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**Differential detection of B virus and rhesus cytomegalovirus in rhesus macaques**

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**Non-human primate herpesviruses establish and maintain a lifelong persistent infection in immunocompetent hosts in the absence of clinical signs of disease. A fundamental issue for understanding the natural history of non-human primate herpesviruses is whether the viruses are maintained in a truly latent state or one characterized by a low level of chronic expression. To address this issue, a real-time PCR assay was developed to quantify *Cercopithecine herpesvirus type 1* (B virus) DNA in mucosal fluids of rhesus macaques. This assay was rapid, sensitive (10 genome copies) and specific for B virus obtained from multiple species of macaques. The shedding profile of B virus was compared to another endemic herpesvirus, rhesus cytomegalovirus (RhCMV), in colony-reared monkeys. Mucosal swabs or saliva samples were taken daily from two groups of seropositive monkeys undergoing either a stressful relocation (group 1) or daily chair restraint (group 2). B virus DNA was detected in mucosal fluids from four animals relocated during the breeding season (group 1) but not from 10 animals moved at other times of the year. No B virus DNA was detected in any group 2 monkey. In contrast, RhCMV DNA was detected in the majority of animals of both groups 1 and 2. Detection of B virus DNA shedding is a relatively rare event associated with the breeding season, while RhCMV DNA is persistently detected in mucosal fluids of most monkeys.**

## INTRODUCTION

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The number of non-human primates, especially rhesus macaques, used in biomedical research has greatly increased and will continue to do so for many years, due largely to expanded AIDS-related studies (Cohen, 2000). Approximately 90 % of adult macaques at the California National Primate Research Center (CNPRC; California, USA) are infected with B virus (*Cercopithecine herpesvirus type 1*) and

rhesus cytomegalovirus (RhCMV). These infection rates are similar to those observed in other captive populations (Kessler & Hilliard, 1990; Vogel *et al.*, 1994; Weigler *et al.*, 1990, 1993; Zwartouw *et al.*, 1984). While programmes are in place in a number of primate centres and breeding facilities to expand the number of specific-pathogen-free (SPF) animals for B virus (Hilliard & Ward, 1999; Ward & Hilliard, 1994), demand for rhesus macaques will exceed the supply of SPF animals for the foreseeable future.

B virus is an alphaherpesvirus that is closely related to herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) of humans. Zoonotic human infection with B virus can be fatal and working with B virus-infected animals or culture of the virus carries substantial occupational risk (Holmes *et al.*, 1995; Palmer, 1987). The few studies of B virus shedding in rhesus macaques have been based on virus detection in cell culture (Lees *et al.*, 1991; Weigler *et al.*, 1993; Weir *et al.*, 1993; Zwartouw & Boulter, 1984). Although B virus isolation is generally considered the standard for virus detection, studies with other viruses indicate that virus isolation is a less sensitive method than other techniques such as PCR (Cone *et al.*, 1994; Wald *et al.*, 1995). Non-quantitative PCR techniques have been described for B virus (Black & Eberle, 1997; Scinicariello *et al.*, 1993; Slomka *et al.*, 1993; Weigler *et al.*, 1995). A sensitive, quantitative PCR-based assay to determine the presence of B virus DNA would help assess both the frequency and the titre of shed viral DNA. In addition, a PCR-based test would eliminate lengthy culture detection and the inherent risks of working with the virus in the laboratory.

RhCMV is another endemic herpesvirus of rhesus macaques that is shed at mucosal surfaces. RhCMV is closely related to human CMV (HCMV) by DNA sequence, life cycle and natural history (Barry *et al.*, 1996; Kravitz *et al.*, 1997; Vogel *et al.*, 1994). In contrast to B virus, RhCMV does not pose any known risks to humans working with macaques. However, infection of rhesus macaques with RhCMV provides an excellent model for HCMV persistence and pathogenesis (Lockridge *et al.*, 1999; Sequer *et al.*, 2002; Tarantal *et al.*, 1998). HCMV is the most common congenital infection in the world (Daniel *et al.*, 1995; Stagno *et al.*, 1982) and a significant cause of morbidity and mortality in immunocompromised patients (Flo *et al.*, 1995). Use of this method to determine the frequency and quantify the magnitude of RhCMV shedding would expand our understanding of the natural history of this infection.

