

Kaposi Sarcoma in Mantled Guereza

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We identified a novel Kaposi's sarcoma herpesvirus–related rhadinovirus (Colobine gammaherpesvirus 1) in a mantled guereza (*Colobus guereza kikuyensis*). The animal had multiple oral tumors characterized by proliferation of latent nuclear antigen 1–positive spindle cells and was not co-infected with immunosuppressive simian viruses, suggesting that it had Kaposi sarcoma caused by this novel rhadinovirus.

Kaposi's sarcoma herpesvirus (KSHV), a member of the genus *Rhadinovirus*, is the causative agent of Kaposi sarcoma (1), an endothelial neoplasm of the dermis, oral cavity, and intestinal organs. The tumors are highly vascularized and characterized by proliferation of spindle cells that contain KSHV DNA and antigen (2,3). Predisposing factors for Kaposi sarcoma include immunodeficiency, especially infection with HIV (4). Nevertheless, a major portion of Kaposi sarcoma cases in Africa occurs in HIV-negative persons (5).

Clinically, Kaposi sarcoma is divided into 4 forms: classical Kaposi sarcoma, African endemic Kaposi sarcoma, Kaposi sarcoma caused by iatrogenic immunosuppression, and HIV-associated Kaposi sarcoma (6,7). Lesions of classical Kaposi sarcoma initially occur on the lower extremities, progress slowly, and affect visceral organs at a late stage (6,7). In contrast, the remaining Kaposi sarcoma forms affect lymph nodes, mucosa, and visceral organs at early stages, progress rapidly, and encompass symptoms in the hard palate and oral mucosa (6,7).

Rhadinoviruses with high similarity to KSHV have been detected in Old World monkeys, including chimpanzees (8), macaques (9–11), and African green monkeys (12). The rhadinoviruses have split into 2 lineages, RV1 and RV2, and

many Old World monkeys harbor viruses of both lineages. In contrast, humans harbor only KSHV, which belongs to the RV1 lineage. Kaposi sarcoma–like disease has been observed in rhadinovirus-infected nonhuman primates (NHP), but only in the presence of immunodeficiency, induced, for instance, by co-infection with simian immunodeficiency virus (13,14).

We report Kaposi sarcoma in a simian immunodeficiency virus– and simian retrovirus–negative mantled guereza (*Colobus guereza kikuyensis*) that was infected with a novel rhadinovirus that had high homology to KSHV. This new virus is called Colobine gammaherpesvirus 1 (CbGHV1).

The Study

A 13-year-old female mantled guereza who was born in a zoological garden in Germany showed development of swelling on the inner aspects of the lower lips; several circumscribed masses were found on the inner upper and lower labial mucosa. The masses were pink to light red and had a smooth and shiny surface, coarse consistence, and a diameter of 1–2 cm (Figure 1, panel A). After incision of 1 mass, the surface of the cut appeared to be cavernous and highly vascularized.

The mucosal masses were removed by surgery. Subsequently, bilateral cataract, progressive weight loss, and recurrence of the mucosal masses developed in the animal, and it had to be euthanized (Appendix, <https://wwwnc.cdc.gov/EID/article/25/8/18-1804-App1.pdf>). Necropsy showed several flattened and smooth tumorous lesions on the inner aspects of the upper and lower lips, as well as multiple small, partly ulcerated nodules at the gingival margin of the upper and lower jaw (Figure 1, panel B).

Histologically, masses and nodules consisted of a collagen-rich fibrous stroma with multifocal areas of increased cellularity represented by spindle cell proliferations with moderate lymphoplasmacellular infiltrates (Figure 1, panel C). The tonsils and mandibular and axillary lymph nodes showed similar foci of fibrovascular tissue. In the perinodal adipose tissue of 1 mandibular lymph node, we found distinct formation of caverns lined by endothelial cells, filled with erythrocytes, and surrounded by spindle cells (Figure 1, panel D).

