

## SHORT REPORT

# PREVALENCE OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS INFECTION IN SEX WORKERS AND WOMEN FROM THE GENERAL POPULATION IN SPAIN

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**Transmission routes of Kaposi's sarcoma-associated herpesvirus (KSHV) in the general population are poorly understood. Whereas sexual transmission appears to be common in homosexual men, the evidence for heterosexual transmission is less convincing. In our study, prevalence of KSHV infection was examined among women in the Spanish general population and among sex workers. Subjects consisted of 100 prostitutes and 100 women randomly sampled from the general population and age-matched to the prostitutes. Women had a personal interview and gynecologic examinations in which a blood sample, cervical cells and oral cells were obtained. Peripheral blood mononuclear cells (PBMC), oral and cervical samples were tested for KSHV DNA by quantitative real-time PCR. Sera were tested for antibodies against human immunodeficiency virus (HIV) by ELISA and against KSHV by latent IFA and K8.1 ELISA. Women who were positive in either serologic assay or PCR were considered infected by KSHV. Human papillomavirus (HPV) DNA in cervical scrapes were evaluated using the Hybrid Capture System™. The study population had an average age of 30 years and were HIV-negative. Women from the general population were largely of Spanish nationality, and 61% reported lifetime monogamy. The majority of the prostitutes (76%) were immigrants, primarily from South America. Sex workers were twice as likely to be infected with KSHV than women in the general population (16% vs. 8%, prevalence odds ratio [OR] = 2.2). KSHV was more prevalent among HPV DNA-positive women (OR = 2.5) and among women with an early age at first sexual intercourse (OR = 2.7,  $p < 0.05$ ). KSHV DNA was detected by PCR in 3% of the oral cavity samples, in 2% of the cervical samples of the prostitutes and in 1% of the cervical samples of women in the general population. All PBMC samples were negative. These results suggest that in low-risk countries for KSHV, oral shedding and heterosexual contacts are potential pathways for KSHV transmission.**

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Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), has been shown to be causally associated with Kaposi's sarcoma and other diseases.<sup>1–3</sup> The natural history of KSHV and its routes of transmission are not well understood. Epidemiologic studies have shown that seroprevalence of KSHV antibodies is more than 25% in African countries, whereas in the United States and Europe is lower than 10%.<sup>4</sup> Data from Latin America are limited but suggest a wide variability within the region.<sup>5,6</sup> Routes of transmission may vary in different geographic areas either reflecting or possibly leading to variations in background prevalence. Nonsexual transmission of KSHV has been reported to be the major route of transmission in countries with high KSHV prevalence rates where infection in children is commonly reported.<sup>6–9</sup> In countries with lower background prevalence, sexual transmission, at least among homosexual men, may

be predominant. Among homosexual men, KSHV infection correlates with sexual promiscuity and history of other sexually transmitted diseases (STDs).<sup>10–12</sup> However, evidence for heterosexual transmission of KSHV is conflicting. The prevalence of KSHV is reported to be higher in heterosexual STD clinic attendees than in the general population in the United States and the United Kingdom.<sup>13,14</sup> A weak association between KSHV and number of sexual partners has been reported in a large study in South Africa.<sup>15</sup> However, in a study of KSHV risk factors in an STD clinic in London, no association was seen between KSHV and markers of sexual promiscuity in heterosexuals.<sup>12</sup> KSHV DNA sequences have been detected in the prostate,<sup>16,17</sup> semen,<sup>18–21</sup> oral cavity<sup>6,22–25</sup> and in the female genital tract.<sup>24,26,27</sup>

To further explore the heterosexual route of transmission of KSHV in a nonendemic region, we studied the serologic and molecular prevalence of KSHV in a sample of female sex workers and women from the general population in Spain.

