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One-Step PCR To Distinguish B Virus from Related Primate Alphaherpesviruses

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By adding betaine to the PCR mixture, we previously established a PCR method to amplify a DNA segment of the glycoprotein G gene of B virus (BV) derived from a rhesus macaque. We have found that DNA of other BV strains derived from cynomolgus, pigtail, and lion-tailed macaques can also serve as the template in our PCR assay. Under the same conditions no product was obtained with DNA of simian agent 8 of green monkeys and *Herpesvirus papio* 2 of baboons, or the human herpes simplex viruses types 1 and 2. Thus, this PCR method is useful to discriminate BV from other closely related primate alphaherpesviruses.

Cercopithecine herpesvirus 1 (*Herpesvirus simiae* or monkey B virus [BV]) is a member of the alphaherpesvirus subfamily and a common pathogen in macaque monkeys (3, 15). BV infection is usually asymptomatic in macaques, but there are ca. 40 cases in which BV transmission to humans led to severe encephalomyelitis with high mortality (4, 10, 22). In these cases BV was transmitted to humans from rhesus (*Macaca mulatta*) or cynomolgus (*M. fascicularis*) macaques, but there is no report about the infectivity of BV derived from other macaque species for humans. At present all BV isolates are classified as level 4 pathogens. Besides BV, the alphaherpesvirus subfamily includes *Cercopithecine herpesvirus* 2 (simian agent 8 [SA8]) of green monkeys and *Cercopithecine herpesvirus* 16 (*Herpesvirus papio* 2 [HVP2]) of baboons, as well as human herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2) (6, 13). SA8 and HVP2 are categorized as level 2 pathogens, because there is no evidence that either virus is lethal to humans. It was recently reported that BV transmitted from lion-tailed macaques (*M. silenus*) caused an outbreak in a colony of DeBrazza's monkeys (*Cercopithecus neglectus*) with high mortality (20). In addition, infectious BV was successfully isolated from one of the surviving monkeys after 11 years, suggesting that nonmacaque monkeys can survive BV infection and continue to shed infectious BV after recovery (20). Therefore, a simple method to discriminate BV from other primate alphaherpesviruses has practical significance for the safety of animal care staff dealing with monkeys.

PCR has been applied to in vitro diagnosis to detect viral DNA with rapidity and safety. Several two-step PCR methods, which make use of restriction fragment length polymorphism (RFLP) after PCR amplification, have been developed to detect and identify BV (1, 16, 17, 18). As previously reported, we established a PCR method to amplify a DNA segment of the glycoprotein G (gG) gene (US4 gene) from a BV strain isolated from a rhesus macaque by adding betaine (1-carboxy-*N*,*N*,*N*-trimethylmethanammonium inner salt) to the PCR mixture (9).

BV specificity. The BV strains used in this study included strain E2490 from rhesus, E90-136 from cynomolgus, strain Kumquat from pigtail, and strain 8100812 from lion-tailed macaques (20). Other primate alphaherpesviruses used in this study included SA8 strain B264, HVP2 strain OU1-76, HSV-1 strain KOS, and HSV-2 strain 186. Confluent monolayers of Vero cells were infected with virus in serum-free medium. Infected cells were harvested and dispersed in extraction buffer (10 mM Tris [pH 8.0], 0.1 M EDTA [pH 8.0], 0.5% sodium dodecyl sulfate) containing 20 μg of RNase A and 100 μg of proteinase K/ml and then incubated at 56°C for 2 h and overnight at 37°C. DNA was purified by extraction once with Tris-saturated phenol, three times with phenol-chloroform-isoamyl alcohol, and once with chloroform-isoamyl alcohol. Finally, DNA was recovered by ethanol precipitation. To detect the BV DNA, we performed PCR assays to amplify a DNA segment of the gG gene (the legend of Fig. 1A). PCR mixtures (50 μl) contained 1.5 M betaine and 1 U of ExTaq DNA Polymerase (TaKaRa Shuzo, Kyoto, Japan). The G+C content of the BV gG gene is so high that the DNA segment was refractory to the PCR amplification with 10% dimethyl sulfoxide (9).

