of A3 into virions[16,34]. These residues are involved in the formation of nucleic acid–binding grooves primarily consisting of loops 1, 3, 5 and 7 near the coordinated zinc ions of A3G and A3C proteins. The A3G DPD motif for Vif interaction is immediately adjacent to the four YYFW residues (124–127) necessary for A3G incorporation into virions[16]. Therefore, we must use caution to not perturb the contributions of the neighboring YYFW residues that are necessary for A3G incorporation into virions. In contrast, the Vif-binding interfaces that we identified in A3C, A3F and A3DE are mapped to a position distal to the nucleic acid–binding pocket that is important for A3's encapsidation into virions. Therefore, during drug discovery and development, it could be advantageous to target the interaction of Vif with A3C, A3F or A3DE, particularly that with A3F, without disturbing the nucleic acid–binding capability. Taken together, these findings on the structural features of Vif-binding interfaces may aid in our understanding of Vif-A3 interactions and lead to the development of new pharmacologic anti-HIV-1 compounds that could restore the activity of the intrinsic antiviral factor in the context of HIV-1 infection.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited into the Protein Data Bank, with the accession code 3VOW.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.K., H.O., M.N., T.K., T.Y., N.W., A.S. and Y.I. performed experiments and analysis for the crystal structure determination; S.K., M.N., M.I., Y.N., T.K., Y.Y. and Y.I. performed biochemical experiments; S.K., M.N., M.I., Y.N., Y.Y., W.S. and Y.I. analyzed the biochemical data; Y.I. directed the project; S.K., H.O. and Y.I. wrote the manuscript with all authors' help.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plasmids and antibodies. To construct a plasmid to express A3C in bacteria, a DNA fragment (573 base pairs) was inserted into pET41a(+) (Novagen), which has an N-terminal GST tag and an enterokinase cleavage site. This construct was designated pET41 GST-A3C. A3 expression plasmids for mammalian cells were constructed by replacing the A3G gene of pcDNA A3G (Myc-His)[35] with the cDNA fragments of the A3 derived from pCAGGS APOBEC3 (ref. 36). Substitutions of the A3 residues were introduced into the A3 expression plasmids as previously described[25]. pcDNA-HVif SLQ→AAA was generated by site-directed mutagenesis from pcDNA-HVif[37]. For all mutants, the sequences of both the insert and the boundary regions were verified by DNA sequencing. The pNL4-3 WT and pNL4-3*vif*(−) plasmids were described previously[25]. Anti-p24 (CA) rabbit serum (4250) and a peptide antibody (C-17 rabbit serum; 10082) for human A3G were obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, US National Institutes of Health, Germantown, Maryland, USA. Anti-His mAb (D291-3) and anti-His rabbit serum (PM032) (Medical & Biological Laboratories Co.) and anti-Vif mAb (ab66643) and anti–β-tubulin rabbit polyclonal antibody (ab6046; Abcam) were purchased.

Protein expression and purification. Rosetta2(DE3)pLysS bacterial cells (Novagen) transformed with pET41 GST-A3C, were grown at 37 °C in Luria-Bertani medium containing 25 μg ml^{-1} of kanamycin and 34 μg ml^{-1} of chloramphenicol until reaching an optical density at 600 nm (OD_{600}) of 0.6. After the addition of 1 mM IPTG and 1 μM $ZnSO_4$, the cells were further incubated to express the GST-A3C at 20 °C for 20 h. The bacterial pellets were collected by centrifugation and resuspended with a lysis buffer (1,000 mM NaCl, 10 mM $CaCl_2$, 1 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol (2-ME) and 25 mM HEPES, pH 7.0). The lysed cells were disrupted by sonication and French press and then subjected to centrifugation and filtration. The supernatant was applied to a glutathione Sepharose 4 FF column (GE Healthcare) for affinity purification. The bound GST-A3C was eluted by an elution buffer (500 mM NaCl, 10 mM $CaCl_2$, 10% glycerol, 5 mM 2-ME, 40 mM reduced L-glutathione and 50 mM Tris HCl, pH 8.0). The eluate was digested by recombinant enterokinase (Novagen) in a cleavage buffer (350 mM NaCl, 12 mM $CaCl_2$, 2% glycerol, 1 mM 2-ME, 1% Triton X-100 and 30 mM Tris HCl, pH 7.4) at 20 °C overnight. The digested A3C was purified by using SPXL cation-exchange chromatography (GE Healthcare) with IEX start buffer (10% glycerol, 5 mM 2-ME and 50 mM Tris HCl, pH 8.0) and IEX elution buffer (IEX start buffer containing 1,000 mM NaCl and 200 mM L-arginine hydrochloride (L-Arg HCl)). A3C was further purified by using Superdex-75 gel filtration chromatography (GE Healthcare).