Immunohistochemical examination showed distinct immunoreaction of most spindle cells with endothelial cell markers CD31 and von Willebrand factor. We found variable expression of Ki67 in $\leq 20\%$ of spindle cells (Figure 1, panel E), and $\approx 50\%$ – 60% of spindle cells reacted with

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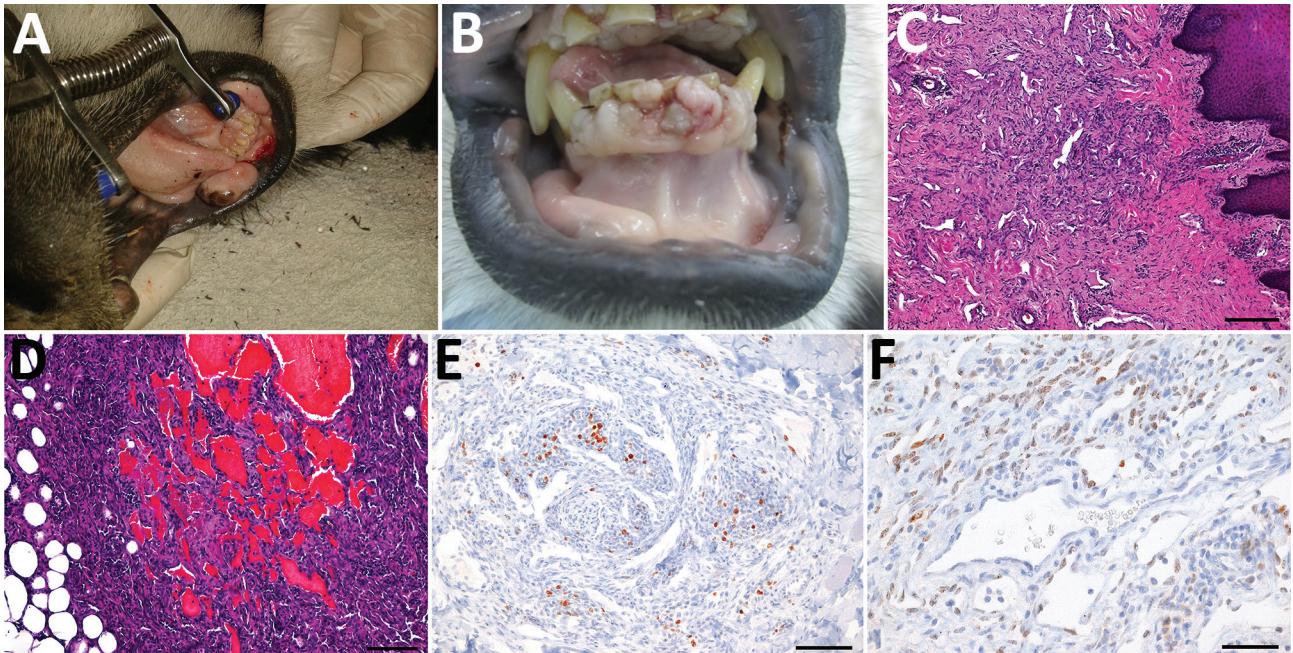


Figure 1. Disease manifestations in mantled guereza with Kaposi sarcoma. A) Oligofocal flattened masses on the inner aspects of the lower lip. B) Multinodular fissured masses at the gingival margin. C) Fibrovascular stroma in the subepithelial propria of the lower lip with spindle cell proliferations delineating narrow vascular clefts and containing lymphoplasmacytic inflammatory cell infiltrates, hematoxylin and eosin stained; scale bar indicates 200 μ m. D) Spindle cell proliferation with cavern formation in the perinodal adipose tissue of the mandibular lymph node; hematoxylin and eosin stained; scale bar indicates 100 μ m. E) Immunohistochemical staining showing variable Ki67 expression in $\leq 20\%$ of spindle cells, streptavidin-biotin complex method–diaminobenzidine tetrahydrochloride; scale bar indicates 100 μ m. F) Immunohistochemical staining showing nuclear expression of latent nuclear antigen 1 in $\approx 50\%$ – 60% of spindle cells, streptavidin-biotin complex method–diaminobenzidine tetrahydrochloride; scale bar indicates 50 μ m.

