome was re-sequenced. Twelve strains were isolated from captive bred rhesus macaques, two isolates from pigtail macaques (*M. nemistrina;* BVpt), one isolate from a lion-tailed macaque (*M. Silenus;* BVlt), one isolate from a Japanese macaque (*M. fuscata;* BVfu), and one isolate made from primary kidney cells. One additional BV strain was isolated in 2005 from necropsy tissues of a 23 year old bonnet macaque (*M. radiata;* BVbn) that developed typical herpetic oral lesions shortly before being humanely euthanized for cardiopulmonary complications due to congestive heart failure. (Scharf et al., 2008).

2.2. Viral genome sequencing

Viral DNA was purified from infected Vero cells on NaI gradients and used for both PCR and MiSeq genomic sequencing (600 cycle paired-end runs) as described. (d'Offay et al., 2013; Fulton et al., 2013) For all sequencing runs total read counts were 2.9 – 18.2 x 10^{6} (avg. 8.2 x 10^{6}), average read length was 165 – 284 bp (avg. 250 bp), and average depth of coverage was 4.3–31.1 x 10^{3} (avg. 13.1 x 10^{3}). Where very poor MiSeq results were obtained, new DNA preparations were made and sequenced. Analysis of sequence data, assembly of sequences, alignments and phylogenetic analyses were all conducted using the CLC Main Workbench 7.0.3 programs.

Sequence data of all strains were initially assembled both *de novo* and by alignment to a reference sequence. Coding sequences were readily assembled by both methods, but some intergenic and much of the R_L and R_S regions required manual extension of assembled sequences by searching unassembled reads. The initial two BVrh isolates sequenced (24105-G & 32425-G) were each assembled *de novo* and using both the published sequence for BVrh E2490 (Genbank NC_004812.1) and BVcy E90-136 sequence (Genbank KJ566591) as a scaffold/reference. Since there were a number of sequence discrepancies between published E2490 sequences and between E2490 and that of 24105-G, the 24105-G sequence was used as a reference sequence for assembly of all other BV isolates except those from pigtail or lion-tailed macaques. In addition to *de novo* assembly, both the 24105-G and E90-136 sequences were used as references to assemble one BVpt isolate (1504-11). Once the 1504-11 sequence was completed and confirmed (PCR/sequencing of all questionable areas), it was used (in addition to *de novo* assembly) as a reference to assemble the remaining BVpt and BVlt sequences.

Once Illumina generated sequences were assembled, sequence assemblies and sequence data of each isolate were manually reviewed to identify areas of low coverage, sequence discrepancies, manually extended areas of concern, or other areas where there was any question regarding the veracity of the draft sequence assembly. The sequence of all such areas in each isolate were determined/confirmed by PCR amplification and dideoxy sequencing of the products. Deep vent polymerase (New England BioLabs, Ipswich, MA) and 32% betaine (Sigma Chemical Co, St Louis, MO) were used in PCR reactions. Primers having T_ms of 70–74 °C were used to amplify and sequence areas of repetitive sequence. When completed, genome sequences were aligned and all conflicts resolved with additional PCR/sequencing. All genome sequences have been deposited in GenBank and their accession numbers are listed in Table 1.

2.3. Neurovirulence testing

Neurovirulence of BV isolates was assessed using the mouse skin scarification model as described previously. (Black et al., 2014; Brush et al., 2014; Rogers, 2006) Briefly, the left flank of 10–12 g female Balb/c mice was shaved, lightly scarified with an 18 Gg needle, and 10 ul of virus applied to the scarified area. Ten-fold dilutions of virus ranging from 10^{6}–10^{3} PFU/10 ul were tested. Mice were observed twice daily for signs of neurological involvement and scored on a scale of 0–5. (Brush et al., 2014) Any mice showing signs of brain involvement (ataxia, tremors) were humanely euthanized. Blood was collected when surviving mice were euthanized at 14 days PI (DPI). Whether or not survivors were infected was determined by testing serum for anti-BV IgG. For each isolate the dose required to induce neurological symptoms ($CNSD_{50}$), death/euthanasia (LD_{50}) or infection (ID_{50}; based on clinical signs plus positive serology in survivors) in 50% of inoculated mice was determined as described. (Black et al., 2014; Ritchey et al., 2005).