Protein crystallization. The A3C was dialyzed with crystallization buffer (50 mM NaCl, 300 mM L-Arg HCl, 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 10 mM HEPES, pH 8.0) and then concentrated to approximately 10 mg ml^{-1} by using Amicon Ultra-0.5 ml (Millipore). The initial crystallization screening was performed at 20 °C by using a JCSG-*plus* Screen solution (Molecular Dimensions) containing 300 mM L-Arg HCl by the hanging-drop vapor-diffusion method. A crystal formed in a condition containing 85 mM bicine (pH 9.0), 17% PEG6000 and 300 mM L-Arg HCl. A better-shaped crystal was grown at 20 °C with 300 μl of reservoir solution (14% PEG6000, 300 mM L-Arg HCl and 85 mM bicine, pH 9.0) by the hanging-drop vapor-diffusion method.

Data collection and processing. The crystal was soaked in a cryoprotectant solution (30% PEG6000, 300 mM L-Arg HCl and 85 mM bicine, pH 9.0) for a few seconds and flash cooled in nitrogen gas. Diffraction data sets were collected at 95 K by using synchrotron radiation (λ = 0.98 Å) on beamline BL-17A at the Photon Factory, KEK (Tsukuba, Japan). The data sets were indexed, integrated and scaled using the HKL2000 program package[38].

Analysis of structure data and construction of structure models. The structure was determined by the molecular replacement method, starting with the A3G 191–384-2K3A crystal structure (PDB 3IR2)[21] by using the program MolRep[39] and was manually built with the program COOT[40]. The structure refinement was performed by using REFMAC5 (ref. 41). The refinement statistics data are summarized in **Table 1**. Structure models of the A3F and DE CTDs were constructed on the basis of our A3C crystal structure by using Discovery Studio 3.0 (Accelrys).

Assays for Vif-dependent degradation of A3. A3C, A3F, A3DE or the mutant A3 expression plasmids (2 μg) and pcDNA-HVif or pcDNA 3.1 (−) control (that is, empty) vector (4 μg) were cotransfected into human embryonic kidney cells (293T) in 6-well plates by using FuGENE HD (Roche). At 48 h after transfection, cell lysates were prepared with Laemmli buffer (Bio-Rad) containing 2.5% 2-ME. Cell lysates were subjected to SDS-PAGE, and the proteins were transferred to Immobilon-P membranes (Millipore). The membranes were first incubated with appropriate antibodies as specified and were then incubated with horseradish peroxidase–conjugated secondary antibodies (Pierce). Protein bands were visualized by enhanced chemiluminescence by using SuperSignal West Dura (Pierce) and analyzed using ImageQuant TL (GE Healthcare).

Coimmunoprecipitation assays. To assess the Vif-A3 interaction *in vivo*, a coimmunoprecipitation assay was performed. Briefly, 293T cells were cotransfected with an A3 plasmid containing a lysine-free Myc-His tag and with pcDNA-HVif SLQ→AAA. At 48 h after transfection, the cells were harvested and then lysed in lysis buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 μg ml^{-1} of RNase A in PBS) plus a protease-inhibitor cocktail (Sigma). Protein complexes were immunoprecipitated with anti-His rabbit serum and Protein G-Dynabeads (Invitrogen) at 4 °C. The beads were washed with lysis buffer and then analyzed with anti-His mAb and anti-Vif mAb for A3 protein and Vif, respectively.

Infectivity assays with LuSIV cells. Virus production and analysis of virus infectivity were performed as reported previously[25]. Briefly, to obtain virus particles, HeLa cells were cotransfected with 4 μg of pNL4-3 WT or pNL4-3*vif*(−) plus 2 μg of pcDNA-A3 or pcDNA 3.1 (−) (vector control). Virus infectivity was determined by single-cycle replication assays with LuSIV cells[42], obtained from the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, US National Institutes of Health, Germantown, Maryland, USA (originally from J.W. Roos and J.E. Clements). Infectivity was calculated by normalizing for the amount of input CA, determined by p24 antigen ELISA (ZeptoMetrix).