B virus and RhCMV infect rhesus macaques for life (Vogel *et al.*, 1994; Weigler, 1992). Both viruses are spread by periodic reactivation and asymptomatic shedding from mucosal sites (Lees *et al.*, 1991; Lockridge *et al.*, 1999; Weigler, 1992). Neither virus normally causes significant disease in immunocompetent macaques during primary or recurrent infection. These viruses have different temporal patterns of acquisition and different reservoirs of persistent viral genomes (Alford & Britt, 1993; Whitley, 1996). Little is known about the frequency with which B virus and RhCMV are shed in healthy adult animals and what factors contribute to shedding.

To increase the understanding of non-human primate herpesviruses, a sensitive assay for the detection of B virus DNA was developed using real-time PCR methodology and compared to a RhCMV real-time PCR assay described recently (Sequar *et al.*, 2002). Real-time PCR provides a rapid, safe and accurate method of screening for these viruses. Results of this and future studies may have important implications for colony management and occupational safety.

## METHODS

**Viral DNA.** DNA purified from the prototype B virus, strain E2490, isolated originally from a rhesus macaque (Hilliard *et al.*, 1987) was used for the development and optimization of real-time PCR. DNA from other B virus field isolates was also used, including rhesus (*Macaca mulatta*; strain 12930), cynomolgus (*M. fascicularis*; strain E90-136) and pigtail (*M. nemestrina*; strain 1504-11) macaques and the related baboon *Herpesvirus papio type 2* (HVP-2; strain OU1-76) (Smith *et al.*, 1998). All viruses were propagated on Vero cell monolayers as described previously (Eberle & Hilliard, 1984).

**Real-time PCR.** Primers and probe for real-time PCR were designed for the conserved glycoprotein B (gB) gene of B virus (Table 1). Sequences were based on the sequence of B virus rhesus strain E2490 and designed using the PRIMER EXPRESS software (Applied Biosystems). Characterization of the RhCMV primers and probe combination has been presented elsewhere (Sequar *et al.*, 2002). The Taqman probe for RhCMV gB detection was fluorescently labelled with TET at the 5' end and TAMRA at the 3' end. The Taqman probe for B virus gB detection was fluorescently labelled with FAM at the 5' end and TAMRA at the 3' end. All primers and probes were synthesized by Applied Biosystems.

**Table 1.** Sequences of primers and probes for Taqman assay

Oligonucleotide	Sequence	Label	Position*
RhBVgB forward	5'-GGTGATCGACAAGATCAACGC-3'	None	817–837
RhBVgB reverse	5'-GCCGTGCTCTCCATGTTGTT-3'	None	875–894
RhBVgB probe	5'-TCTGCCGCTCGACGGCAAAGTAC-3'	5' FAM, 3' TAMRA	846–867
RhCMVgB forward	5'-TGCGTACTATGGAAGAGACAATGC-3'	None	1533–1556
RhCMVgB reverse	5'-ACATCTGGCCGTTCAAAAAAAC-3'	None	1583–1604
RhCMVgB probe	5'-CCAGAAGTTGCGCATCCGCTTGT-3'	5' TET, 3' TAMRA	1558–1580

\*GenBank accession numbers U14664 [rhesus B virus gB (UL27) gene] and U41526 (RhCMV gB).

Real-time PCR was performed with a Perkin-Elmer model 7700 Sequence Detection system. Each PCR reaction contained 12.5 µl Taqman Universal PCR Master mix, 2.5 pmol probe, 5 µl purified DNA and either 17.5 (RhCMV) or 12.5 (B virus) pmol of each virus-specific primer in a total reaction volume of 25

µl. All samples were run in triplicate and repeated at least twice. Results are reported as average copy number ml<sup>-1</sup> mucosal fluid.

A standard curve was generated using a plasmid containing either the full-length B virus (pND-BVgB) (Loomis-Huff *et al.*, 2001) or RhCMV gB (Sequar *et al.*, 2002). Serial 10-fold dilutions of each plasmid containing 10<sup>6</sup> to 10<sup>0</sup> copies per 5 µl were made in 20 µg calf thymus DNA ml<sup>-1</sup> (Sigma) in water. Both assays reproducibly detected between 1 and 10 copies of RhCMV or B virus DNA.