antibodies against KSHV latent nuclear antigen (Figure 1, panel F). These findings were compatible with Kaposi sarcoma. A pan herpesvirus PCR amplified DNA fragments in all tested samples (blood, swabs, pathologic tissues of upper and lower lips) that contained identical sequences of a novel herpesvirus (CbGHV1). This virus is most closely related to retroperitoneal fibromatosis herpesvirus from *Macaca nemestrina* (the pig-tailed macaque) (Figure 2, panel A) and was also identified in a sibling of the animal we studied (15).

We found high viral loads in the tumorous masses of the oral cavity, in swabs from cut surfaces of mucosal masses, and in tumorous lesions on the inner upper and lower lips, as well as in nodules at the gingival margin (Table 1). A lower viral load was detected in blood and was identical to that measured in the blood of a healthy offspring of the animal (Table 1). Viral load in all remaining organs was in the range of that measured for blood, potentially because of circulation of positive blood cells through these organs. Moreover, mucosal, anal, and fluid swab samples were clearly positive for viral genomes and the high viral load in the mucosa of the eye and in the lacrimal glands might be explained by Kaposi sarcoma in unusual locations (16). Finally, CbGHV1-negative samples

were not available for calibration by PCR; their inclusion might have altered overall, but not relative, CbGHV1 genome copies measured.

Serologic analysis showed that the guereza had antibodies against lymphocryptovirus, cytomegalovirus, and simian foamy virus but, somewhat counter intuitively, not against rhesus rhadinovirus (RRV) (Table 2). In contrast, serum from the animal was reactive against KSHV antigen in an ELISA (Figure 2, panel B) and an immunofluorescence-based assay (data not shown). Finally, we did not detect antibodies reactive against KSHV in a CbGHV1-positive healthy offspring, potentially because CbGHV1 antibody levels were higher in diseased compared with healthy animals.

Conclusions

The animal we studied had several characteristic features of Kaposi sarcoma, including tumorous lesions in the buccal mucosa and proliferation of spindle cells, which harbored viral antigen. Although the disease symptoms did not fully match those of Kaposi sarcoma in humans (3,4), in part because of absence of initial symptoms in the lower extremities, an animal model based on CbGHV1 might still provide major insights into Kaposi sarcoma/KSHV infection of humans.

Table 1. Viral loads of CbGHV1 genomes in various organs of mantled guereza with Kaposi sarcoma*

Category	Copies of CbGHV1 DNA/ μ g total DNA \pm SD
Sampling during anesthesia	
Blood	$4 \times 10^4 \pm 8 \times 10^3$
Blood	$7 \times 10^4 \pm 2 \times 10^4$ †
Mucosal masses	
Swab specimen from cut surface	$6 \times 10^7 \pm 3 \times 10^7$
Buccal	$9 \times 10^6 \pm 1 \times 10^6$
Upper labial	$3 \times 10^7 \pm 6 \times 10^6$
Lower labial	$2 \times 10^7 \pm 3 \times 10^6$
Sampling during necropsy	
Mucosal masses	
Gingiva	$9 \times 10^5 \pm 1 \times 10^5$
Upper labial	$6 \times 10^6 \pm 1 \times 10^6$
Lower labial	$1 \times 10^7 \pm 5 \times 10^6$
Oral swab specimen	$5 \times 10^3 \pm 2 \times 10^3$ ‡ $5 \times 10^4 \pm 2 \times 10^4$ ‡
Genital swab specimen	$6 \times 10^4 \pm 1 \times 10^4$
Anal swab specimen	$3 \times 10^5 \pm 5 \times 10^4$
Lacrimal fluid swab specimen	$3 \times 10^6 \pm 6 \times 10^5$
Spleen	$5 \times 10^5 \pm 9 \times 10^4$
Kidney	$6 \times 10^3 \pm 3 \times 10^3$ ‡
Kidney	$2 \times 10^5 \pm 2 \times 10^4$ ‡
Liver	$3 \times 10^3 \pm 2 \times 10^3$
Lung	$1 \times 10^4 \pm 6 \times 10^3$
Heart	$9 \times 10^3 \pm 3 \times 10^3$
Brain	$1 \times 10^4 \pm 5 \times 10^3$