Incorporation of A3 proteins into virions. A3 incorporation was analyzed as previously described[25].

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Seroprevalence of Kaposi's Sarcoma-Associated Herpesvirus Among Men Who Have Sex With Men in Japan

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Kaposi's sarcoma-associated herpesvirus (KSHV), the etiologic agent of Kaposi's sarcoma, causes malignancies frequently in patients with acquired immunodeficiency syndrome. In the United States and Europe, KSHV infection is common among men who have sex with men. However, the seroprevalence of KSHV among men who have sex with men in Japan is unknown. In the present study, the seroprevalence of KSHV was investigated among 230 men who have sex with men and 400 age- and area of residence-matched men (controls) using a mixed-antigen (KSHV-encoded K8.1, open reading frame 59, 65, and 73 proteins) enzyme-linked immunosorbent assay and an immunofluorescence assay. Among the Japanese men who have sex with men, serological assays revealed that 27 (11.7%) were seropositive for KSHV; 20 (5%) of the men in the control group were also KSHV seropositive. The seroprevalence of KSHV among men who have sex with men was significantly higher than in the control group (odds ratio = 2.52, 95% confidence intervals = 1.38–4.62, $P = 0.0019$, Chi-square test). Infection with the human immunodeficiency virus, *Treponema pallidum*, or hepatitis B and C virus did not correlate with KSHV infection. Furthermore, the association of KSHV seropositivity with specific sexual activities was not statistically significant. In conclusion, a higher KSHV seroprevalence was found among Japanese men who have sex with men than among the controls, suggesting that the circulation of KSHV infection is more efficient among men who have sex with men in Japan than among men who do not engage in such sexual activities. ***J. Med. Virol. 85:1046–1052, 2013.*** © 2013 Wiley Periodicals, Inc.

KEY WORDS: KSHV; seroprevalence; men who have sex with men

INTRODUCTION

Kaposi's sarcoma (KS) is a malignancy observed frequently in patients with acquired immunodeficiency syndrome (AIDS). KS occurs not only in human immunodeficiency virus 1 (HIV-1)-positive men who have sex with men, but also in immunocompromised hosts like transplant patients, elderly people in the Mediterranean region, and young African patients [Antman and Chang, 2000]. Kaposi's sarcoma-associated herpesvirus (KSHV) has been detected in all cases of KS, and the serum of KS patients is positive for anti-KSHV antibodies [Antman and Chang, 2000; Ganem, 2005]. Thus, it is clear that KSHV is associated with the pathogenesis of KS, but its infection route and mechanism remain unknown. Among the general

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population, a high seroprevalence of KSHV has been shown in African countries; a medium seroprevalence in countries around the Mediterranean Sea; and a low seroprevalence in other regions, such as North America, Europe, and Asia, suggesting that the KSHV infections are spreading globally [Ganem, 2005]. Although serum antibodies to KSHV are detected in healthy individuals at various rates around the world, including 1.4% in the general Japanese population [Katano et al., 2000], they have been detected more frequently in men who have sex with men than in the general population in the United States and other countries. In previous studies, the seropositivity of KSHV in men who have sex with men ranged from 8% to 24% [Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007]. Furthermore, there is a higher rate of KSHV seropositivity (i.e., >50%) in men who have sex with men and who are infected with HIV-1 [Katano et al., 2000; Casper et al., 2002]. These studies have argued that KSHV infection spreads effectively among men who have sex with men.

In Japan, the incidence of AIDS-KS has been increasing for several years. KS was found in 2.5% of AIDS patients in 1998, and increased to 5.6% in 2008. Similarly, the prevalence of individuals infected with HIV-1 has been increasing, with 70% of the total affected Japanese population being comprised of men who have sex with men (AIDS Surveillance Committee 2011, http://api-net.jfap.or.jp/status/index.html, Japanese). An earlier study reported that 60% of Japanese men who have sex with men infected with HIV-1 were also seropositive for KSHV [Katano et al., 2000]. However, the incidence of KSHV seropositivity among the total population of Japanese men who have sex with men is unknown. Despite the 1997 introduction of highly active antiretroviral therapy (HAART) in Japan, the number of KS cases has not decreased, due to the increasing number of men who have sex with men infected with HIV-1. In the present study, the seroprevalence of KSHV was measured and compared between Japanese men who have sex with men and age- and area of residence-matched control men; the investigation was conducted using enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays (IFAs).