**Animal selection and screening.** Study protocols were approved by the Institutional Animal Use and Care Committee of University of California, Davis, USA (UC Davis), prior to implementation. UC Davis is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Juvenile rhesus macaques were selected for the group 1 study on the basis of B virus and RhCMV seropositivity. Outdoor-housed 3- to 4-year-old animals were bled during routine physical examination. Plasma was screened by ELISA for reactivity to B virus and RhCMV (Lockridge *et al.*, 1999; Loomis-Huff *et al.*, 2001). A total of 13 seropositive animals (4 females, 9 males) with a range of antibody titres were brought indoors and same-sex pair-housed with an animal unknown to them. Changes in housing conditions and pairing with an unknown animal are known stressors for macaques (Capitanio & Lerche, 1998). One animal, 29937, was included in two phases of the group 1 study (described below). Group 2 animals included 43 adult males (age 6–10 years), seropositive for both RhCMV and B virus, from which saliva samples were obtained during a screening phase of animals entering an unrelated study (described below).

**Sample collection and processing.** Group 1 monkeys were sampled in three phases. The first phase occurred in December, during the breeding season. The second and third phases occurred in April and July. During the third phase, daily mucosal swab samples were obtained for 8 days, then, after a 5 day rest period, mucosal swab samples were collected daily for 10 days during dexamethasone treatment. A low to moderate intravenous dose of dexamethasone (2 mg kg<sup>-1</sup>) was administered daily for 7 days, then tapered off over the next week.

Mucosal swab samples were taken at the same time every day by one of two technicians and all samples for a single day were obtained by the same technician. Mild ketamine anaesthesia was used to facilitate sample collection. Animal weights were monitored routinely to assure the animals were not losing weight due to multiple days of anaesthesia.

For group 1 animals, mucosal swab samples were taken of the oral cavity, each eye and the genital region using sterile Dacron swabs (Fisher). Oral swabbing was done by running the swab inside the lower lip,

into the buccal pouch and along the gumline. Separate swabs of each eye were taken by swabbing the upper and lower conjunctival surface. For the genital samples, the vaginal mucosa was swabbed in females; the swab was inserted in the prepuce of male animals. Each swab was placed into a tube containing 1 ml DMEM (Invitrogen) with 10 % foetal bovine serum, 200 units penicillin ml<sup>-1</sup> (Sigma), 200 µg streptomycin ml<sup>-1</sup> (Sigma) and 2.5 µg fungizone ml<sup>-1</sup> (Invitrogen). Only the oral samples were of sufficient volume to saturate the swab with mucosal fluid prior to placement in media.

Swab samples were processed on the day of collection. Samples were vortexed and aliquotted for DNA extraction (400 µl) and virus isolation (volume ranged from 350 to 600 µl). Samples for DNA extraction were mixed with an equal volume of AL Lysis buffer (Qiagen) and both aliquots were stored at -80 °C.

Group 2 monkeys were part of another study assessing the psychosocial aspects of disease progression. A baseline saliva sample was obtained and then monkeys were subjected to seven daily chair restraint sessions. Animals were not anaesthetized during chair restraint. At the end of each chair session, a saliva sample was obtained by allowing the animal to chew a length of braided cotton rope that had been saturated with a sugar solution. After 1 min of chewing, the distal 1 cm of saliva-saturated rope was cut off and placed into the barrel of a 6 ml syringe. Saliva was expressed into a sterile tube by compressing the rope sample with the plunger of the syringe (approximately 400 µl). An aliquot of each saliva sample was mixed 1 : 1 with AL Lysis buffer and stored at -80 °C for batch processing.

**DNA extraction.** DNA was extracted from mucosal swab and saliva samples using the QIAmp Blood kit in a 96-well plate format (Qiagen). Each sample was processed according to the manufacturer's instructions. The final elution volume was 200 µl. Samples were stored at -20 °C until PCR analysis was performed.

**Virus isolation for B virus.** Virus isolation was performed on subconfluent monolayers of Vero cells grown in 24-well tissue culture plates. Samples (100–500 µl) were inoculated onto cells and incubated at 37 °C for 8–18 h. The inoculum was then removed and fresh media added (1 ml per well). Media was changed every other day for 1 week. Cultures were checked twice daily for cytopathic effect. No culture for RhCMV was attempted.