## MATERIAL AND METHODS

Subjects were recruited in Oviedo and Barcelona, Spain, and included 100 practising prostitutes and 100 women randomly selected from the general population. Prostitutes were invited to participate at their regular visits to a specialised STD clinic. All prostitutes declared actively working as prostitutes and reported more than 100 sexual partners in the previous year. Women from the general population were extracted from a larger follow-up study that included a random sample from the electoral registry of the metropolitan area of Barcelona. Of all invited women, 50% agreed to participate ( $n = 1127$ ). Of them, 100 women matched by age to the group of prostitutes were selected for our study. After informed consent, women had a gynecologic examination that included the collection of cervical cells by means of an Ayre spatula and a cervixbrush to scrape the endocervix. Cells were

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suspended in saline medium and sent to be processed for HPV DNA detection. Oral cells were obtained using brush scrapes. A blood sample was obtained and separated by density gradient centrifugation for serologic and molecular studies. All women were interviewed by specially trained nurses. Structured questionnaires explored in detail sexual and reproductive histories and use of intravenous drugs. All protocols were cleared by the institution's ethics committees.

#### Serology: KSHV and HIV

Sera were tested for antibodies against KSHV by using 2 different serologic assays. A latent IFA measured antibodies to the latent nuclear antigen (LANA or LNA-1) encoded by orf73, while a recombinant protein ELISA measured antibodies in the lytic-phase glycoprotein K8.1 as previously described,<sup>28</sup> using a cutoff of OD 1.0. For the multivariate statistical analysis, women with a positive result to either of the studied antigens were considered seropositive. HIV infection status was determined by testing sera with a licensed commercial ELISA (Abbot Diagnostics, North Chicago, IL).

#### KSHV quantitative real-time PCR

Oral and cervical samples and peripheral blood mononuclear cells (PBMC) were tested for KSHV DNA by quantitative real-time PCR. DNA quality and cell quantitation was determined using real-time PCR for endogenous retrovirus 3 (ERV-3).<sup>29</sup> Quantitative testing for KSHV was performed in triplicate using a primer and probe set that amplifies a 176-base pair fragment of the K6 region (viral macrophage inflammatory protein  $\alpha$ ). The primers used were K6-10F 5'-CGCCTAATAGCTGCTGCTACGG-3' (nucleotides 27309–27330) and K6-10R 5'-TGCATCAGCTGCCTAACCCAG-3' (nucleotides 27159–27330). The probe sequence was p-K6-10 5'-R-CACCCACCGCCCGTCCAAATTC-Q-3' (nucleotides 27277–27298, GenBank accession number U75698). The amplification consisted of 45 cycles performed at 95°C for 15 sec, 55°C for 30 sec and 60°C for 60 sec. All PCR reactions were performed in 50  $\mu$ l. The PCR master mix contained 3 mM magnesium chloride, 0.05% EIA reagent-grade gelatin (Sigma, St. Louis, MO), 0.01% Tween 20, 200  $\mu$ M each dATP, dCTP, dGTP, 400  $\mu$ M dUTP, 1  $\mu$ M forward and reverse primers, 200 nM probe, 0.1 U/ $\mu$ l AmpErase uracil-N-glycosylase and 1.25 U/ $\mu$ l AmpliTaq Gold polymerase (Perkin-Elmer, Foster City, CA) in TaqMan buffer A. Repeatability assays, using known viral DNA input have demonstrated that as few as 3 copies of target DNA can be reliably detected per 10  $\mu$ l of input sample in 100% of experiments (data not shown). All reactions were performed using an Applied Biosystems Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA).

#### HPV DNA

The Hybrid Capture 2<sup>TM</sup> assay (Digene, Gaithersburg, MD) was used in exfoliated cervical cells to detect and quantify HPV DNA. In the assay, the cellular DNA is denatured in an alkaline solution and then hybridised with complementary RNA probes to produce DNA-RNA hybrid molecules that are then captured and quantitated using a chemoluminescence reaction system. The assay includes a cocktail probe for the detection of high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The analytical sensitivity of the assay to identify positivity was set at 1 pg/ml.

#### Statistical analysis

Logistic regression was used to estimate the prevalence odds ratios (OR) for KSHV infection and 95% confidence intervals (CI). A combined variable between study group and country of birth (general population and prostitutes; Spanish and foreigners) was used to control for the colinearity of both variables. Models were adjusted for age and by study group-country of birth. Likelihood ratio tests were used to evaluate the significance of terms in the models.

