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# Discovery and biological characterization of two novel pig-tailed macaque homologs of HHV-6 and HHV-7

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ABSTRACT

Human herpesvirus-6 (HHV-6) and -7 (HHV-7) are Roseoloviruses within the Betaherpesvirus family, which have a high prevalence and suspected involvement in a number of diseases. Using CODEHOP-based PCR, we identified homologs of both viruses in saliva of pig-tailed macaques, provisionally named MneHV-6 and MneHV-7. This finding supports the existence of two distinct Roseolovirus lineages before the divergence of humans and macaques. Using specific qPCR assays, high levels of MneHV-6 and MneHV-7 DNA were detected in macaque saliva, although the frequency was greater for MneHV-7. A blood screen of 283 macaques revealed 10% MneHV-6 DNA positivity and 25% MneHV-7 positivity, with higher prevalences of MneHV-6 in older females and of MneHV-7 in younger males. Levels of MneHV-6 were increased in animals coinfected with MneHV-7, and both viruses were frequently detected in salivary gland and stomach tissues. Our discovery provides a unique animal model to answer unresolved questions regarding Roseolovirus pathology.

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## Introduction

Herpesviruses comprise a family of relatively large double-stranded DNA viruses, which are widespread among humans and other animals. Both the *Cytomegalovirus* and the *Roseolovirus* genera in the *Betaherpesvirinae* subfamily include viruses found in humans. The *Roseolovirus* genus is composed of three human viruses: the closely related human herpesvirus 6A (HHV-6A) and human herpesvirus 6B (HHV-6B) and the more distantly related human herpesvirus 7 (HHV-7) (Pringle, 1998). Although HHV-6A and HHV-6B were originally considered as two variants of the same virus ( Ablashi, 1993), evidence of genetic, biological and epidemiologic differences between HHV-6A and HHV-6B has led to their recent classification as distinct virus species (Adams and Carstens, 2012).

Roseoloviruses are among the most prevalent viruses in the human population with primary infection occurring during early childhood (Hall et al., 2006; Zerr et al., 2005). Primary infections with HHV-6B and HHV-7 have been reported to cause exanthema subitum (Tanaka et al., 1994; Yamanishi et al., 1988) while the clinical symptoms associated with HHV-6A are still unclear. These clinical features associated with roseolovirus infections are generally characterized by low morbidity, as the virus infection becomes latent. However, severe pathological conditions can arise due to active viral replication, often in immunocompromised hosts. In transplant patients HHV-6B reactivation is associated with frequent complications including encephalitis, acute graft versus host disease, CMV reactivation and bone marrow suppression (Zerr, 2012). In this setting, the causal involvement of HHV-7 reactivation is less well documented. HHV-6B is also implicated in a majority of pediatric cases of febrile status epilepticus (Epstein et al., 2012). HHV-6A active replication on the other hand is associated with autoimmune diseases such as Hasimoto's thyroiditis (Caselli et al., 2012) and enhances disease progression in HIV infected individuals (Ablashi et al., 1998; Boutolleau et al., 2004; Lusso et al., 2007).

Both HHV-6B and HHV-7 are believed to be transmitted by exposure to saliva. Infectious virus particles can be isolated from saliva samples and both viruses have been detected in salivary glands (Di Luca et al., 1995). In contrast HHV-6A is rarely detected in saliva (Aberle et al., 1996) and it is likely that transmission occurs following other types of exposure including sexual contact (Leach et al., 1994). To date, no significant differences in prevalence of roseolovirus infections in men and women have been reported. However, large

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variations in prevalence have been observed in different geographic locations (Krueger et al., 1998; Magalhaes et al., 2010).

All roseoloviruses were initially characterized by their tropism for lymphocytes (Frenkel et al., 1990; Salahuddin et al., 1986). However, since these viruses can infect and replicate in a wide range of tissues, a broader cellular tropism is likely. Although HHV-6 and HHV-7 have been detected in a similar panel of tissues (Chen and Hudnall, 2006; Kempf et al., 1998; Mori, 2009), it is likely that they infect different cell types since each virus relies on a different membrane receptor for viral entry (Lusso et al., 1994b; Santoro et al., 1999; Tang et al., 2013).

To our knowledge, no roseolovirus homolog has been identified in any of the three major macaque species used in research, including long-tailed macaques (Macaca fascicularis (Mfa)), Rhesus macaques (Macaca mulata (Mmu)), and pig-tailed macaques (Macaca nemestrina (Mne)). The only non-human primate roseoloviruses discovered to date are a HHV-6 homolog in chimpanzee (PanHV6) (Lacoste et al., 2005) and a roseolovirus in drill monkeys (MndHVbeta) (Lacoste et al., 2000). Both viral homologs were identified in DNA isolated from blood by nested PCR using the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) approach. Nonetheless, HHV-6 reactive antibodies have been detected in a number of primate species, including macaque species, orangutans and African green monkeys, suggesting the existence of additional roseolovirus homologs (Higashi et al., 1989).