**ELISA.** ELISA assays were performed according to protocols published previously (Lockridge *et al.*, 1999; Loomis-Huff *et al.*, 2001). Briefly, a Triton X-100 extract of HVP-2-infected Vero cells was used as antigen for B virus antibody detection (Loomis-Huff *et al.*, 2001; Ohsawa *et al.*, 1999). Reactivity to uninfected Vero cell extract was subtracted from the absorbance value (measured at 450 nm) for HVP-2 reactivity. RhCMV ELISAs were performed using a Triton X-100 extract of RhCMV-infected primary

skin fibroblasts. Little to no reactivity to uninfected primary skin fibroblast extract was detected. Plasma samples were screened initially at 1 : 100 and 1 : 500. Plasma samples of selected animals were serially diluted to determine the antibody titre. Titres were calculated by taking the inverse of the last dilution for which the absorbance value was greater than 0.1 absorbance units above negative control sample absorbance values (measured at 450 nm).

## RESULTS

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### Real-time PCR development

Initial testing of the B virus primer set confirmed the amplification of B virus E2490 DNA. The amplicon size was verified by agarose gel electrophoresis and ethidium bromide staining (data not shown). To determine the sensitivity and accuracy of the real-time PCR assay, the number of genome equivalents of NaI gradient-purified B virus DNA was calculated from the optical density value at 260 nm and 10-fold serial dilutions of this DNA were analysed by real-time PCR (Table 2). For the standard B virus rhesus strain E2490, the real-time PCR assay detected 75–100 % of the genome copies calculated by measuring the optical density at 260 nm.

Sequence variation has been demonstrated in B virus isolates obtained from different macaque species (Smith *et al.*, 1998; Thompson *et al.*, 2000). The ability of this primer/probe set to detect field isolates of B virus obtained from different macaque species was evaluated. As expected from sequence data (GenBank accession no. 7109250 and unpublished data), DNA of rhesus, cynomolgus and pigtail B virus field isolates was detected efficiently. In contrast, HVP-2 DNA was detected inefficiently, giving copy number values much lower than calculated by spectrophotometric analysis (Table 2). Although the two B virus primer sequences are identical to that of HVP-2 (accession no. U14662), a 2 bp mismatch is present in the probe sequence; this might account for its inefficient detection.

A plasmid containing the HSV-2 gB gene was undetectable even at a very high concentration by the B virus real-time PCR assay. Consistent with the lack of detection of HSV-2 with this assay, sequence alignments of the B virus primer and probe with the published sequence of HSV-2 (accession no. Z86099) indicated that there is one mismatched base pair in the forward and two each in the reverse primer and probe sequences. Though SA8 DNA from African green monkeys was not tested with this assay, one primer and two probe base pair mismatches suggest that there would be inefficient amplification with this primer/probe set as well.

**Table 2.** Genome equivalent calculations for 10-fold serial dilutions of DNA by spectrometry and real-time PCR

The optical density reading at 260 nm was used to calculate genome equivalents based on a genome size of 162 kb for B virus strains and HVP-2 and 6 kb for an HSVgB plasmid. Genome equivalents determined by real-time PCR are averages for triplicate wells of 10-fold serial dilutions.

DNA	Genome equivalents	
	Spectrometry	Taqman
Rhesus B virus E2490	3 350 000	2 538 733
	335 000	285 693
	33 500	36 590
Rhesus B virus 12930	9 620 000	3 225 233
	962 000	485 903
	96 200	68 687
Cynomolgus B virus	5 080 000	2 601 900
	508 000	350 070
	50 800	44 520
Pigtail B virus	7 340 000	1 719 000
	734 000	190 133
	73 400	22 438
HVP-2	5 510 000	1 286
	551 000	419
	55 100	61
HSVgB	8 360 000	0
	836 000	0
	83 600	0

### **B virus DNA detection in mucosal samples**

After demonstrating the sensitivity and specificity of the quantitative real-time B virus PCR assay, experiments to evaluate clinical mucosal swab samples for the presence of B virus were conducted. In captive rhesus macaques B virus infection usually occurs at 3–4 years of age (Weigler, 1992; Whitley, 1996). Studies of HSV-2 infection in humans have demonstrated that reactivation frequency is greater in the period immediately following infection (Benedetti *et al.*, 1994, 1999; Koelle & Wald, 2000). This age group was selected to identify recently infected seropositive animals that would be likely to reactivate and shed virus under stress.