## RESULTS

The women had a mean age of 30.3 years (range 19–49). Women from the general population were largely monogamous (61%), all but 3 were of Spanish nationality and had an average age at first sexual intercourse of 22. A large majority of the prostitutes were immigrants (76%), mostly from South America. None of the women had HIV antibodies. Prostitutes reported more than 100 occasional sexual partners and an average age of sexual initiation of 16 years old. Prostitutes were more likely than women in the general population to report a medical history of sexually transmitted disease (51% vs. 10%,  $p < 0.001$ ) and having had sexual intercourse with a partner affected by an STD (27% vs. 10%,  $p = 0.012$ ).

Table I summarizes the detection of KSHV infection in the general population and in the prostitutes. Prostitutes were more likely to have evidence of KSHV infection (antibodies to KSHV LANA or K8.1 or PCR positivity) than women in the general population (16% vs. 8%,  $p = 0.08$ ). The concordance between tests was low, as expected in a low-prevalence population.

KSHV DNA was not detected in any of the 200 PBMC samples. KSHV DNA was detected by PCR in 3 oral cavity samples and 2 cervical samples from prostitutes and in 1 cervical sample from the general population (Table II). None was KSHV DNA-positive in both oral and cervical cells. Of the 6 KSHV DNA-positive samples, 5 were from women without a serologic response to KSHV. Of the samples from the oral cavity, one came from a woman who was seropositive by the K8.1 ELISA and who had an IFA titre of 1:800. This sample had the highest viral load observed in our study, 1,044,444 copies per 10<sup>6</sup> cells. The KSHV viral load in the other 5 PCR-positive samples ranged from 19–15,873 copies per million cells and was higher in the oral exfoliated cells than in the cervical samples.

To determine the relationship between sexual behavior and KSHV prevalence, several surrogates of sexual behavior were explored. Table III shows the age-adjusted OR and the age- and study group/country of birth-adjusted OR for KSHV infection. KSHV was significantly more prevalent among foreign prostitutes (OR = 2.8) and among those with an early sexual debut (OR = 2.7). This last association remained almost unchanged after adjustment for study group and country of birth, although it lost its statistical significance. KSHV was more prevalent among Spanish prostitutes and among foreign women from the general population (only 3 women) compared to women in the general population. Women with cervical HPV DNA and those who reported a medical treatment for STD had also an increased prevalence of KSHV, although none of the OR reached statistical significance.

KSHV was not related to number of regular sexual partners (1 regular partner 8.5% vs. more than 1 regular partner 14.4%,  $p$ -value 0.20) or to number of sexual partners before age 20 (1 partner 13.4% vs. more than 1 partner 10.7%,  $p$ -value 0.55).

Other variables explored were frequency and age at starting anal/oral sexual relations, smoking habits, use of drugs and regular use of condoms (data not shown). None of these variables showed any statistically significant association with KSHV infection.

TABLE I—KSHV INFECTION IN THE GENERAL POPULATION AND IN PROSTITUTES IN SPAIN

	General population positive/total	Prostitutes positive/total	$p$ -value <sup>1</sup>
K8.1	4/100	7/100	0.35
IFA	3/100	9/100	0.07
PCR	1/100	5/100	0.21
K8.1 or IFA or PCR	8/100	16/100	0.08

<sup>1</sup>Comparison between groups based on either  $\chi^2$  or Fisher test.

TABLE II – CHARACTERISTICS OF KSHV DNA-POSITIVE WOMEN

Sample	Risk group	Age	IFA titer	K 8.1 ELISA	KSHV copies per 10 <sup>6</sup> cells
Saliva	Prostitute	36	Negative	Negative	15,873
Saliva	Prostitute	34	Negative	Negative	9,615
Saliva	Prostitute	29	800	Positive	1,044,444
Cervix	Prostitute	40	Negative	Negative	15,038
Cervix	Prostitute	23	Negative	Negative	2,829
Cervix	General population	23	Negative	Negative	19

