A population based study of herpes simplex virus 2 seroprevalence in rural Costa Rica

A C Rodríguez, P E Castle, J S Smith, C Bratti, A Hildesheim, M Schiffman, R Viscidi, R D Burk, R L Ashley, X Castellsagué, R Herrero

Objectives: To determine seroprevalence and determinants of herpes simplex virus 2 (HSV-2) seropositivity, in a random sample of a population based cohort of 10 049 women of Guanacaste, Costa Rica, using a highly sensitive and specific serological assay.

Methods: Seroprevalence was determined by a type specific HSV-2 ELISA assay in an age stratified random sample of 1100 women. Univariate and multivariate logistic regression was used to calculate odds ratios and 95% confidence intervals for risk factors of seropositivity.

Results: Overall age adjusted HSV-2 seroprevalence was 38.5% (95% CI, 37.5 to 39.5), and it was strongly associated with increasing age (p trend < 0.0001), both among monogamous women and women with multiple sexual partners. A greater number of lifetime sexual partners increased the risk of seropositivity, with a 28.2% (95% CI, 24.4 to 32.2) seroprevalence among monogamous women and 75% (95% CI, 65.6 to 83.0) seroprevalence for those with four or more partners (OR = 7.6 95% CI, 4.7 to 12.4 p trend < 0.0001). Barrier contraceptive use was negatively associated with HSV-2 seropositivity (OR 0.54, 95% CI, 0.31 to 0.94). Women with antibodies against HPV 16, 18, or 31 were 1.6 times more likely to be HSV-2 seropositive (OR 1.6, 95% CI, 1.2 to 2.1).

Conclusions: HSV-2 infection is highly endemic in Guanacaste, even among lifetime monogamous women, suggesting a role of male behaviour in the transmission of the infection. Until vaccination against HSV-2 is available, education to prevent high risk sexual behaviour and the use of condoms appear as preventive measures against HSV-2.

Methods

Study population and sample design

One sixth of 1038 censal segments (comprising 40–60 households each) of Guanacaste were randomly selected and, between June 1993 and December 1994, all resident women were invited to participate in a cohort study of the natural history of cervical neoplasia. The study enrolled 10 049 women with 93.6% participation. Details have been described elsewhere. Briefly, after signing informed consent, participating women were given a questionnaire on sociodemographic, reproductive, contraceptive, sexual, and smoking history. A pelvic examination was performed including collection of exfoliated cervical cells with a Cervex brush (Unimar, CT, USA) for conventional and liquid based cytology (ThinPrep, Cytyc Corporation, MA, USA). Additional cells were collected with a Dacron swab and stored in a DNA transport medium (Virapap, Digene Corporation) for human papillomavirus (HPV) testing. Immediately afterwards the cervix was rinsed twice with 5% acetic acid and two images of the cervix were taken (Cervigrams, National Testing Laboratories, MI, USA). A ~15 ml heparinised blood sample was drawn, and plasma aliquots were frozen at −70°C. Plasma was shipped to the United States on dry ice and stored in liquid nitrogen. Of the 10 049 women, 9967 (99.2%) donated blood and plasma aliquots were frozen at −70°C. Plasma was shipped to the United States on dry ice and stored in liquid nitrogen. Of the 10 049 women, 9967 (99.2%) donated blood and plasma aliquots were frozen at −70°C.

African American groups to 74 years, and 75 years or older. Plasma samples were shipped to the United States on dry ice and stored in liquid nitrogen. Of the 10 049 women, 9967 (99.2%) donated blood and plasma aliquots were frozen at −70°C.

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Table 1  Univariate analysis of HSV-2 antibody seroprevalence by selected reproductive and sociodemographic risk factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number*</th>
<th>% Positive</th>
<th>OR</th>
<th>95% CI</th>
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<tr>
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<td>183</td>
<td>25.1</td>
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<td>0.9 to 2.7</td>
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<td>5.4</td>
<td>3.2 to 9.1</td>
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<td>335</td>
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<td>1.7</td>
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<td>538</td>
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<td>1.3</td>
<td>0.6 to 2.9</td>
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<td>1.0 to 5.5</td>
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<td>145</td>
<td>46.9</td>
<td>1.9</td>
<td>0.8 to 4.4</td>
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<td>Trend p&lt;0.001</td>
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<td>HPV 16, 18, or 31 serological status</td>
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<td>1.4 to 2.4</td>
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<td>Oral/injectable contraceptive use</td>
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<tr>
<td>Former</td>
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<tr>
<td>Trend p&lt;0.001</td>
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</table>

*Women without sexual experience or with missing HSV-2 results are excluded.
†One missing case.
‡34 missing HPV DNA results.
were selected irrespective of sexual experience and cervical diagnosis.