*Values are mean \pm SD for 3 independent quantitative PCRs. CbGHV1, Colobine gammaherpesvirus 1.
†Results for healthy offspring.
‡Two samples were obtained during necropsy.

CbGHV1 exhibited a higher similarity to KSHV and retroperitoneal fibromatosis herpesvirus from *M. nemestrina*, which are RV1 rhadinoviruses, when compared with RRV, a RV2 rhadinovirus. Consistent with these findings,

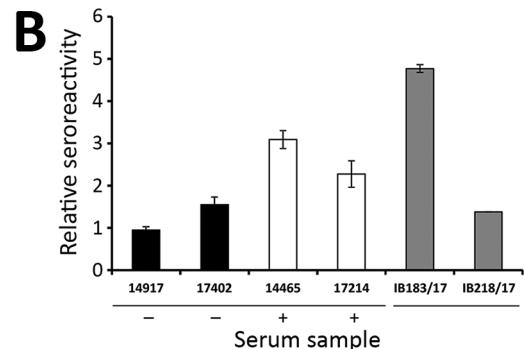
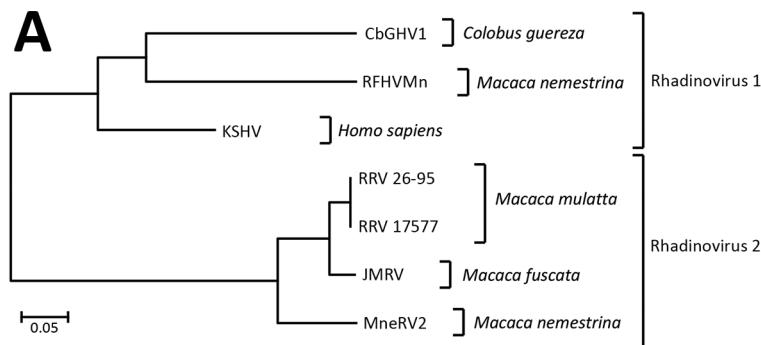


Figure 2. Analysis of CbGHV1 and seroreactivity in mantled guereza with Kaposi sarcoma. A) Phylogenetic analysis of partial sequences of the polymerase gene. Analysis was performed by using the neighbor-joining method. The distance between CbGHV1 and selected viruses was analyzed by using the maximum composite-likelihood method and MEGA6 (<https://www.megasoftware.net>). The PCR sequence of CbGHV1 was compared with KSHV (GenBank accession no. NC_009333.1); RFHVMn (KF703446.1); RRV 26–95 (AF210726.1); RRV 17577 (NC_003401.1); JMRV (AY528864.1); and MneRV2 (KP265674.2). Scale bar indicates nucleotide substitutions per site. B) Antibodies from mantled guereza with Kaposi sarcoma showing cross-reactivity against KSHV. Reactivities of KSHV antibody-positive human serum samples (14465 and 17214), KSHV antibody-negative human serum samples (14917 and 17402), and serum sample from the Kaposi sarcoma-affected mantled guereza (IB183/17) and its healthy offspring (IB218/17) were analyzed by ELISA. Relative reactivities of serum samples with KSHV-positive and KSHV-negative cell lysates are shown. The sum of relative errors is used as an error estimate for the ratio and is indicated by error bars (mean \pm half error). Reactivity of human serum samples against KSHV is indicated. CbGHV1, Colobine gammaherpesvirus 1; JMRV, Japanese macaque rhadinovirus; KSHV, Kaposi's sarcoma herpesvirus; MneRV2, *Macaca nemestrina* rhadinovirus 2; RFHVMn, retroperitoneal fibromatosis-associated herpesvirus *M. nemestrina*; RRV, rhesus rhadinovirus; –, negative; +, positive.

