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Prevalent Herpes Simplex Virus Type 2 Infection is Associated with Altered Vaginal Flora and an increased Susceptibility to Multiple Sexually Transmitted Infections

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Background. Prevalent herpes simplex virus type 2 (HSV-2) infection increases human immunodeficiency virus acquisition. We hypothesized that HSV-2 infection might also predispose individuals to acquire other common sexually transmitted infections (STIs).

Methods. We studied the association between prevalent HSV-2 infection and STI incidence in a prospective, randomized trial of periodic STI therapy among Kenyan female sex workers. Participants were screened monthly for infection with *Neisseria gonorrhoeae* and *Chlamydia trachomatis,* and at least every 6 months for bacterial vaginosis (BV) and infection with *Treponema pallidum, Trichomonas vaginalis,* and/or HSV-2.

Results. Increased prevalence of HSV-2 infection and increased prevalence of BV were each associated with the other; the direction of causality could not be determined. After stratifying for sexual risk-taking, BV status, and antibiotic use, prevalent HSV-2 infection remained associated with an increased incidence of infection with *N. gonorrhoeae* (incidence rate ratio [IRR], 4.3 [95% confidence interval {CI}, 1.5–12.2]), *T. vaginalis* (IRR, 2.3 [95% CI, 1.3–4.2]), and syphilis (IRR, 4.7 [95% CI, 1.1–19.9]). BV was associated with increased rates of infection with *C. trachomatis* (IRR, 2.1 [95% CI, 1.1–3.8]) and *T. vaginalis* (IRR, 8.0 [95% CI, 3.2–19.8]).

Conclusion. Increased prevalences of HSV-2 infection and BV were associated with each other and also associated with enhanced susceptibility to an overlapping spectrum of other STIs. Demonstration of causality will require clinical trials that suppress HSV-2 infection, BV, or both.

Herpes simplex virus type 2 (HSV-2) infection is an important cofactor in the global HIV-1 (HIV) pandemic. A recent meta-analysis of all published studies concluded that HSV-2 infection increases the risk of HIV acquisition in women by just over 3-fold [1]. In Africa, over 50% of adult women are infected by HSV-2 [2], and statistical modeling suggests that at this prevalence, almost half of all HIV transmission may be attributable to HSV-2 infection [3]. The increase in the risk of HIV acquisition may be due to mucosal macro-ulceration or micro-ulceration during HSV-2 reactivation [4, 5], but may also relate to HSV-2 infection–induced genital inflammation and increases in the number of HIV-susceptible target cells in the genital tract mucosa, with the latter seen even in the absence of HSV-2 reactivation [6].

We hypothesized that HSV-2 infection–induced alterations in the mucosal immune milieu might also en-

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hance host susceptibility to other sexually transmitted infections (STIs) and to alterations in vaginal bacterial flora (ie, bacterial vaginosis [BV]). HSV-2 reactivation has been shown to induce a number of proinflammatory cytokines and chemokines in the female genital tract [6], and nuclear factor κ-B (NFκB) upregulation, a common final pathway for many inflammatory stimuli, has been demonstrated to enhance infection by *Neisseria gonorrhoeae* in vitro [7]. In HIV-infected individuals, both coinfection by bacterial STIs and altered vaginal flora have been associated with increased levels of HIV in the genital tract, which likely enhances secondary sexual transmission of HIV. Furthermore, in individuals who are not infected with HIV, both bacterial STIs and altered vaginal flora may increase susceptibility to HIV infection [8]. Therefore, if HSV-2 infection were to increase susceptibility to bacterial STIs and/or alterations of vaginal flora, this would imply that HSV-2 infection enhances the sexual transmission of HIV both directly (through mucosal macroulceration and/or micro-ulceration and alterations in the mucosal immune milieu) and indirectly (by increasing the incidence of other genital infections that act as HIV cofactors). This would emphasize the need to test HSV-2 control strategies as a means to reduce the transmission not only of HIV, but also of other STIs.