Seropositive 3- to 4-year-old monkeys (four females and nine males, designated group 1) were stressed by relocation to new housing and pairing with a new animal in three phases over 1 year. Each phase involved new animals, with one exception, at a different time of the year: December (breeding season,

$n=4$ ), April ( $n=4$ ) and July ( $n=6$ ). Animal 29937 was included in both the December and July phases. B virus DNA was detected only in mucosal swab samples obtained from animals moved during the breeding season. Each of the four breeding season animals yielded a unique pattern of B virus-positive swabs (Table 3). Although swabs were collected up to 28 days after relocating the animals, all B virus-positive swabs occurred within the first 10 days of sampling. B virus DNA was detected from multiple sites on multiple days for two animals (29937 and 30200). The highest copy number of B virus DNA was in the genital tract samples. Intermittent detection of B virus was seen also in both ocular and oral samples. The other two animals had only a single site that was B virus DNA positive and was confined to a single day. The overall frequency of positive swabs during the first 10 days of sampling during the breeding season was 14.6 % (21/144) as compared to 0 % (0/376) in April and July. For the two animals with multiple positive swab samples (29937 and 30200), 26.4 % (19/72) of swabs were positive for B virus DNA during the first 10 days.

**Table 3.** B virus genome copy number in mucosal swab samples from group 1 (breeding season)

Mucosal swab samples were taken daily for 7 days, then every other day until day 28. There were no positive swab samples after day 10. Results are from mucosal swabs taken in December. All mucosal swabs taken in April and July were negative for B virus DNA. Results are reported as copy number  $\text{ml}^{-1}$  mucosal fluid. The number of each animal is given together with its gender (in parentheses). Paired animals are next to each other in the table. RE, right eye; LE, left eye; G, genital; O, oral.

Animal no. (gender)...	29937 (M)				30200 (M)				30016 (F)				30141 (F)			
	RE	LE	G	O	RE	LE	G	O	RE	LE	G	O	RE	LE	G	O
0	0	0	0	0	0	0	5 237	0	0	0	0	0	0	955	0	0
1	0	0	0	0	0	3 471	47 945	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	65 809	0	0	0	0	0	0	0	0	0
3	0	0	0	0	1061	0	7 406	3 095	0	0	0	0	0	0	0	0
4	838	0	0	0	2 299	1 653	253 726	0	0	0	0	5 083	0	0	0	0
5	0	0	6 130	0	0	0	25 842	0	0	0	0	0	0	0	0	0
6	1 542	0	0	0	2 126	0	4 761	0	0	0	0	0	0	0	0	0
8	0	1 424	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	5288	1 381	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Group 2 animals included 43 adult males seropositive for both RhCMV and B virus from which saliva samples were collected during daily chair restraint sessions. No B virus was detected from the saliva samples collected from the animals of group 2. Virus isolation was performed on samples collected during the breeding season but infectious B virus was not detected in any samples. Virus isolation was not attempted on samples collected in April and July or samples collected from group 2 monkeys.

### RhCMV detection in mucosal samples

To demonstrate key differences in the natural history of two endemic macaque herpesviruses, the 0001-8808 © 2003 SGM

shedding profile of RhCMV DNA was assayed with the same mucosal samples and compared with that of B virus. RhCMV was detected more frequently than B virus DNA, though there was wide variability between animals within a group and between group 1 and group 2 animals. Results of RhCMV DNA detection for the four animals from which B virus DNA was detected are shown in Table 4. The variability seen in these four animals is representative of that seen from other animals in group 1. RhCMV DNA detection occurred more frequently and at higher copy number in the younger group 1 animals compared to group 2 animals (Table 5).

**Table 4.** RhCMV genome copy number in mucosal swab samples from group 1 (breeding season)

Mucosal swab samples were taken daily for 7 days, then every other day through day 28. Results are shown for only the first 10 sample days. Results are reported as copy number ml<sup>-1</sup> mucosal fluid. The number of each animal is given together with its gender (in parentheses). Paired animals are next to each other in the table. G, genital; O, oral (no ocular shedding of RhCMV was detected).