**HSV-2 serological testing**

Plasma samples were shipped to Dr Rhoda Ashley’s laboratory for HSV-2 serological testing using type specific HSV-2 and HSV-1 ELISA assays (Focus Technologies, CA, USA). All testing was conducted blinded to other data. All HSV-2 positive sera were retested for confirmation with another Focus Technologies HSV-2 ELISA; 18 specimens without valid result were excluded from analyses.

As a validation of the ELISA, 50 random positive specimens and 50 negative specimens by the HSV-2 ELISA were tested with a western blot test, the current reference standard for HSV-2 serological testing. The kappa was 0.88 (95% CI 0.68 to 1.0), after exclusion of three pairs with missing results. The ELISA had a sensitivity of 100%, a specificity of 89.5%, a positive predictive value (PPV) of 87%, and a negative predictive value (NPV) of 100% compared to western blot.

**Human papillomavirus (HPV) testing**

VLP based ELISA

VLPs were prepared in Trichoplusia ni (High five) cells (Invitrogen, Carlsbad, CA, USA) from recombinant baculoviruses expressing L1 and L2 genes of HPV 16 or 31 or L1 gene alone of HPV 18 and purified by density gradient ultracentrifugation and column chromatography techniques. HPV-16, 18, and 31 specific ELISAs were performed as previously described, except for the use of an automatic plate washer (Skatron, Norway) and a MultiProbe II robotic liquid handling system (Packard Instruments, CT, USA) to dilute serum samples in 1:10 in 0.5% polyvinyl alcohol (PVA, MW 30 000–70 000, Sigma, St Louis, MO, USA) and 10 μl of the diluted serum sample was added to antigen coated plates containing 10 μl per well of 0.5% PVA.

The cut-off point for positive results was determined from the reactivity of concurrently tested plasma samples from self reported virgins. The mean and standard deviation (SD) of optical density (OD) values for controls were calculated and values greater than the mean plus 3 SD were excluded. The analysis was repeated until no further OD values could be excluded by this criterion. A positive cut-off point of 3 OD above the mean of this distribution was chosen. Using this threshold of seropositivity and three HPV types combined (HPV 16, 31), 97% of seropositive women were HPV DNA positive for either of the types and among those HPV DNA positive 78% were seropositive.

**Polymerase chain reaction (PCR)**

Details of the MY09/11 L1 consensus primer PCR with AmpliTaq Gold have been previously reported. After an aliquot of the DNA transport medium (Virapap, Digene Corporation) specimen was lysed, the DNA was precipitated by ammonium acetate/ethanol solution, by centrifugation, resuspended in 10 mM TRIS, pH 7.5, 0.1 mM EDTA, and stored frozen until used. Quality control, amplification, and thermocycling conditions were as previously described.

PCR products were analysed by gel electrophoresis and then transferred to Nylon filters. The filters were hybridised overnight with radiolabelled generic probes for HPV (HPV 11, 16, 18, 51, 73, and 81 combined) as has been described previously. Dot-blot hybridisation for HPV type specific detection (HPV types 2, 6, 11, 13, 16, 18, 26, 31–35, 39, 40, 42–45, 51–59, 61, 62, 64, 66–70, 71 (AE8), AE2 (IS39), AE9, AE10, and the combination of 2, 13, 34, 42–44, 57, 62, 64, 69, 74, 82 (W13B), and AE9) was conducted on specimens that were positive for the generic probes. Three experienced investigators interpreted each dot-blot result, and discrepancies were resolved by consensus.

**Statistical analysis**

Overall prevalence in the cohort was estimated using the direct standardisation method with the cohort and the world standard population as reference populations. Univariate and age stratified analyses (18–24, 25–34, 35–44, 55–64, 65+ years) were carried out to examine associations between HSV-2 seropositivity and the different variables. Odds ratios (ORs) with 95% exact binomial confidence intervals were calculated and Pearson χ² test (p<0.001) was used for significance (STATA 7.0, Stata Press, TX, USA). Continuous variables were categorised.