Interactions between genital infections are complex and challenging to study, because the precise timing of infection acquisition may be difficult to elucidate, many or most genital infections are asymptomatic, and some, particularly BV, tend to relapse frequently. In addition, BV may predispose individuals to HSV-2 acquisition [9], as well as to the acquisition of other STIs [10]. This raises the possibility that underlying alterations in vaginal flora may confound observed associations between HSV-2 infection and other genital infections.

To address these questions, we examined the association of prevalent HSV-2 infection with BV and with the acquisition of several different STIs. This work was performed in the context of a randomized, controlled trial of periodic presumptive STI therapy in high-risk Kenyan sex workers as a possible strategy to prevent HIV acquisition [11].

METHODS

Study participants and procedures. HIV IgG–seronegative Kenyan female sex workers were recruited into a randomized, double-blind, placebo-controlled trial of monthly oral azithromycin therapy. The primary study end point was HIV acquisition, and secondary end points were the acquisition of sexually transmitted infections (STIs) [11]. Ethical approval for the trial was obtained from institutional review boards at the Kenyatta National Hospital (Nairobi, Kenya) and the University of Manitoba (Winnipeg, Canada). Women received 1 g of oral azithromycin or identical placebo monthly, as directly observed therapy. The primary outcome was the incidence of HIV infection, and secondary outcomes were rates of infection with *N. gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Treponema pallidum, Haemophilus ducreyi,* and of BV. Urine samples were obtained monthly for polymerase chain reaction for *N. gonorrhoeae* and *C. trachomatis* (Amplicor PCR Diagnostics; Roche Diagnostics); these assays were run in a batched fashion after study completion.

Detailed behavioral data and blood samples for HIV-1 IgG ELISA and HSV-2 ELISA (Kalon Biological) were collected every 3 months. Full STI screening was performed at enrolment, every 6 months thereafter, and to investigate any symptomatic genital infection; screening included culture for *T. vaginalis* (In Pouch TV culture; Biomed Diagnostics), a Gram stain of vaginal swab sample, and serological testing (rapid plasma reagin) for syphilis. Bacterial vaginosis was diagnosed if there was a Nugent score of 7–10, incident syphilis was diagnosed if there was an increase in rapid plasma reagin titer to ⩾1:8, and vaginal candidiasis was diagnosed if yeast was found on Gram staining [11]. If a genital ulcer was present, a swab sample of the ulcer base was taken for *H. ducreyi* culture on an activated charcoal medium [12]. Any STI diagnosed was treated according to Kenyan national guidelines, whether symptomatic or asymptomatic; symptomatic BV was treated, while asymptomatic BV was not. Self-reported condom use was reported on a scale of 0–5, where "0" represented no condom use, and "5" represented condom use with all clients.

Statistical methods. Poisson regression was used to calculate rate ratios and 95% confidence intervals for the comparison of STI incidence rates in participants with and without HSV-2 infection. Both the receipt of monthly azithromycin therapy and the level of sexual risk-taking (ie, with "increased risk" defined as reduced reported condom use and increased client numbers) were previously found to be associated with an increased incidence of bacterial STIs [11, 13], and so stratified adjustment was performed to control for both factors. Sexual risk-taking was grouped into 4 levels on the basis of the weekly number of unprotected sexual contacts, based on self-reported condom use and weekly client numbers: level 1 was defined as <0.5 contacts; level 2, 0.5–1 contact; level 3, 1–2 contacts; level 4, >2 contacts. BV has been associated with an increased incidence of bacterial STIs [10] and HSV-2 infection [9]. However, because BV was only treated if symptoms were present and may be rapidly recurrent even if treated appropriately [14], we felt that when studying BV as a risk factor for the acquisition of other STIs, it was inappropriate to compare STI rates before and after incident BV in an individual. Rather, participants were stratified either as "ever" having had BV, either at enrolment or during clinical follow-up, or as "never" having had BV.