Animal no. (gender)...	29937 (M)		30200 (M)		30016 (F)		30141 (F)	
	G	O	G	O	G	O	G	O
0	0	44 998	3 987	2 328	13 164	24 201	0	0
1	0	161 972	0	23 981	3 319	53 469	0	0
2	0	13 809	0	0	39 142	13 960	0	0
3	0	49 035	0	2 699	3 539	3 329	0	0
4	0	18 673	20 544	3 243	2 818	17 930	0	0
5	0	11 974	617	1 527	2 645	23 975	0	0
6	0	18 922	5 612	417	8 148	6 615	0	0
8	0	7 439	0	619	12 924	17 386	0	0
10	0	23 118	3 397	1 643	13 351	21 861	0	0

Particularly noteworthy was the frequency of animals that were positive for RhCMV DNA at baseline testing (on the day of movement from the outside corrals). Shedding of RhCMV was detected in oral swabs of 64.3 % ( $n=9/14$ ) of group 1 monkeys and 40 % ( $n=12/30$ ) of saliva samples from group 2 monkeys at baseline (Table 5). For group 1 animals, the eye and genital tract swab samples were also assayed for RhCMV DNA. No RhCMV shedding was detected in the eye swab samples and less virus was detected in the genital swab samples compared to oral samples (representative data in Table 4). Mucosal swab and saliva samples were not cultured for RhCMV.

**Table 5.** RhCMV genome copy number in oral swab and saliva samples

DNA detection	Group	Frequency (%)	Average copy number (range)
All samples positive	1	36 (5/14)*	100 379 (2 748–490 050)
	2	5 (2/43)	59 559 (4 463–213 861)
Majority positive	1	36 (5/14)	11 463 (0–161 972)
	2	26 (11/43)	5 839 (0–95 245)
Minority positive	1	14 (2/14)	1 324 (0–7 185)
	2	26 (11/43)	558 (0–10 152)
All samples negative	1	14 (2/14)	NA
	2	44 (19/43)	NA
Positive at baseline	1	64 (9/14)	6745 (0–60 858)
	2	40 (12/30)‡	6993 (0–70 128)

\*Number of animals in the category over the total number in each group.

‡Only 30 group 2 animals had baseline samples available.

NA, Not applicable.

### Dexamethasone treatment

Reactivation of latent HSV and shedding of infectious virus in response to glucocorticoid administration has been well documented in clinical settings (Mosimann *et al.*, 1994; Shane *et al.*, 1994) and in animal models (Cook *et al.*, 1991; Kaufman *et al.*, 1999). Real-time PCR was used to determine whether dexamethasone treatment resulted in increased detection of B virus DNA. Six of the monkeys of group 1 sampled in July were kept indoors for a second round of sample collection while being treated with dexamethasone. After a 5 day rest period, swab samples were obtained for 10 days during daily dexamethasone treatment (2 mg kg<sup>-1</sup>).

B virus DNA was not detected in any swab samples taken while the monkeys were being treated with dexamethasone. The frequency of RhCMV DNA detection was also unchanged with dexamethasone treatment (Table 6) and the number of positive days remained unchanged. An increase in the amount of virus shed did occur for one animal (29938), with 2.8×10<sup>6</sup> genome copy numbers ml<sup>-1</sup> mucosal fluid being detected (Table 6).

**Table 6.** RhCMV genome copy number in oral swab samples with or without dexamethasone

Mucosal swab samples were taken daily for 8 days, followed by a 5 day rest period, then for 10 days under dexamethasone (dex) treatment. Results are reported as copy number ml<sup>-1</sup> mucosal fluid. The number of each animal is given together with its gender (in parentheses). Paired animals are next to each other in the table. O, oral; G, genital.