Table 2. Antibodies against selected viral antigens in mantled guereza with Kaposi sarcoma*

Antigen source	Test result	
	Monkey with Kaposi sarcoma	Healthy offspring
Herpes simplex viruses	–	–
Simian immunodeficiency virus	–	–
Simian retrovirus	–	–
Simian T-cell leukemia virus	–	–
Measles virus	–	–
Rhesus rhabdovirus	–	–
Lymphocryptovirus	+	+
Cytomegalovirus	+	+
Simian foamy virus	+	–

*–, negative; +, positive.

serum from the guereza cross-reacted with KSHV but not RRV. However, we cannot exclude that assay specificity was moderate and confirmation with independent tests is pending. Apart from the animal having Kaposi sarcoma, 4 genetically related animals were also PCR-positive for CbGHV1, raising questions regarding the route of transmission. We detected high copy numbers of the viral genome in swab specimens of the oral cavity and the anogenital mucosa, suggesting that transmission might occur by close contact, including sex, the route of KSHV transmission between humans (3,4).

CbGHV1 was most likely involved in tumorigenesis because high numbers of the viral genome were found within tumorous tissues. Moreover, viral antigen was detected in spindle cells. However, it was unclear that infection by CbGHV1 was sufficient to induce Kaposi sarcoma. A link between immunosuppression and Kaposi sarcoma has been established for human patients and cannot be excluded for

CbGHV1/NHP (3,4). A younger male sibling of the guereza analyzed in this study was PCR positive for CbGHV1 and showed development of primary effusion lymphoma, another disease caused by KSHV, without evidence for immunosuppression (15). Thus, a genetic component might contribute to disease development. Finally, 1 offspring of the animal infected with Kaposi sarcoma and 2 genetically related animals were also CbGHV1-positive but healthy; it remains to be examined whether they will show development of disease in the future.

We report a case of spontaneous Kaposi sarcoma in an NHP. Our findings might aid the development of an NHP model for KSHV/Kaposi sarcoma in humans. For development of this model, it is critical to isolate CbGHV1; those efforts are under way.

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About the Author

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Appendix

Methods

Anesthesia and Biopsies

General anesthesia was performed by using 0.7 mg/kg xylazine (2% Xylazin; Serumwerk Bernburg AG, <http://www.serumwerk.com>) and 10 mg/kg ketamine (Narketan [100 mg/mL]; Vétoquinol GmbH, <https://www.vetoquinol.de>) intramuscularly and was maintained with isoflurane (isoflurane CP [1 mL/mL]; CP-Pharma Handelsgesellschaft mbH, <https://www.cp-pharma.de>). Local vasoconstriction in the mucosa of the oral cavity was achieved by subcutaneous administration of ultracain plus adrenalin (Ultracain D-S [2 mL]; Sanofi-Aventis Deutschland GmbH, <https://www.sanofi.deGmbH>) at a ratio of 1:200,000.

Biopsy specimens from different tumorous masses were obtained and fixed in 10% buffered formaldehyde for routine histologic examination. In addition, serum samples, blood (in EDTA), and aerobic, anaerobic and native swab specimens from the cut surface of the masses, as well as tumorous tissue of the buccal area (upper lip and lower lip) were obtained for viral testing that included a pan herpesvirus nested PCR. Biopsy sites were closed intracutaneously by using a resorbable intracutaneous suture. The animal was treated with 0.2 mg/kg meloxicam (Metacam [5 mg/mL]; Boehringer Ingelheim Vetmedica GmbH, <https://www.bi-vetmedica.com>) and 10 mg/kg long-acting amoxicillin (Duphamox LA [150 mg/mL]; Zoetis Deutschland GmbH, <https://www.zoetis.de>) intramuscularly.