HSV-2 seroconversion that occurred between enrolment and the first follow-up visit was potentially due to HSV-2 infection acquired prior to enrolment, and therefore participants who met this criterion were excluded from analysis of HSV-2 infection associations.

Table 1. Characteristics of Kenyan female sex workers at study enrollment.

Variable	Value
Age, years	28.8 (18–52)
Duration of prostitution, years	5.3 (0–34)
Clients per week	15.7 (1–100)
Charge for sex, Kenyan shillings	133.3 (10–1500)
Condom use[a]	2.4 (0–5)
Ever practice sex during menses	78 (18.8%)
Ever practice anal sex	62 (14.9%)
Ever use injection drugs	18 (4.3%)
Infection	
Vaginal candidiasis	46/393 (11.7%)
Bacterial vaginosis	199/393 (50.6%)
Neisseria gonorrhoeae	41 (9.9%)
Chlamydia trachomatis	32 (7.7%)
Treponema pallidum	16 (3.8%)
Trichomonas vaginalis	48 (11.5%)
Herpes simplex type 2	322 (77.4%)

NOTE. Data are no. (%) of participants or mean value (range).

[a] Condom use was reported on a semiquantitative scale, from 0–5 (see Methods for details).

RESULTS

HSV-2 infection status and baseline demographic characteristics. Study enrollment took place from 1998 through 2002, and a total of 466 HIV-seronegative female sex workers were enrolled [11]. Serological testing for HSV-2 was performed post hoc, and enrollment plasma samples were available for 443 (95%) of 466 HIV-seronegative female sex workers. The seroprevalence of HSV-2 infection was 72.7% (322 of 443 female sex workers). HSV-2 seroconversion occurred in 24 (22.3%) of 121 HSV-2–uninfected participants; most HSV-2 infections (in 15 of 24 female sex workers) occurred between enrollment and the first follow-up visit, perhaps because of rapid postenrollment reductions in sexual risk-taking [13]. Participants excluded from analysis included all of those who seroconverted to HSV-2 positive status following the baseline visit ($N = 24$) and 3 participants with inconsistent HSV-2 ELISA results. HSV-2 infection status was unchanged through clinical follow-up for 416 participants (322 [77%] were HSV-2 infected, and 94 [23%] were not infected with HSV-2), and this population served as the basis for subsequent analyses of the impact of HSV-2 infection on the acquisition of other genital infections.

The mean age of participants was 28.6 years (range, 18–52 years) (table 1). At enrollment, women had been engaged in commercial sex work for a mean of 5.3 years; participants reported a mean of 15.7 paying clients per week and a mean charge of 133.3 Kenyan shillings ($2.19 USD; table 1). STIs and BV were common at baseline. The mean duration of clinical follow-up for participants was 2.1 years (773.7 days; range, 0.5–1,608 days).

Associations between genital herpes and BV. At enrollment, the prevalence of BV was higher among participants with chronic HSV-2 infection, compared with those without (37 [42%] of 89 vs. 162 [53%] of 304; likelihood ratio, 3.8; $P = .05$). After stratifying for sexual risk-taking and antibiotic use, chronic HSV-2 infection was also associated with an increased incidence of laboratory-confirmed BV (incidence rate ratio [IRR], 1.4 [95% CI, 1.1–1.8]; $P = .006$). Overall, 288 (69%) of 416 participants had at least 1 episode of BV during follow-up, and HSV-2–infected participants were more likely to ever have had BV, compared with those who were not HSV-2 infected (232 [72.5%] of 320 vs. 56 [60.9%] of 92 ; $P = .04$). A subgroup analysis including only those participants who were negative for BV at enrolment ($N = 215$) found a similar association between chronic HSV-2 infection and incident BV (IRR, 1.4), although the 95% CI now included 1 (95% CI, 0.94–2.11; $P = .1$), likely because of decreased participant numbers. However, the frequent recurrence of BV may confound the analysis of chronic HSV-2 infection and BV incidence: an earlier BV episode may have predisposed the individual to the initial acquisition of HSV-2 , or prior HSV-2 acquisition may have predisposed the individual to the current BV episode(s). Therefore, it might be more accurate to report that the prevalence of HSV-2 infection and the prevalence of recurrent BV were associated, rather than to analyze BV incidence.