Accession numbers. The nucleotide sequences of the PCR products of strain Kumquat (pigtail macaque) and strain 8100812 (lion-tailed macaque) have been deposited in the DNA Data Bank of Japan under accession numbers AB062748 and AB062749, respectively.

As shown in Fig. 1A, lane 1, a 209-bp amplicon was detected with template DNA of a rhesus macaque-derived BV strain in the presence of 1.5 M betaine as previously described (9). When a cynomolgus macaque-derived BV strain was used, a product of the same size was obtained (Fig. 1, lane 2). This result was expected since the primers had been designed based on the published gG sequence of a BV strain isolated from a cynomolgus macaque (19). DNA of strain 8100812, derived

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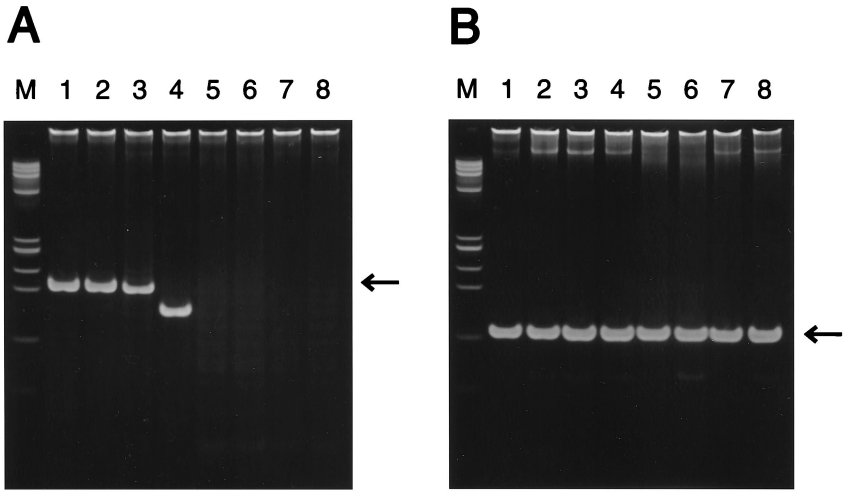

FIG. 1. Detection of BV and other alphaherpesviruses by PCR. (A) PCR with primers gGS4 (forward, 5′-CCGCGTACGACTACGAGATC C-3′) and gGAS4 (reverse, 5′-GTTCGCGGCCACGATCCA-3′) in the presence of 1.5 M betaine. After an initial denaturation step (94°C, 5 min), PCR was conducted for 35 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min), followed by a final extension (72°C, 7 min). PCR products were subjected to electrophoresis on a 5% polyacrylamide gel, and DNA bands were visualized under illumination at 254 nm after being stained with SYBR Green I (9). The arrow at the right indicates the position of the 209-bp fragment in lanes 1 and 3. (B) PCR with primers BV1 and BV2 tested according to the method of Scinicariello et al. (16, 17). The arrow indicates the 128-bp fragment. Lane M, *Hae*III-digested ϕX174 replicative form (RF) DNA. Lanes 1 to 4, BV DNA as the PCR template. Lane 1, strain E2490 (rhesus macaque); lane 2, strain E90-136 (cynomolgus macaque); lane 3, strain 8100812 (lion-tailed macaque); lane 4, strain Kumquat (pigtail macaque); lanes 5 to 8, DNA of primate alphaherpesviruses other than BV as the PCR template; lane 5, human HSV-1 (strain KOS); lane 6, human HSV-2 (strain 186); lane 7, SA8 (strain B264) of green monkeys; lane 8, HVP2 (strain OU1-76) of baboons.

from a lion-tailed macaque, yielded a product of 203 bp, while a PCR product of a smaller size (161 bp) was obtained with DNA of the Kumquat strain derived from a pigtail macaque (Fig. 1A, lanes 3 and 4). Thus, we could detect amplicons of various BV strains with DNA extracted from virus-infected cells as the template. The HSV-1 and HSV-2 strains used in this study (KOS and 186, respectively) were different strains from those used in our previous report (HF and UW268, respectively) (9). However, as previously reported, we detected no specific PCR products with DNA from either of these two viruses (Fig. 1A, lanes 5 and 6). Furthermore, using these same conditions no amplicon was detected with the template DNA of either SA8 or HVP2 (Fig. 1A, lanes 7 and 8). Thus, our PCR assay does not amplify the gG genes of primate alphaherpesviruses other than macaque BV.