MATERIALS AND METHODS

Study Subjects

The study protocol was approved by the Institutional Review Board of the National Institute of Infectious Diseases (Approval Nos. 228 and 303). Sera were obtained during KSHV testing from participants at a free and anonymous HIV-1 test clinic for men who have sex with men. All participants in this study were also participants in the 2011 annual Nagoya Lesbian & Gay Revolution festival, one of the largest annual events for Japanese sexual minorities, held on June 4–5, 2011. The HIV-1 test was organized especially for the participants of the festival at a nearby public health center. A total of 257 individuals visited the public health center for the HIV-1 test; 237 agreed to provide informed consent and participate in the study. All participants completed questionnaires, including data on age, gender, area of residence, and sexual behavior. For the purposes of this study, men who have sex with men were defined as men who have insertive anal or oral sex with other men. Individuals who practiced both homosexual and heterosexual activities were also classified as men who have sex with men. Seven participants were excluded from the analysis: four were women, and three were men who described themselves as heterosexual in the questionnaire. Thus, 230 men who have sex with men were included in the study (Fig. 1).

Sera from 400 age-, gender-, and area of residence-matched individuals were collected as controls (Table I). The control sera were obtained from the World Health Organization and the National Serum Reference Bank/Tokyo, the National Institute of Infectious Diseases (http://idsc.nih.go.jp/yosoku/index-E.html). These sera were collected from healthy donors across all districts of Japan and across all age groups in order to survey the prevalence of various infectious diseases. Blood samples were collected in serum-separating tubes from individuals who visited public health centers for medical checks between 2008 and 2010. Collected sera were frozen, shipped to the serum bank, and stored at −80°C until use. There is no information regarding the sexual orientation of the control sera donors.

KSHV Serology

Serum KSHV antibodies were detected using both mixed-antigen ELISAs and IFAs, with a positive

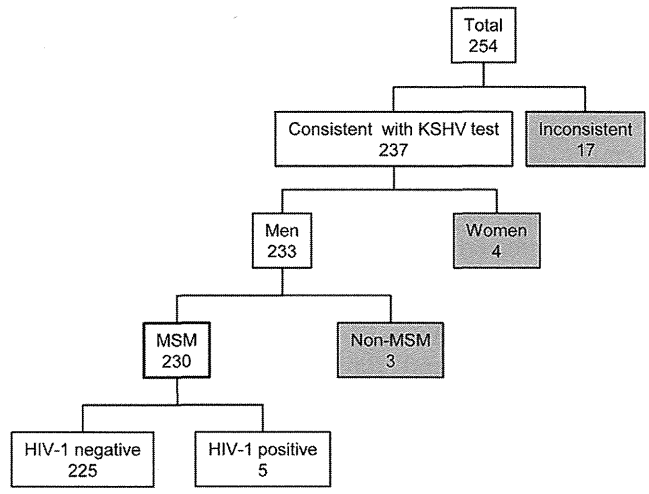

Fig. 1. Study flow diagram. Of the 257 individuals attending the free and anonymous HIV-1 test clinic, 237 agreed to participate in the study. According to the participants' responses to a questionnaire, three men who described themselves as heterosexuals and four women were excluded. Thus, 230 men who have sex with men were enrolled in the study. Five of them were HIV-1-positive.

TABLE I. Kaposi's Sarcoma-Associated Herpesvirus Seropositivity Among Men Who Have Sex With Men and Controls

	Men who have sex with men[a]	Control[a]	OR	(95% CI)	P^*
Total	27/230 (11.70%)	20/400 (5.00%)	2.52	1.38–4.61	0.003
ELISA	6/230 (2.61%)	2/400 (0.50%)	5.33	1.07–26.63	0.057**
IFA	26/230 (11.3%)	18/400 (4.50%)	2.70	1.45–5.05	0.001
Both	5/230 (2.17%)	0/400 (0.00%)	—		0.013**
Age					
18–29	5/75 (6.67%)	8/150 (5.33%)	1.23	0.39–3.90	0.957
30–39	11/81 (13.58%)	9/150 (6.00%)	2.46	0.97–6.22	0.087
40–60	6/46 (13.04%)	3/100 (3.00%)	4.85	1.16–20.35	0.048**
No answer	5/25 (20.00%)	—	—	—	—
Area					
Chubu	22/200 (11.00%)	16/319 (5.02%)	2.34	1.20–4.57	0.018
Other	5/30 (16.67%)	4/81 (4.94%)	3.85	0.96–15.46	0.105**