Association of prevalent HSV-2 infection with other genital infections. The association of prevalent HSV-2 infection with the incidence of classical STIs was then analyzed, using a stratified Poisson regression model (table 2). After stratifying for sexual risk-taking, assigned study arm (i.e., azithromycin use), and BV at any time, prevalent HSV-2 infection was associated with an increased incidence of infection with *N. gonorrhoeae* (IRR, 4.3 [95% CI, 1.5–12.2]; $P = .006$), *T. vaginalis* (IRR, 2.3 [95% CI, 1.3–4.2]; $P = .004$), and syphilis (IRR, 4.7 [95% CI, 1.1–19.9]; $P = .035$). As would be expected, genital ulcers were almost 4-fold more common in the HSV-2–infected group, although this finding did not reach statistical significance because clinical ulcers were rare (rate, 1.5 vs 0.4 ulcers per 100 person-years; adjusted IRR, 2.2 [95% CI, 0.3–16.3]). No association was seen between HSV-2 infection and either vaginal candidiasis (IRR, 1.1 [95% CI, 0.7–1.7]; $P = .6$) or infection with *C. trachomatis* (IRR, 1.0 [95% CI, 0.6–1.7]; $P = .99$).

C. trachomatis infection has consistently been associated with younger age [15], and in keeping with this the mean age of female sex workers with chlamydial cervicitis at enrollment was lower than that of female sex workers without (26.1 vs 28.9 years; $P = .03$). HSV-2 infection is a persistent infection, and its prevalence would be expected to increase with age, potentially confounding its associations with other infections more common in younger women. An additional analysis was therefore performed, with age added to the stratified model (as age less than the mean cohort age of 28.8 years or age greater than or equal to

Table 2. Association of prevalent herpes simplex virus type 2 (HSV-2) infection and altered vaginal flora with the incidence of sexually transmitted infections.

Coinfection	Incidence, no. of cases / 100 person-years (no. of cases[a]) HSV-2 IgG seronegative ($N = 94$)	HSV-2 IgG seropositive ($N = 322$)	Adjusted IRR	95% CI
Vaginal candidiasis	12.2 (30)	18.9 (129)	1.1[b]	0.7–1.7
Neisseria gonorrhoeae	7.5 (13)	13.1 (67)	4.3[b]	1.5–12.2
Chlamydia trachomatis	12.6 (22)	14.2 (73)	1.0[b]	0.6–1.7
Trichomonas vaginalis	5.7 (14)	17.7 (121)	2.3[b]	1.3–4.2
Treponema pallidum	0.8 (2)	5.1 (35)	4.7[b]	1.1–19.9
	Never had bacterial vaginosis ($N = 128$)	Ever had bacterial vaginosis ($N = 288$)		
Vaginal candidiasis	15.5 (30)	20.4 (129)	1.3[c]	0.9–1.9
N. gonorrhoeae	13.6 (18)	12.1 (52)	0.8[c]	0.5–1.4
C. trachomatis	8.8 (12)	15.3 (79)	2.1[c]	1.1–3.8
T. vaginalis	2.4 (5)	20.0 (129)	8.0[c]	3.2–19.8
T. pallidum	3.3 (7)	4.1 (30)	1.0[c]	0.5–2.4

NOTE. CI, confidence interval; IRR, incidence rate ratio,

[a] Parentheses show the total number of cases per group observed during follow-up.

[b] Stratified for sexual risk-taking, antibiotic use (ie, azithromycin), and ever having had bacterial vaginosis during clinical follow-up.

[c] Stratified for sexual risk-taking, antibiotic use (ie, azithromycin), and HSV-2 infection status.

28.8 years). Again, no association was found between HSV-2 infection and *C. trachomatis* acquisition (IRR, 1.1 [95% CI, 0.6–1.9]; $P = .7$).