In the current study, we investigated whether pig-tailed macaques maintained at the Washington National Primate Research Center (WaNPRC) were naturally infected with Roseolovirus homologs using a CODEHOP PCR approach specifically designed to detect novel roseoloviruses. We identified multiple DNA fragments in pig-tailed macaques with strong sequence homology to HHV-6A, HHV-6B, and HHV-7. Phylogenetic analysis revealed that these DNA fragments corresponded to two novel Roseoloviruses belonging to the beta-herpesvirus subfamily. One viral sequence clustered closely with HHV-6A and -6B and panHV-6 suggesting that it was derived from the pig-tailed macaque homolog of HHV-6, provisionally termed MneHV-6. The other viral sequence clustered closely with HHV-7, suggesting that it was derived from the pig-tailed macaque homolog of HHV-7, provisionally termed MneHV-7. Using specific quantitative TaqMan PCR assays, we determined that the prevalence and tissue tropism of MneHV-6 and MneHV-7 were similar to HHV-6 and HHV-7 in humans. Our studies suggest that pig-tailed macaques naturally infected with MneHV-6 and MneHV-7 can be used as biologically relevant animal models to study HHV-6 and HHV-7 infections and associated pathologies in the human host.

## Results

### *Development of CODEHOP PCR assays to identify novel roseoloviruses*

In order to identify novel members of the *Roseolovirus* genus among the Betaherpesvirinae subfamily in Old World monkey species, we used the Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) technique (Rose et al., 1998) to develop PCR primers targeting the highly conserved DNA polymerase (DNA pol) and glycoprotein B (gB) genes, as done previously (Rose, 2005). Amino acid motifs conserved within the known member species of the Betaherpesvirinae subfamily were used to design CODEHOP primer pairs that would preferentially amplify members of the betaherpesvirus family. Several primer pairs were designed using the iCODEHOP software (Boyce et al., 2009) targeting multiple conserved sites within the DNA pol and the gB genes. CODEHOP primers with low degeneracy and with a sequence bias for the known roseolovirus sequences were selected. Inosine was introduced into the degenerate cores of some primers to further lower degeneracy, and some primer consensus sequences were adjusted to more specifically target roseolovirus sequences. Primers were then paired based on compatible annealing temperatures and suitable amplicon lengths. Forty-six DNA pol-specific and 16 gB-specific CODEHOP primer pairs targeting different regions of the DNA pol and gB genes were tested with DNA from the HBS-2 cell line infected with the HHV-6A strain U1102 (Downing et al., 1987). Since the optimal annealing temperature of each CODEHOP primer pool to a novel template is not known, initial PCR reactions were performed using a temperature gradient from 50 °C to 70 °C. PCR products were then resolved on agarose gels (data not shown). Five DNA pol-specific and fifteen gB-specific CODEHOP primer pairs displayed robust amplification of HHV-6A over the entire temperature gradient and showed minimal non-specific amplification.

### *Identification of a pig-tailed macaque homolog of HHV-6*

Since HHV-6 is often detected in human saliva, whole saliva from macaques was used as a source of DNA to screen for the presence of a macaque roseolovirus homolog. Archived saliva samples were available from five pig-tailed macaques that had been challenged with SHIV in a previous vaccine study at the WaNPRC (Polacino et al., 2008). Two of the animals (M03126 and M03182) had detectable viral loads of SHIV in their plasma and three animals (M02383, M02156 and L02393) were negative. DNA isolated from the five saliva samples was tested by PCR using the CODEHOP primers SLYP-F (forward) and YGD-R (reverse). These primers, which target the highly conserved “SLYP” and “YGD” amino acid motifs within the DNA pol gene (Fig. 1), had given a strong amplification signal with DNA from the HHV-6A infected HSB-2 cell line. This primer pair is similar to CODEHOP primers we have used previously to identify unknown macaque gammaherpesviruses (Rose, 2005), but is biased towards roseoloviruses. Using the SLYP-F / YGD-R primer pair with an annealing temperature of 60 °C, we detected a PCR amplicon of the expected size (~550bp) in one of the five macaques tested (Table 1). Following gel purification and Sanger sequencing, we obtained a 478bp nucleotide sequence of the amplified DNA fragment between the flanking primer sequences. We compared the nucleotide sequence to the non-redundant GenBank nucleotide database (NCBI) using BLASTN. The closest matches were partial DNA pol nucleotide sequences from different herpes 6 viruses (Pan troglodytes herpesvirus 6 (PanHV6), HHV-6B (strain Z29) and HHV-6A (strain U1102)) with at least 75% identity (Table 2). This suggested that we had identified a novel roseolovirus DNA pol sequence corresponding to a pig-tailed macaque homolog of HHV-6, provisionally named MneHV-6.

To obtain additional sequence for MneHV-6, additional CODEHOP primer pairs were used to amplify DNA from the same previously positive animal (M02156). The DNA pol-specific CODEHOP primer pair DIEC-F/CNS-R produced an amplicon at 60 °C, which contained a sequence that overlapped the previous MneHV-6 DNA fragment, generating a 1313bp long contiguous sequence of the MneHV-6 DNA pol gene (Fig. 1). Alignment of this sequence with other roseolovirus DNA pol sequences revealed highest nucleotide identity with known human and chimpanzee herpesviruses 6 (Table 2). We also used a CODEHOP primer pair (NPFG/PLEN) targeting motifs conserved in the roseolovirus gB sequences. The NPFG/PLEN assay produced 326bp PCR amplicons at both 50 °C and 60 °C annealing temperatures from the saliva of only one animal (M02156). No PCR products were detected in the other macaque samples. Both amplicons were gel-purified and sequenced revealing the same 262bp sequence flanked by the primers (Fig. 1, Table 1). Nucleotide sequence comparisons with known roseolovirus gB sequences showed again the highest identity with HHV-6B, HHV-6A, and with the chimpanzee PanHV6 gB sequences. Sequence analysis from two independently

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