KSHV, Kaposi's sarcoma-associated herpesvirus; ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence assay; OR, odds ratio; CI, confidence interval.
[a]n/N (%): where n is the number KSHV seropositives, N is the total number of participants, and (%) is the percent of KSHV seropositive individuals in each category.
*Chi-square test for comparison of KSHV positivity between men who have sex with men and controls.
**Chi-square test with Yates correction was used because of sparse data.

result from either test indicating a positive serum sample. The mixed-antigen ELISA and IFA were performed as reported previously [Katano et al., 2000]. All of the serum samples were heat-incubated at 55°C for 30 min to inactivate any viruses in the serum. Mixed antigens, including K8.1 and open reading frames 59, 65, and 73 proteins, were employed as the immunogens in the ELISA. These proteins were identified as antigenic proteins encoded by KSHV using an expression library-based analysis [Katano et al., 2000]. These recombinant proteins were produced as glutathione *S*-transferase fusion proteins in *Escherichia coli*, as described previously [Smith and Johnson, 1988]. The cut-off value for the mixed-antigen ELISA was determined as the mean value plus 5× SD for 43 normal serum samples. The ELISA was validated by 100% (24/24) positivity in KS patients and 1.4% (14/1,004) in the general Japanese population [Katano et al., 2000]. Sera, diluted at 1:100, were used in the assay and all positive sera were tested in duplicate to confirm their positivity.

In the IFA, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced TY-1 cells, a KSHV-infected cell line, were initially used as antigen cells. Positive sera were then examined in TPA-induced BCBL-1, a KSHV-infected PEL cell line, BJAB, a KSHV-negative B-cell line, and Raji, a KSHV-negative, EBV-positive B-cell line [Renne et al., 1996; Katano et al., 1999]. Sera, positive in BCBL-1 and TY-1 but negative in BJAB and Raji cells, were categorized as positive.

Human Immunodeficiency Virus, *Treponema pallidum*, and Hepatitis B (HBV) and C (HCV) Virus Infections

Serum HIV-1 RNA was measured by reverse transcription-polymerase chain reactions (COBAS AmpliPrep/COBAS TaqMan HIV-1 Test; Roche Diagnostics, Boehringer Mannheim, Germany). The presence of *T. pallidum* (TP) infection was determined using a Latex suspension (a rapid plasma regain, Sekisui Medical, Tokyo, Japan). HBV and HCV antigens were identified using Architect HBsAg QT and HCV (Abbott, Abbott Park, IL).

Statistical Analysis

Chi-square tests, with Yates correction, were used to compare KSHV seropositivity between men who have sex with men and controls. A multivariable logistic regression analysis, with a forced entry method, was performed to determine the independent role of the variables (answers in the participants' questionnaires). All of the statistical analyses were conducted using SPSS (IBM, Armonk, NY).

RESULTS

The median ages (mean, range) of the men who have sex with men and controls were 33.0 (33.1, 18–60) and 32.0 (33.4, 20–49) years, respectively. Twenty-seven (11.7%) of the 230 Japanese men who have sex with men were seropositive for KSHV, as determined by ELISA or IFA (Figs. 2 and 3, and Table I). Five serum samples were found to be positive by both ELISA and IFA, and one serum sample, positive by ELISA in the men who have sex with men group, was negative by IFA. In the control group, 20 (5%) of the 400 age- and area of residence-matched Japanese men were seropositive by ELISA or IFA; none of the ELISA-positive control sera were positive by IFA. Compared to the controls, the seroprevalence among men who have sex with men was significantly higher (odds ratio [OR] = 2.52, 95% confidence intervals (CI) = 1.38–4.61, $P = 0.003$, Chi-square test) than among the control men. In an examination of seroprevalence by age groups, 40–60 year-old men who have sex with men showed significantly higher positivity for KSHV than did the age-matched