Anesthesia was partly antagonized by administration of atipamezol (Nosedorm [5 mg/mL]; Alfavet Tierarzneimittel GmbH, <https://www.alfavet.de/startseite.html>). After recovery from anesthesia, drug therapy was supplemented orally with 20 mg omeprazole, 1×/d (Antra Mups [20 mg]; AstraZeneca GmbH, <https://www.astrazeneca.de>), 0.2 mg/kg meloxicam (Metacam [1.5 mg/mL]; Boehringer Ingelheim Vetmedica GmbH), and 1 dose of Zylexis (Zoetis Deutschland GmbH) on days 0, 2, and 7 after surgery.

Immunohistochemical Analysis

Immunohistochemical analysis was performed on paraffin-embedded sections by using the following primary antibodies: Ki67 antibody (monoclonal mouse antihuman Ki67 antigen, clone MIB-1 [1:50 dilution]; DakoCytomation GmbH, <https://www.agilent.com>) CD31 antibody (monoclonal mouse antihuman endothelial cell antibody, clone JC70A [1:10 dilution]; DakoCytomation GmbH), von Willebrand factor antibody (monoclonal mouse antihuman von Willebrand factor antibody, clone F8/86 [1:25 dilution]; DakoCytomation GmbH), and antihuman herpesvirus 8 antibody (monoclonal rat antihuman herpesvirus 8 HHV8 antibody monoclonal, clone LN35 [1:10 dilution]; Abcam plc, <https://www.abcamplc.com>). Immunohistochemical staining was performed by using an automated immunostaining system (Discovery XT; Roche Diagnostics GmbH, Mannheim, Germany), the streptavidin-biotin complex method, diaminobenzidine tetrahydrochloride for signal detection (DAB Map Kit, Roche Diagnostics GmbH, <https://www.roche.com>), and appropriate positive and negative controls.

Extraction of Total DNA from Samples

Total DNA was isolated from the indicated tissue samples by using the First-DNA All-Tissue Kit (GEN-IAL GmbH, <https://www.gen-ial.de>) according to the manufacturer's instructions. Concentration of extracted DNA was quantified by means of optical density (OD) measurements, and total DNA was adjusted to a concentration of 0.1 µg/µL. Subsequently, all DNA concentrations were verified by 3 OD measurements.

Qualitative PCR and Sequencing

Viral sequences were amplified by PCR from total DNA isolated from blood (in EDTA), a swab specimen obtained from cut surface of the masses, and tumorous tissues of the buccal area, upper lip, and lower lip. PCR amplification was performed by using previously reported pan herpesvirus PCR nested primer sets (1). The final volume of the PCR was 25 µL and contained the following components: 0.5 mmol/L MgCl₂, 2.5% (vol/vol) dimethylsulfoxide, 0.2 mmol/L deoxynucleoside triphosphates, 1.2 µmol/L of each primer, 1× PCR buffer, and 0.25 µmol/L HotStarTaq DNA Polymerase (QIAGEN, <https://www.qiagen.com>). For amplification of the target sequences, samples were first heated to 95°C for 4.5 min; 55 cycles were then run with 1 cycle consisting of melting (20 s at 95°C), annealing (30 s at 46°C or 20 s at 46°C for nested PCR amplification), and extension (30 s at 72°C or 20 s at 72°C for nested PCR amplification).

Samples were then incubated for 10 min at 72°C. PCR products were extracted from agarose gels after electrophoresis by using a commercial kit (NucleoSpin Gel and PCR Clean-Up; Macherey-Nagel, <https://www.mn-net.com>) according to the manufacturer's instructions and sequenced by using Sanger sequencing.