As reported elsewhere [11], prevalent HSV-2 infection was strongly associated with HIV acquisition in a Cox regression model adjusting for study arm (ie, azithromycin use) and sexual risk-taking (rate ratio [RR], 5.8 [95% CI, 1.5–27.1]; $P < .001$).

BV and STI incidence. The impact of ever having had BV on STI incidence was examined among participants with stable HSV-2 serostatus. A total of 288 (69%) of 416 participants with at least 1 episode of laboratory-confirmed BV were compared with 128 (31%) of 416 participants who had never had BV (table 2). Again, no association was seen with vaginal candidiasis. In contrast to HSV-2 infection, BV was not associated with an increased incidence of infection with *N. gonorrhoeae* (IRR, 0.8 [95% CI, 0.5–1.4]; $P = .6$) or syphilis (IRR, 1.0 [95% CI, 0.5–2.4]; $P = .9$). However, BV was associated with increased rates of infection with both *C. trachomatis* (IRR, 2.1 [95% CI, 1.1–3.8]; $P = .02$) and *T. vaginalis* (IRR, 8.0 [95% CI, 3.2–19.8]; $P < .0001$). These associations remained significant when the analysis was restricted to participants who did not have BV at enrollment and had stable HSV-2 serostatus ($N = 190$); an increased incidence was still observed for both *C. trachomatis* infection (IRR, 2.4 [95% CI, 1.2–4.8]; $P = .02$) and *T. vaginalis* infection (IRR, 9.8 [95% CI, 3.5–27.1]; $P < .001$) in those participants with incident BV during follow-up.

Subgroup analysis of BV associations restricted to those participants who were not infected with HSV-2 at baseline and who remained so throughout follow-up ($N = 94$) was not possible, because no participants in the HSV-2–negative, BV-negative group acquired *C. trachomatis* infection or syphilis during follow up, precluding a regression analysis using these endpoints.

DISCUSSION

It is increasingly apparent that HSV-2 infection acts as an important cofactor in the HIV/AIDS pandemic. HSV-2 infection increases the frequency of HIV transmission from HIV–HSV-2 coinfected individuals [16], as well as increasing susceptibility to HIV acquisition by approximately 3-fold in HSV-2–infected men and women from the general population [1], and by as much as 6-fold in HSV-2-infected female sex workers [11]. This may relate either to micro-ulceration or macro-ulceration of the genital mucosa, or to HSV-2 infection–associated increases in the number of mucosal HIV target cells, specifically CCR5-expressing $CD4^+$ T cells and/or DC-SIGN–expressing dendritic cells [6]. On this basis, clinical trials of HSV-2 suppression as a strategy to reduce HIV sexual transmission are ongoing [17]. Other genital tract infections may also enhance the risk of sexual HIV acquisition and secondary transmission [8]; these include BV and *N. gonorrhoeae, T. pallidum, H. ducreyi,* and *T. vaginalis* infections, although treatment and/or prevention of these infec-

tions as a strategy to prevent HIV acquisition has met with mixed success [11, 18–20].

Our data clearly show, for the first time, to our knowledge, that chronic HSV-2 infection is not only associated with an increased incidence of HIV infection, but also with an increased incidence of both sexually transmitted and non–sexually transmitted genital infections, specifically *N. gonorrhoeae* infections, *T. pallidum* infections, and *T. vaginalis* infections. BV, a disturbance in the vaginal microflora that tends to be recurrent, has itself been associated with increased susceptibility to HSV-2 infection [9], as well as to HIV infection [8] and other STIs [10], perhaps by altering the genital tract cytokine milieu or by enhancing HIV replication [20]. We observed an association between prevalent HSV-2 infection and the presence of BV at enrollment, and also a prospective association between HSV-2 infection and new BV episodes. However, we believe that the nature of BV as a waxing and waning imbalance of vaginal flora makes any analysis of the causes of incident BV difficult, if not impossible, because the direction of causality cannot be determined with confidence. Nonetheless, there is clearly an association between rates of HSV-2 infection and BV, and between each of these and the incidence of several STIs, so that each may confound analysis of the impact of the other. We therefore stratified our analysis of HSV-2 infection status and STI incidence on the basis of BV at any time (either symptomatic or asymptomatic) during clinical follow-up, as well as on the basis of reported sexual risk-taking and study arm (ie, azithromycin vs placebo), and we continued to find strong associations between HSV-2 infection and the subsequent acquisition of *N. gonorrhoeae, T. pallidum,* and *T. vaginalis.*