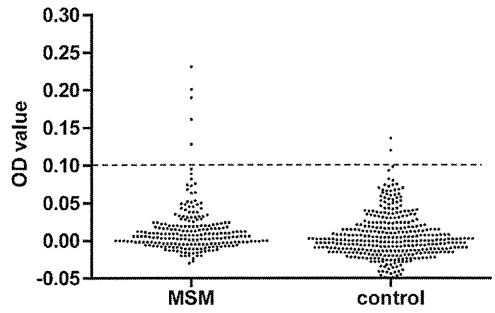

Fig. 2. Group scatter diagrams for enzyme-linked immunosorbent assay (ELISA) results. The scatter diagrams show the results of reactions of sera from men who have sex with men and controls in the mixed Kaposi's sarcoma-associated herpesvirus antigen ELISA. Optical density (OD) values were calculated as follows: (sample OD – negative control OD)/(positive control OD – negative control OD) [Katano et al., 2000]. A horizontal broken line indicates the cut off value.

controls ($P = 0.048$, Chi-square test with Yates correction), indicating a higher seroprevalence of KSHV among older men who have sex with men. Furthermore, men who have sex with men from the Chubu area showed significantly more prevalent KSHV positivity than was observed in controls ($P = 0.018$, Chi-square test), but did not in any other area. This may have been due to the small number of samples from other areas.

The presence of serum antibodies against HIV-1, TP, HBV, and HCV was also tested in all samples from men who have sex with men. Of the five men who have sex with men and who were also HIV-1-positive, KSHV antibodies were detected in one. HIV-1 positivity among KSHV seropositive men who have sex with men (1/27, 3.7%) was 1.91 (95% CI: 0.21–17.78) times higher than among KSHV seronegative men who have sex with men (4/203, 2.0%). Of the 12 test subjects with TP antibodies, three were KSHV seropositive. The rate of TP positivity among KSHV seropositive men who have sex with men (3/27, 11.1%) was 2.69 (95% CI: 0.68–10.64) times higher than that among KSHV seronegative men who have sex with men (9/203, 4.4%). However, there was no significant difference between HIV-1 or TP infection rates and KSHV seropositivity ($P = 0.14$ and 0.56, respectively, Chi-square test). Two HBV-positive and 1 HCV-positive men who have sex with men were negative for KSHV; there was no association between KSHV infection and the presence of these antibodies.

The association between the infections and sexual behaviors, determined using the participants' questionnaires, is shown in Table II. KSHV seropositivity was not correlated with the possibility of HIV-1 infection (subjects' perceived potential HIV-1 infection status) or with their sexual behaviors during the previous 6 months. There were no statistical differences between the use of condoms during anal sex and the rate of KSHV seropositivity, regardless of whether the subjects were performing or receiving