Quantitative PCR

For quantitative analysis of viral loads, primers and probes were designed that were specific for the polymerase gene. A GenScript (<https://www.genscript.com>) real-time PCR (TaqMan) primer design was used as a tool for design of the following oligonucleotides: sense primer: 5'-CCGAGACAGTAACCCTCCAA-3', antisense primer: 5'-TTAGCAGGCAGGCTAAGTGT-3', and probe of antisense polarity: 5'-FAM-TGGCTTCCACGAAGACCTGTGACT-BHQ-1-3' (Microsynth, <https://www.microsynth.ch>). Two microliters of DNA extractions were used for quantitative PCR analysis by using Rotor-Gene Q (QIAGEN). Amplifications were conducted in duplicate and PCRs were repeated 3 times. For PCR, samples were first heated to 95°C for 15 min; 45 cycles were then run with each cycle consisting of melting (15 s at 95°C) annealing and extension (1 min at 60°C). The total volume of the reaction mixture was 25 µL and contained the following components: 3 mmol/L MgCl₂, 0.4 mmol/L deoxynucleoside triphosphates, 0.266 µmol/L probe, 0.6 µmol/L (each) sense and antisense primers, 1× PCR buffer, and 0.25 µL HotStarTaq DNA Polymerase (QIAGEN).

Chip-Based Technology for Detection of Antibodies

For simultaneous detection of serum antibodies to herpes simplex viruses, simian immunodeficiency virus, simian retrovirus of type D, simian T-cell leukemia virus, measles virus, rhesus rhadinovirus, lymphocryptovirus, cytomegalovirus, and simian foamy virus, we used a commercial microarray multiplexing technology (Simian Panel E Kit; Intuitive Biosciences, <http://intuitivebio.com>). All steps were performed according to the manufacturer's instructions. Subsequently, microarrays were scanned and analyzed by using the image capture and analysis system AQ 1000 (Intuitive Biosciences).

Preparation of Antigens and Kaposi's Sarcoma Herpesvirus ELISA

ELISA coating material was prepared by induction of either empty iSLK cells or iSLK cells harboring Kaposi's sarcoma herpesvirus BAC16 with 2.5 mmol/L sodium butyrate and 1 µg/mL doxycycline for 2 days. Cells were then harvested by brief trypsinization and collected by centrifugation. Pelleted cells and debris were lysed in phosphate-buffered saline (PBS) containing

1% NP40 for 1 h on ice. The lysate was clarified by centrifugation for 2 h at 4,200 rpm in a TX-1000 swing bucket rotor (ThermoFisher Scientific, <https://www.thermofisher.com>) in 50-mL tubes, adjusted to a concentration of 0.7 mg/mL, as measured by using the Bradford assay, aliquoted, and stored at -80°C . A total of 50 μL of this material per well was used for coating ELISA plates overnight at 4°C . Before use, plates were washed 3 times (5 min/wash) with 200 μL PBS containing 0.5% Tween 20 (PBS-T) per well. The wells were blocked with 300 μL blocking buffer (10% fetal calf serum in PBS-T) for 2 h at 37°C . Serum samples were diluted 1:40 in blocking buffer, and 50 μL were added per well in duplicates after removal of the blocking buffer, followed by incubation for 2 h at 37°C . After 3 washes with 200 μL PBS-T, 100 μL of goat antihuman IgG (heavy + light chain) horseradish peroxidase conjugate (Southern Biotech, <https://www.southernbiotech.com>) at a 1:12,000 dilution in blocking buffer was added, and plates were incubated for 1 h at 37°C . Plates were then washed 3 times with 200 μL PBS-T, 100 μL substrate solution (3,3',5,5' tetramethylbenzidine; Sigma-Aldrich, <https://www.sigmaaldrich.com>) was added, and incubated for 20 min at 37°C . After addition of 100 μL stop solution (1 mol/L HCl), OD was determined at 450 nm. All samples were measured in duplicate for each coating. The ratio of the ODs after background subtraction was calculated. The relative range of each duplicate was calculated and used as an error estimate. The sum of the 2 relative errors was used as an error estimate for the ratio and is represented by error bars (mean \pm half error).

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