Multivariate logistic regression models, adjusting for lifetime number of sexual partners and age, were then constructed for each risk factor identified in the univariate analysis. To test for statistical significance of dose-response relations (p trend), categorical variables were treated as continuous in the logistic models. The risk factor analysis was restricted to sexually active women with valid HSV-2 results (n = 1003). In accordance with the sexual mode of transmission, seroprevalence among virgins was low (n = 80, 79 with valid HSV-2 result; 2.5%).

**RESULTS**

The mean age of the entire cohort was 41.1 years (range 18–97). Among sexually active women, 54% reported having had only one lifetime sexual partner and 76% had fewer than three partners. Elementary schooling is common (89%), but few (27%) received 9 or more years of education. Guanacaste is a rural, agricultural province with relatively good public services; 50% of the women reported to have all household amenities (refrigerator, running water, in-house toilet, electricity, television) and only 9% declared having none.

The age standardised HSV-2 antibody prevalence for the entire Guanacaste cohort was estimated to be 38.5% (95% CI, 37.5 to 39.5). The prevalence adjusted for the world standard population was 38.4% (95% CI, 38.1 to 38.7).

In univariate analysis, age and number of lifetime sexual partners were the major predictors of HSV-2 seropositivity (table 1). Seroprevalence ranged from 17.7% (95% CI, 11.8 to 25.0) among women 18–24 years old to 58.8% (95% CI, 48.3 to 68.7) among those 65 years or older with a 6.6 (95% CI, 3.7 to 11.9) fold increase in risk (p trend<0.0001). A strong trend of increasing prevalence was observed with increasing number of sexual partners (p trend<0.0001). Sexually inexperienced women had a seropositivity of 2.5% (95% CI, 0.3 to 8.4) while women reporting four or more partners had a seroprevalence of 75% (95% CI, 65.6 to 83.0) with a 115.5-fold (95% CI 26.5–503.4) increase in risk compared to virgins and 7.6 (95% CI, 4.7 to 12.4) compared to monogamous women. Only two HSV-2 seropositive women (0.5%) reported a history of genital herpetic ulcers.

Additional univariate determinants of HSV-2 seropositivity include limited education, separation or divorce, smoking, low socioeconomic status, sexual initiation before age 17, number of live births, abortion, higher vaginal pH, and seropositivity for HPV 16, 18, or 31. Use of oral/injectable contraceptive methods, having had a cytology, hysterectomy, or being positive for HPV DNA were not significantly associated, but use of barrier methods showed a significant decrease in risk that was even stronger for current users compared to never users (OR 0.3, 95% CI, 0.2–0.5).