To confirm that viral DNA was extracted from virus-infected cells, control experiments were performed with primers BV1 and BV2 according to the method of Scinicariello et al., which amplified a 128-bp segment of the ICP 18.5 (UL28) gene of monkey BV and human HSV (16, 17). Fragments of 128 bp were detected with template DNA of all four BV strains, as well as with HSV-1 and HSV-2 (Fig. 1B, lanes 1 to 6). We could also detect amplicons of a similar size with SA8 and HVP2 DNA (Fig. 1B, lanes 7 and 8). These results exclude the

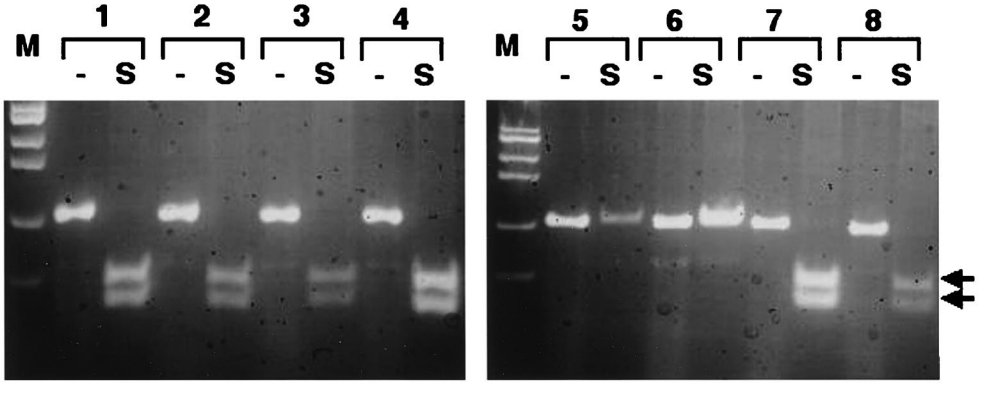

FIG. 2. RFLP assays according to the method of Scinicariello et al. (16, 17). Lanes: −, PCR products prior to restriction digestion; S, digests of the 128-bp products with *Sac*II (the arrows indicate the 72- and 56-bp fragments); M, *Hae*III-digested ϕX174 RF DNA. Lanes 1, BV strain E2490 (rhesus macaque); lanes 2, BV strain E90-136 (cynomolgus macaque); lanes 3, BV strain 8100812 (lion-tailed macaque); lanes 4, BV strain Kumquat (pigtail macaque); lanes 5, human HSV-1 (strain KOS); lanes 6, human HSV-2 (strain 186); lanes 7, SA8 (strain B264) of green monkeys; lanes 8, HVP2 (strain OU1-76) of baboons.

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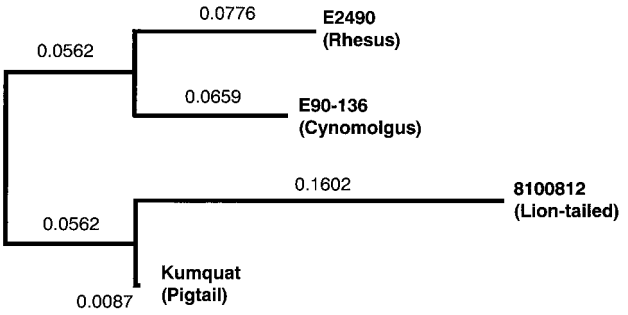

FIG. 3. Phylogenetic tree using the neighbor-joining method based on the partial amino acid sequences of gG of strain E2490 (rhesus macaque), strain E90-136 (cynomolgus macaque), strain 8100812 (lion-tailed macaque), and strain Kumquat (pigtail macaque).

possibility that the negative results shown in Fig. 1A, lanes 5 to 8, were caused by insufficient template DNA.