TABLE III – CHARACTERISTICS ASSOCIATED WITH KSHV INFECTION

Comparison categories	KSHV		OR <sup>1</sup> (95% CI)	OR <sup>2</sup> (95% CI)
	Negative	Positive		
Study group and country of birth				
Spanish general population	90	7	1	1
Spanish prostitutes	22	3	1.7 (0.4–7.0)	1.7 (0.4–7.0)
Foreign general population	2	1	5.9 (0.5–75.5)	5.9 (0.5–75.5)
Foreign prostitutes	62	13	2.8 (1.0–7.4)	2.8 (1.0–7.4)
Age				
<30	93	10	1	1
≥30	83	14	1.6 (0.7–3.7)	1.6 (0.7–3.9)
Age at first sexual intercourse				
>16	112	10	1	1
≤16	64	14	2.7 (1.1–6.5)	2.4 (0.9–6.2)
HPV DNA cervix				
Negative	156	19	1	1
Positive	21	5	2.5 (0.8–7.7)	1.8 (0.6–5.8)
Previous treatment of STD				
No	136	21	1	1
Yes	40	3	2.1 (0.6–7.4)	1.2 (0.3–5.2)

<sup>1</sup>OR adjusted for age.–<sup>2</sup>OR adjusted for age and study group and country of birth.

## DISCUSSION

KSHV infection was more common among foreign sex workers and among Spanish prostitutes compared to Spanish women from the general population. KSHV infection in the age-adjusted analysis was higher among women with presumable high-risk behavior for acquiring an STD (*i.e.*, early age at first sexual intercourse, HPV cervical infection). The majority of foreign prostitutes came from Latin America (Colombia, Venezuela and Brazil were the most common countries of origin). Although some communities in Brazil seem to have high prevalence rates of KSHV,<sup>6</sup> the prevalence in other communities may be low.<sup>5</sup>

The lack of association between KSHV and some parameters of high-risk sexual behaviour (number of sexual partners, frequency of oral/anal sex) may be due to the contrasting sexual behavior of both study groups under evaluation and to the low prevalence of KSHV in the general population. Since the association between KSHV infection and history of STDs or serologic evidence for HSV-2 infection has been inconsistent in previous studies of heterosexuals,<sup>12–14</sup> our observation deserves further investigation.

The lack of detection of KSHV in the PBMC DNA was not unexpected and is consistent with previous findings that KSHV detection is rare in populations at low risk for KS.<sup>2,27</sup> The detection of KSHV DNA in oral or cervical cells was observed in only 6 women, only 1 of whom was seropositive for KSHV. All but 1 were sex workers. There are several possible explanations for these discordant results. The most straightforward would be false-positive KSHV DNA results. Although possible, this explanation is unlikely because of the high specificity and sensitivity of the real-time PCR assay and the considerably lower risk of PCR contamination using this technique compared to nested PCR. The second possible explanation is that these KSHV DNA-positive, antibody-negative individuals were falsely seronegative. This is more likely given the limited sensitivity of KSHV serologic assays.<sup>28</sup> A third explanation is that these women were acutely

infected with KSHV and had not yet seroconverted. Since follow-up samples are not available, it is not possible to test this possibility. A fourth explanation is that KSHV can, like HPV, infect at a peripheral site without eliciting a detectable humoral response. This last explanation is the most controversial and has the greatest implications for KSHV biology and epidemiology. Further studies are needed to investigate this possibility.

The markers for KSHV infection we have used in our study are highly specific and sensitive. The lack of agreement between tests for KSHV infection has been previously reported to be highest in populations with low KSHV prevalence and correspondingly low antibody titre.<sup>30</sup> One explanation for the disagreement between tests is that subjects differ in their immunologic response to KSHV infection. In our analyses therefore, we classified subjects as KSHV infected if they were positive by any of the 3 very specific assays.

Our results suggest that KSHV may be transmitted by heterosexual contacts and that foreign prostitutes working now in Spain are a potential reservoir of KSHV infection.

Within Europe, Spain is a country with high HIV-prevalence rates. None of the women studied were HIV-positive, but those who work as prostitutes are at high risk of acquiring HIV. In Spain, KS in young adults has increased dramatically in the last 10 years.<sup>31</sup> The identification of sexual routes of KSHV transmission may help to reinforce the importance of safe sex practices among women who have high-risk or numerous sexual partners.

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