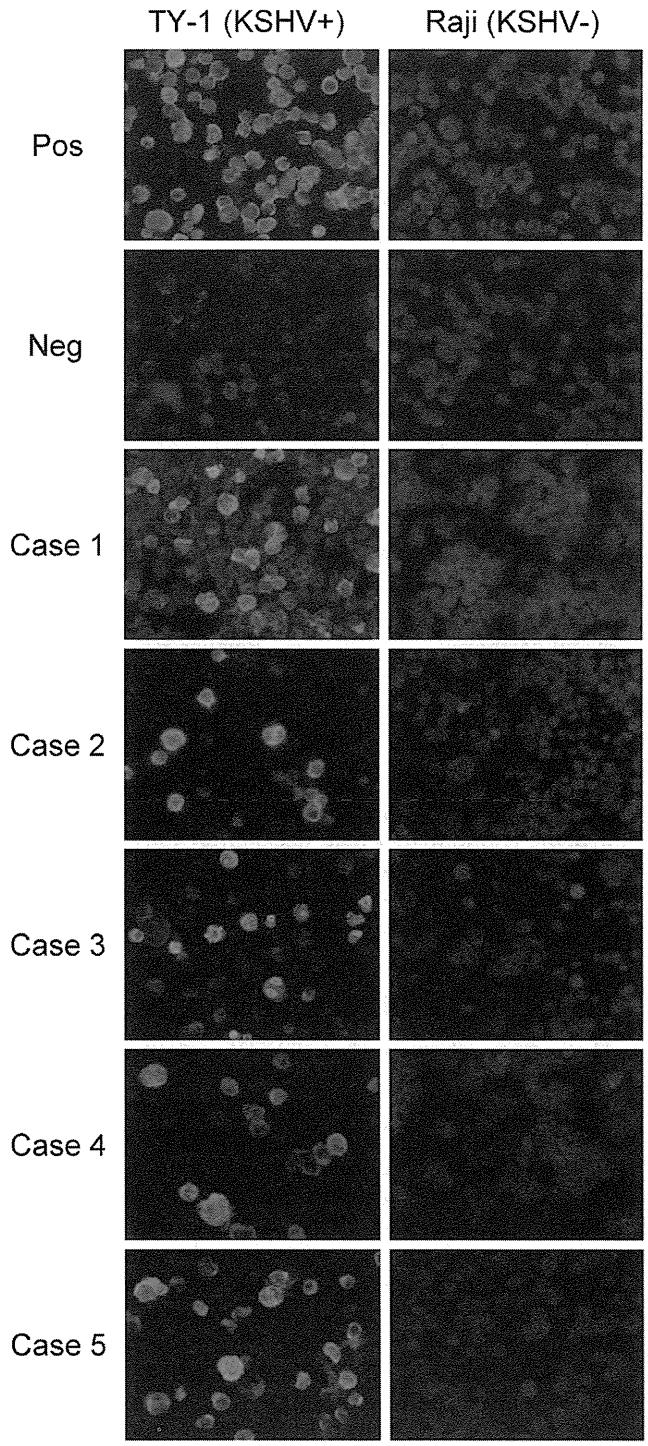

Fig. 3. Immunofluorescence images for Kaposi's sarcoma-associated herpesvirus (KSHV) immunofluorescence assay (IFA). Five positive samples from men who have sex with men are shown. The positive sera reacted with antigens in TY-1 (KSHV-positive, Epstein-Barr virus-negative lymphoma cell line), but not in Raji (KSHV-negative, Epstein-Barr virus-positive lymphoma cell line). Positive control serum from a Kaposi's sarcoma patient and negative control serum from a healthy individual are also shown.

TABLE II. Multivariate Model of Predictors of Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Seropositivity in Sexual Behaviors

Question	Answer	KSHV+	Total	%	AOR (95% CI)*	P
Sexual orientation	Homosexual	25	196	12.76	Reference	
	Bisexual	2	34	5.88	0.431 (0.088–2.117)	0.300
Possibility of HIV infection	No	14	144	9.70	Reference	
	Yes	13	86	15.10	1.867 (0.767–4.544)	0.169
Sexual behaviors in last 6 months	No	2	10	20.00	Reference	
	Yes	25	216	11.60	0.356 (0.59–2.144)	0.260
Performance of insertive anal sex with main partner	Not wearing condom	4	30	13.30	Reference	
	Sometimes wearing condom	4	39	10.30	1.077 (0.141–8.224)	0.943
	While wearing condom	6	56	10.70	0.737 (0.095–5.724)	0.771
Receipt of anal sex with main partner	Partner not wearing condom	3	29	10.30	Reference	
	Partner sometimes wearing condom	2	30	6.70	1.467(0.123–17.574)	0.762
	Partner wearing condom	8	50	16.00	3.676 (0.365–36.975)	0.269
Performance of insertive anal sex with casual partner(s)	Not wearing condom	5	20	25.00	Reference	
	Sometimes wearing condom	1	31	3.20	0.117 (0.008–1.786)	0.123
	While wearing condom	8	68	11.80	0.346 (0.049–2.419)	0.285
Receipt of anal sex with casual partner(s)	Partner not wearing condom	4	14	28.60	Reference	
	Partner sometimes wearing condom	1	31	3.20	0.093 (0.005–1.699)	0.109
	Partner wearing condom	10	48	20.80	0.737 (0.085–6.400)	0.782

*AOR, adjusted odds ratio; CI, confidence interval.

anal sex or whether the anal sex was performed with the subject's main partner or with casual partners. However, condom use was associated with decreased (0.3–0.7 times less) KSHV positivity among subjects performing or receiving anal sex with casual partners than among those who did not use condoms.