Animal no. (gender)...	30045 (F)		30036 (F)		29937 (M)		29955 (M)		29938 (M)		29349 (M)	
	G	O	G	O	G	O	G	O	G	O	G	O
0	0	0	1213	73 960	0	0	60 858	490 050	2 454	26 227	0	0
1	0	0	6 869	16 306	0	2 020	2 712	187 242	0	210 394	0	2 496
2	0	0	1 017	300 472	0	10 289	1 848	153 856	0	413 152	0	2 924
3	0	0	2 309	35 356	0	0	14 312	68 872	0	288 002	0	0
4	0	0	0	20 638	0	1 514	6 098	126 720	0	75 488	0	0
5	0	0	1 127	3 992	0	0	4 714	43 647	0	5 364	0	0
6	0	0	0	21 413	0	7 664	1 725	45 208	0	319 744	0	7 185
7	0	0	315	99 912	0	10 573	7 147	62 013	0	6 752	0	0
13	0	0	349	113 104	0	4 910	10 877	226 320	0	76 258	0	1 857
14	0	0	8 415	25 978	0	13 689	2 478	118 308	0	285 618	0	0
15	0	0	2 817	239 162	0	12 703	3 116	87 538	0	795 740	0	0
16	0	0	0	920	0	1 500	2 478	33 184	0	325 192	0	0
17	0	0	1 158	62 691	0	12 519	0	64 805	0	776 500	0	0
18	0	0	0	99 982	0	3 378	12 027	93 428	0	1 822 440	0	0
19	0	0	476	58 895	0	11 918	3 345	279 350	0	326 946	0	1 858
20	0	0	0	1 470	0	7 537	10 124	187 244	0	2 855 320	0	2 442
21	0	0	7 283	7 593	0	1 832	9 812	327 243	0	242 462	0	0
22	0	0	0	121 326	0	7 681	1 893	162 700	0	580 782	0	0
Mean*	0	0	1 853	72 398	0	6 096	8 642	153 207	136	524 021	0	1 042
Mean without dex†	0	0	1 606	71 506	0	4 008	12 427	147 201	307	168 140	0	1 576
Mean with dex‡	0	0	2 050	73 112	0	7 767	5 615	158 012	0	808 726	0	616

\*Mean copy number ml<sup>-1</sup> mucosal fluid for all sample days.

†Mean copy number ml<sup>-1</sup> without dexamethasone (days 0–7 only).

‡Mean copy number ml<sup>-1</sup> with dexamethasone (days 13–22 only).

## DISCUSSION

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B virus shedding has rarely been documented in colony-housed rhesus macaques (Weigler, 1992; Weir *et al.*, 1993). Most cases detected with virus isolation have been associated with primary infection (Weigler *et al.*, 1993; Zwartouw *et al.*, 1984), immunosuppression (Lees *et al.*, 1991; Zwartouw & Boulter, 1984) or the breeding season (Weigler *et al.*, 1993). This likely reflects both rare shedding of B virus and the lack of sensitivity of the virus isolation method. Comparative studies of HSV detection have demonstrated that virus isolation lacks the sensitivity of PCR for the detection of virus shedding (Coyle *et al.*, 1999; Madhavan *et al.*, 1999; Wald *et al.*, 1997). Traditional PCR assays for B virus detection have been described (Black & Eberle, 1997; Hirano *et al.*, 2000; Scinicariello *et al.*, 1993; Slomka *et al.*, 1993). This study represents the first description of a real-time, quantitative PCR assay for B virus and its use for detection of B virus shedding from mucosal sites in macaques.

The relationship between B virus copy number and infectious virus titre is not known. Studies of HSV shedding demonstrated values 100-fold higher for copy number by traditional PCR than virus titres determined by virus culture (Koelle & Wald, 2000; Wald *et al.*, 1997). No virus was detected by virus isolation in this study. However, the limit of detection of the assay is not known. It is possible that low titres of infectious virus were present in the samples. In addition, inactivation of small amounts of infectious virus may have occurred during the single freeze–thaw cycle samples were subjected to before being inoculated onto Vero cell monolayers. Freezing and thawing samples for B virus culture has been shown to decrease virus detection by 1–2 logs (Krech & Lewis, 1954; Weir *et al.*, 1993). The real-time PCR assay described here is probably not more sensitive for B virus than traditional PCR but it is more specific due to the use of an internal probe that appears to differentiate between B virus and other primate alphaherpesviruses, including HSV. While further study is needed, there is a potential for application of this assay to diagnose B virus infection in an exposed human that is also infected with HSV.