In the multivariate logistic regression, women who were 65 years of age or older were approximately eight times more likely to be HSV-2 seropositive compared to women under 25 years (p trend<0.0001) (table 2). A clear trend of increasing risk with increasing number of partners was observed. The age adjusted OR associated with having had four or more sexual partners was 21.4 (95% CI 7.2 to 63.6) compared to...
Table 2 HSV-2 antibodies adjusted odds ratios (OR) for seropositivity among sexually active women by selected reproductive and sociodemographic risk factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>OR*†</th>
<th>95% CI</th>
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<tr>
<td><strong>Age (years)</strong></td>
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</tr>
<tr>
<td>18–24</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>25–34</td>
<td>1.5</td>
<td>0.8 to 2.7</td>
</tr>
<tr>
<td>35–44</td>
<td>3.8</td>
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<td>55–64</td>
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<td>65+</td>
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</tr>
<tr>
<td><strong>No of lifetime sexual partners</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2–3</td>
<td>3.6</td>
<td>2.7 to 4.9</td>
</tr>
<tr>
<td>4–5</td>
<td>6.0</td>
<td>3.4 to 10.7</td>
</tr>
<tr>
<td>6+</td>
<td>21.4</td>
<td>7.2 to 63.6</td>
</tr>
<tr>
<td>Trend: p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Separated/divorced</td>
<td>1.3</td>
<td>0.8 to 2.3</td>
</tr>
<tr>
<td>Widowed</td>
<td>1.1</td>
<td>0.6 to 2.0</td>
</tr>
<tr>
<td>Single</td>
<td>.84</td>
<td>0.5 to 1.3</td>
</tr>
<tr>
<td>Trend: p = 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>1.4</td>
<td>0.9 to 2.4</td>
</tr>
<tr>
<td>Current</td>
<td>1.4</td>
<td>0.7 to 2.7</td>
</tr>
<tr>
<td>Trend: p = 0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Elementary</td>
<td>0.86</td>
<td>0.6 to 1.4</td>
</tr>
<tr>
<td>High school</td>
<td>0.81</td>
<td>0.4 to 1.5</td>
</tr>
<tr>
<td>Technical/university</td>
<td>0.86</td>
<td>0.5 to 1.6</td>
</tr>
<tr>
<td>Trend: p = 0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Socioeconomic status‡</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.68</td>
<td>0.4 to 1.1</td>
</tr>
<tr>
<td>High</td>
<td>0.98</td>
<td>0.6 to 1.5</td>
</tr>
<tr>
<td>Trend: p = 0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age at first intercourse (years)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1.6</td>
<td>1.1 to 2.1</td>
</tr>
<tr>
<td><strong>Ever pregnant</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>2.2</td>
<td>1.0 to 4.6</td>
</tr>
<tr>
<td><strong>Number of live births</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3–4</td>
<td>0.99</td>
<td>0.7 to 1.5</td>
</tr>
<tr>
<td>5–7</td>
<td>0.73</td>
<td>0.5 to 1.1</td>
</tr>
<tr>
<td>8+</td>
<td>0.77</td>
<td>0.5 to 1.3</td>
</tr>
<tr>
<td>Trend: p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hysterectomy‡</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>1.4</td>
<td>0.9 to 2.4</td>
</tr>
<tr>
<td><strong>Oral/injectable contraceptive use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>1.2</td>
<td>0.9 to 1.7</td>
</tr>
<tr>
<td>Current</td>
<td>1.6</td>
<td>0.9 to 2.6</td>
</tr>
<tr>
<td>Trend: p = 0.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Barrier method use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>0.64</td>
<td>0.4 to 0.9</td>
</tr>
<tr>
<td>Current</td>
<td>0.54</td>
<td>0.3 to 0.9</td>
</tr>
<tr>
<td>Trend: p = 0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IUD use</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>1.7</td>
<td>1.1 to 2.6</td>
</tr>
<tr>
<td>Current</td>
<td>1.5</td>
<td>0.8 to 3.1</td>
</tr>
<tr>
<td>Trend: p = 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HPV 16, 18, or 31 serological status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1.6</td>
<td>1.2 to 2.1</td>
</tr>
<tr>
<td><strong>HPV DNA†</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1.1</td>
<td>0.8 to 1.5</td>
</tr>
</tbody>
</table>

*Women with missing HSV-2 results are excluded.
†ORs adjusted for age and number of sexual partners.
‡One missing case.
§43 missing HPV DNA results.
women with one lifetime partner ($p_{\text{trend}} \leq 0.0001$). Only 10 women reported more than one recent partner and thus this variable could not be evaluated.

The only additional factors that remained statistically significant were age at first intercourse before or at 16 years (OR = 1.6, 95% CI 1.1 to 2.1, compared to those initiating at age 17 or older). Similarly, former intrauterine device users (OR = 1.7, 95% CI 1.1 to 2.6, compared to never users) and HPV 16, 18, or 31 seropositivity (OR = 1.6; 95% CI 1.2 to 2.1) were significant after adjustment. Barrier methods were protective with a 50% reduction in detection of HSV-2 antibodies for current (last month) versus never users (OR = 0.54; 95% CI, 0.31 to 0.94).

Among monogamous women, those separated or divorced had an age adjusted 3.4 (95% CI 1.4 to 8.2) increase in risk of HSV-2 detection.

No statistically significant difference in risk was seen for smoking, education, and socioeconomic status (number of household amenities). Similarly, number of pregnancies, use of oral/injectable contraceptives or positivity for HPV DNA status were not associated with HSV-2 seropositivity.

**DISCUSSION**

HSV-2 antibody seroprevalence in Guanacaste (38.5%) is relatively high for women when compared to other population based studies like the United States (25.6%; aged 12+ years) and Spain (3.6%; age range 5–59).22 However, it is consistent with reported seroprevalences from other Latin American countries, 42% in Brazil and 35.8% in Mexico,23 although the mean age in this cohort (41 years) was younger than in those two studies. Women from Guanacaste have a low risk self reported sexual behaviour, with only one fourth reporting more than two lifetime sexual partners and only 4.2% reporting having had an STD. The high seroprevalence in the context of low risk female behaviour is probably explained by high risk behaviour of their male partners, as suggested by Oberle et al.24 HSV-2 seroprevalence is generally more frequent among women than men, but it is unclear whether women are more susceptible, men are more efficient vectors, or infected men are less likely to seroconvert. Only one study in Jordan reported higher prevalence in males25 and another in Spain reported no difference by sex.26