DISCUSSION

This study showed that KSHV seroprevalence in Japanese men who have sex with men is 11.7%, which is similar to the seroprevalence among a similar population of men in the USA and Europe. The higher seroprevalence of KSHV among men who have sex with men, compared with controls, suggests that the circulation of KSHV infection among Japanese men who have sex with men is more efficient than among heterosexual males, as previously reported [Goudsmit et al., 2000; Casper et al., 2002, 2006; Grulich et al., 2005; Engels et al., 2007; Giuliani et al., 2007]. Although the transmission route of KSHV remains unclear, the higher seroprevalence of KSHV between men who have sex with men than that among the general population suggests that transmission likely occurs through homosexual behaviors in non-endemic areas, such as in the USA, Europe, and Asia [Goudsmit et al., 2000; Diamond et al., 2001]. In contrast, in KSHV endemic areas, such as Africa, a high seroprevalence of KSHV has been found even among children [Bourboulia et al., 1998; Butler et al., 2009]. Since high copy numbers of KSHV have been detected in the saliva of those infected with KSHV, vertical mother-to-child transmission may occur through saliva [Pauk et al., 2000; Mbulaiteye et al., 2006]. In addition, in KSHV endemic areas, sexual transmission has not been associated with KSHV infection [Shebl et al., 2011].

Of the 230 subjects in this study, 12 (5.2%) were positive for TP, suggesting that these were individuals with high levels of sexual activity. There were no significant associations between HIV-1, HBV, HCV, or TP and KSHV infections in Japanese men who have sex with men in the present study. A previous study with a large sample size, on individuals without HIV-1 infection but at high risk for sexually transmitted infections, demonstrated that the incidence of KSHV infection was different from that for HIV-1 and other sexually transmitted infections [Giuliani et al., 2007], suggesting that the routes of KSHV transmission and the opportunity for KSHV infection are different from other infections. The present study showed that the seroprevalence of KSHV is higher than that of the aforementioned sexually transmitted diseases in Japanese men who have sex with men, implying that KSHV infection can be an early marker of sexually transmitted infections in a certain proportion of study subjects.

Japanese men who have sex with men tend to use condoms less frequently for oral sex than for anal sex [Inoue et al., 2006]. Considering that the saliva of KSHV-infected persons contains high loads of KSHV, oral sex is possibly a transmission route of KSHV [Pauk et al., 2000]. There was no statistical difference in the incidence of KSHV positivity between those who did and those who did not use condoms during anal sex with their main partners (Table II). However, in subjects performing or receiving anal sex with

casual partners, the incidence of KSHV positivity was 0.3–0.7 times less among those who used condoms, compared with those who did not use condoms (Table II); this finding suggests that the risk of KSHV infection through anal sex can be reduced by condom use.

A gold standard for KSHV serology testing does not currently exist [Corchero et al., 2001; Pellett et al., 2003]. However, a combination of ELISA and IFA has been found to be more accurate for the detection of serum KSHV antibodies than any individual method. In the present study, 5% of the control sera were positive for KSHV in ELISA or IFA. A previous study demonstrated that by ELISA, alone, 1.4% of the Japanese general population was found to be positive for the KSHV serum antibody [Katano et al., 2000]. However, the findings in the present study are not directly comparable with those in that study as different serological assays were used in the present study and the control sera was obtained predominantly from 30- to 40-year-old men, most of whom resided in the Chubu area. Data, from the current study, using a combination of ELISA and IFA suggests that the seroprevalence of KSHV antibodies among the general, Japanese population is between 2% and 5%. Although information was not available on the sexual habits of those providing the control sera, 2% of adult Japanese men are estimated to have had sex with other men [Ichikawa et al., 2011]. Thus, in the present study involving 400 control subjects, there may have been up to eight participants who have engaged in homosexual sexual activity. If eight are excluded from 380 KSHV-negative controls, the seroprevalence of KSHV among men who have sex with men (11.7%) remains statistically higher than that among controls (OR 2.47, 95% CI 1.35–4.52, $P = 0.002$, Chi-square test), suggesting that the potential inclusion of a small number of men who have sex with men in the control group did not affect the conclusions. However, a more focused investigation, examining sexual orientation-matched samples, would be required to more accurately state the KSHV positivity among men in the control group.

In conclusion, this study revealed that the seroprevalence of KSHV between Japanese men who have sex with men is 11.7%, which is higher than that among controls, suggesting that the circulation of KSHV infection among men who have sex with men in Japan is more efficient than among heterosexual males. In addition, the higher prevalence of KSHV antibodies than those for other infectious diseases that may be sexually transmitted suggests that the KSHV test may be an early maker for sexually transmitted diseases. Nonetheless, the transmission route of KSHV remains unclear. Further detailed studies on sexual behaviors and virus shedding in the saliva will be required to clarify the mechanism of KSHV infection among men who have sex with men.

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