It remains possible that the negative PCR results for HSV, SA8, and HVP2 shown in Fig. 1A, lanes 5 to 8, might have been caused by disruption of the gG genes, e.g., translocation or large deletion. The circumstantial evidence for such an interpretation is that gG-negative HSV mutants have been constructed by in vitro mutagenesis in the gG gene, and this gene appears to be dispensable for viral replication at least in cell cultures (8, 21). However, of 2,400 HSV-2 clinical isolates, only 5 were found to be gG negative (11). Furthermore, all five gG-negative isolates were shown to have a frameshift mutation (single nucleotide insertion or deletion) in the gG gene (12). The fact that no translocation or large deletions that disrupt the gross structure of the gG gene have ever been reported in any primate alphaherpesviruses argues against this being a reason for the BV specificity of the PCR assay. Much more likely is the divergent sequence of the gG genes of HSV, SA8, and HVP2 in the regions where the primers are located (unpublished observations).

Scinicariello et al. reported that the 128-bp amplicon of monkey BV and human HSV can be distinguished by digestion with *Sac*II. Namely, the BV amplicon yields two fragments of 72 and 56 bp, while the HSV amplicon remains 128 bp (16, 17). As shown in Fig. 2, lanes 1S to 4S, we confirmed that *Sac*II cleaved the 128-bp PCR products of all four BV strains used in this study, yielding the 72- and 56-bp fragments. However, the products of SA8 and HVP2 also yielded two fragments of similar sizes (Fig. 2, lanes 7S and 8S). Therefore, SA8 of green monkeys and HVP2 of baboons could not be distinguished from macaque BV according to the two-step method of Scinicariello et al. In contrast, our one-step PCR method is specific for BV and can be used to detect and rapidly discriminate BV from other closely related alphaherpesviruses.

Phylogenetic relationships among BV strains. PCR products were cloned into the pGEM-T Easy Vector (Promega, Madison, Wis.). Nucleotide sequences were determined by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, Calif.) and a Model 310 Genetic Analyzer (Applied Biosystems). Partial amino acid sequences of gG were deduced from the nucleotide sequence of the PCR fragments, and a phylogenetic tree was constructed by using the neighbor-joining method by the GENETYX-MAC version 10.1 software package (Software Development, Tokyo, Japan) (Fig. 3). The overall topology of the tree did not change when other distance analysis and tree drawing were done with the same nucleotide sequences (data not shown). These results are compatible with the phylogeny of primate alphaherpesviruses deduced from the nucleotide sequences of 1.3-kb fragments containing the 3′ end of gG, the full length of gJ, and the 5′ end of gD genes (20).

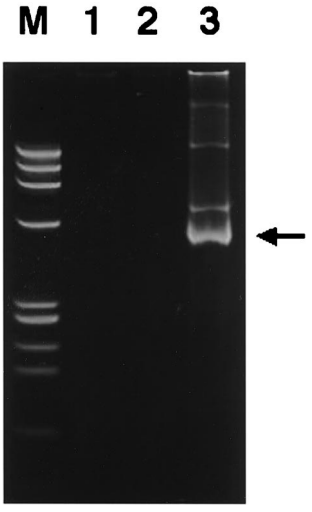

FIG. 4. PCR assays with the purified BHV2 DNA as the template. Lanes: M, *Hae*III-digested ϕX174 RF DNA; 1, PCR with primers S4 and AS4 in the presence of 1.5 M betaine (9); 2, PCR with primers BV1 and BV2 tested according to the method of Scinicariello et al. (16, 17); 3, PCR with primers B3 and B4 tested according to the method of Black and Eberle (1). The arrow indicates the predicted 542-bp fragment in lane 3.

It has been shown that bovine herpesvirus 2 (BHV2) is more related to primate α-herpesviruses than to BHV1 in molecular phylogeny, although the natural hosts of both BHV1 and BHV2 are cattle (2, 5, 7, 14). By our one-step PCR method, we tried to detect the BHV2 gG fragment with viral DNA purified by CsCl centrifugation, but no product was obtained (Fig. 4, lane 1). We could not amplify the 128-bp fragment of the ICP 18.5 gene, either (Fig. 4, lane 2). As shown in Fig. 4, lane 3, the presence of the template DNA was demonstrated by the predicted 542-bp fragment of the glycoprotein B (UL27) gene amplified according to the method of Black and Eberle (1). These results may suggest that BHV2 is relatively divergent from BV as for the gG and ICP 18.5 genes.

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