There are no reported HSV-2 studies in Costa Rican men and we had no concurrent information on partners’ sexual behaviour. The previous HSV-2 study in Costa Rican women reported a seroprevalence of 39.6% in a population with a similar mean age. The provinces of Limón and Puntarenas, which are similar to Guanacaste with regard to rurality and sexual behaviour,27 had higher HSV-2 seroprevalence (~48.7%) than the rest of the country and also a significant association of HSV-2 seropositivity and being positive for another STD was found. We found no association with a history of STDs or current HPV DNA positivity. The negative association of barrier methods (mainly condoms, other methods are rare in Guanacaste) with HSV-2 seropositivity was consistent in both studies, suggesting that condoms may protect against HSV-2 contagion.

Age and lifetime sexual partners were the major determinants of HSV-2 seropositivity. As expected for any STD, high risk sexual behaviour increases the probability of being exposed. The finding of two HSV-2 seropositive virgins (2.5%) may reflect non-sexual transmission (that is, perinatal infection, oral) or false positivity of the ELISA used, which was 90% specific. Among women with multiple sexual partners, HSV-2 prevalence was higher than among monogamous women in all age groups, particularly in the young, and the prevalence peak occurred at earlier ages. In the monogamous group, seroprevalence continued to rise with age, although less steeply for the older age groups (data not shown). The possibility of HSV-2 being acquired among older age women, an age-cohort effect, or both cannot be ruled out.

In contrast with other studies, education, socioeconomic indicators, number of live births, ever being pregnant, use of oral and injectable hormonal contraceptives were not associated with HSV seropositivity. Although hysterectomy was not associated with an increased risk in the general population, menopausal hysterectomised women had higher HSV-2 seropositivity (data not shown). Although smoking is considered as a possible contributor to viral diseases by altering immune function,28 we found no significant association with smoking.

The risk of HSV-2 seropositivity was higher in women with antibodies against a subgroup of high risk HPV types, a partial indicator of HPV exposure. Whether this reflects an interaction that facilitates co-infection or just represents a subgroup at higher risk for both infections is unclear. However, detection of HPV DNA was not associated with HSV-2 seropositivity, probably indicating that HPV DNA represents current infection, which is usually transient and occurs more commonly in younger women.

HSV-2 is an endemic but largely asymptomatic infection in Costa Rican women. Although it is commonly recognised that HSV-2 infection is generally asymptomatic, under-reporting cannot be ruled out. HSV-2 serological screening programmes should be evaluated in selected population groups at especially high risk, either of becoming reservoirs with potential public health impact or in groups at risk of more serious forms of the disease. Identification of antibodies is a reliable method for identifying asymptomatic, never diagnosed population reservoirs for future infections. Pregnant women could be a target group for screening given the silent nature of an infection that can result in neonatal disease.

Control of this viral infection by vaccination may be possible in the future, although many difficulties have to be overcome29 in the interim. Use of condoms and education of adolescents, women of reproductive age, and health related personnel on the importance of the diagnosis, treatment, and prevention may help control the spread of HSV-2 infections.

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**CONTRIBUTORS**

ACK was involved in the statistical analysis, interpretation, and manuscript preparation of the HSV-2 study, also in the overall coordination of the cohort follow up; PEC was involved in the initial idea, study design and overall coordination of the HSV-2 study.
statistical analysis, and manuscript review; JSS was involved in the initial idea, study design, and manuscript review of the HSV-2 study; CR was involved in the design and overall coordination of the cohort and the interpretation and manuscript review of the HSV-2 study; AH was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; JSS was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; RA did the HSV-2 serology and manuscript review; XC was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; CB was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; JSS was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; RDB did the HPV PCR determination and manuscript review; MS was involved in the design and overall coordination of the cohort, as well as in the initial idea, interpretation, and manuscript review of the HSV-2 study; RA did the HSV-2 serology and manuscript review; XC was involved in the statistical analysis and interpretation and manuscript review; RH was involved in the design and overall coordination of the cohort, as well as in the initial idea, study design, statistical analysis, interpretation and manuscript review of the HSV-2 study.

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Informed consent statement: Women who agreed to participate in the population based cohort study of the natural history of cervical neoplasia signed informed consent forms. The protocol was cleared by the institutional review boards of National Cancer Institute, NIH, Maryland, USA and an ad hoc ethics committee of FUCODOCSA, in accordance with the revised Helsinki Declaration of 1983.

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