Multiple factors can trigger reactivation from latency to virus shedding of infectious HSV (Sainz *et al.*, 2001). Examples of the contribution of social stress to virus pathogenesis have been described for both humans (Glaser *et al.*, 1999; Glaser & Kiecolt-Glaser, 1997; Padgett *et al.*, 1998) and non-human primates (Baroncelli *et al.*, 1997; Capitanio *et al.*, 1998b). In this study stress was created by moving, pair-housing with a new animal and submitting each animal to daily sample taking. However, results from this study suggest that factors associated with the breeding season may be more important at inducing B virus reactivation and shedding than stress introduced by the study design. The breeding season has been described as a time of stressful social interaction for rhesus macaques (Weigler *et al.*, 1993; Wilson & Boelkins, 1970). In this study, baseline B virus shedding in two animals (29937 and 30141) may have been a reflection of the stress induced by the breeding season. In addition, animal 29937 was sampled in both December and July and B virus was detected only during the breeding season

(December). B virus-positive mucosal swab samples have also been detected in 1/28 monkeys (3.6 %) in a cross-sectional survey performed during the breeding season (unpublished observations). The timing of B virus detection in this study supports the idea that B virus shedding is relatively rare and is possibly associated more with the breeding season than thought previously. While the study size for group 1 was small ( $n=14$ ) and only a few animals had detectable B virus, the intriguing association between B virus reactivation and the breeding season warrants further investigation.

As with studies of HSV asymptomatic shedding (Koelle *et al.*, 1992; Wald *et al.*, 1995), no differences were apparent in the humoral immune response between animals that were B virus DNA-positive versus those with no B virus detected in this study (data not shown). A clear role for the cellular immune response in controlling HSV reactivation and shedding has been established (Koelle *et al.*, 1998; Posavad *et al.*, 1997, 1998). HSV is also more frequently shed in human immunodeficiency virus (HIV)-infected individuals with low CD4<sup>+</sup> T cell counts (Augenbraun *et al.*, 1995; Schacker *et al.*, 1998). For this reason, dexamethasone immunosuppression was attempted but did not result in the detection of B virus by PCR. The concentration of dexamethasone used in this study has been reported to alter lymphocyte function in rhesus macaques (Capitanio *et al.*, 1998a; Pachner *et al.*, 2001). While other investigators have detected reactivation and shedding of B virus following immunosuppressive treatment, dosing for 3 months was required and only a few animals (3/14) in the highest dose range reactivated (Chellman *et al.*, 1992). Thus, while chronic immunosuppression can probably exacerbate shedding of B virus in some animals, the significance of transient immunosuppression relative to the stress of the breeding season was not defined by this study.

The pattern of RhCMV detection is very similar to what is known for HCMV. Numerous groups have demonstrated clearly that CD34<sup>+</sup> haematopoietic stem cells are reservoirs of truly latent HCMV genomes (Hahn *et al.*, 1998; Soderberg-Naucler & Nelson, 1999). However, recent evidence has demonstrated that mucosal shedding of HCMV occurs commonly in many healthy individuals (do Canto *et al.*, 2000; Lucht *et al.*, 1998; Shen *et al.*, 1996) and very frequently in immunocompromised patients (Diamond *et al.*, 2000; Fidouh-Houhou *et al.*, 2001; Lucht *et al.*, 1998; Mostad *et al.*, 2000). In this study, RhCMV shedding was detected consistently in many animals, indicating low-level, persistent infection. RhCMV was detected most frequently in the oral cavity, which is consistent with what has been reported for CMV in humans. In addition, CMV was not detected in eye swab samples from healthy CMV-infected humans (Lee-Wing *et al.*, 1999), whereas genital shedding of CMV in the semen and cervical fluid has been noted (Gradilone *et al.*, 1996; Yang *et al.*, 1995), especially during HIV-infection (Diamond *et al.*, 2000; Mostad *et al.*, 2000).

The different shedding signatures of RhCMV and B virus in immunocompetent, healthy macaques imply fundamental distinctions in the natural history of these two herpesviruses. The infrequent detection of B virus DNA is consistent with other alphaherpesviruses that establish a true latent infection (Roizman & Sears, 1996). In the case of HSV, latent viral genomes are activated infrequently, resulting in a change from an extremely limited pattern of gene expression to one of active virus production (Roizman & Sears, 1996). In contrast, the continuous detection of RhCMV suggests that this virus establishes a state of persistent virus production. While individual RhCMV-infected cells may be characterized by a non-productive pattern of gene expression (i.e., latency), sufficient numbers of such cells reactivate to generate chronic detection of RhCMV DNA in mucosal fluids.

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