In addition, after controlling for HSV-2 infection status, we were able to confirm the strong association previously demonstrated between BV and the incidence of infection with *C. trachomatis* [10], and a particularly strong association with incident *T. vaginalis* infection was also apparent. However, after controlling for HSV-2 infection, we did not find any association between BV and incidence of infection with *N. gonorrhoeae* . This may relate to differences in cohort or methodology between our study and others [10], but another possibility is that previously described associations may have been confounded by the presence of HSV-2 infection. What is clear is that future cohort studies will need to control for the presence of numerous genital tract infections, including HSV-2 infection, BV, and others. Ultimately, proving causation as it applies to the relationship of either HSV-2 infection or BV with STI susceptibility will require randomized clinical trials that target therapeutic and/or vaccine interventions at a single factor (either BV or HSV-2 infection), and only that factor.

Our study does not permit elucidation of the biological basis for enhanced STI susceptibility due to HSV-2 infection and/or BV. Both infections have been described as inducing quite profound immunological changes in the female genital tract [6, 21–23], in part through the induction of proinflammatory cytokines, and HSV-2 infection is also associated with NFκB induction [24] and alterations in cervical immune cell populations and activation status [6]. In vitro, NFκB induction enhanced infection by *N. gonorrhoeae* [7], suggesting a mechanism for HSV-2–associated increases in rates of gonorrhea acquisition. Increased susceptibility to HIV may relate to direct genital macro-ulceration or micro-ulceration during HSV-2 reactivation, as well as to the increased numbers of mucosal HIV target cells ($CCR5^+$ CD4 T cells, and DC-$SIGN^+$ dendritic cells) present, even in the absence of HSV-2 reactivation [6]. The pathogenesis of *T. vaginalis* infection is less well understood, and how HSV-2 infection may enhance this process is not clear. The complexity of local interactions between mucosal immunology and various genital coinfections in vivo presents a significant obstacle to elucidating the immunopathogenesis of HSV-2–associated increases in STI acquisition in a real-world setting.

Although we adjusted our analysis for reported sexual risk-taking, this is only a proxy for true risk, and it is not possible to entirely rule out residual confounding. However, reported sexual risk-taking has been validated in this cohort as a marker for acquisition of both STIs [13] and HIV infection [11]. In addition, no relationship was seen between HSV-2 infection and *C. trachomatis* acquisition, although the latter was strongly linked to reported sexual risk-taking in this cohort [13]. Therefore, we believe that behavioral confounding is less likely than a true biological effect as an explanation for the strong associations seen between HSV-2 infection and subsequent STI acquisition. Nonetheless, should chronic HSV-2 infection have a broad effect on STI susceptibility, given the important implications of these findings for future research, it will be vital for future confirmatory studies to definitively rule out behavioral confounders by including a careful assessment of sexual risk behavior. In this study, testing for herpes simplex virus type 1 (HSV-1), which is responsible for an increasing proportion of genital herpes cases in developed countries [25], was not performed. We are therefore not able to comment on the prevalence of HSV-1, or on any possible association between HSV-1 and rates of HIV infection and STI acquisition.

In summary, chronic HSV-2 infection in this cohort of Kenyan female sex workers was strongly associated with an increased incidence of several genital infections, including HIV infection, gonorrhea, and trichomoniasis, and with significant perturbations in vaginal microflora. Prevention of HSV-2 infection, or chronic suppression of HSV-2 recurrence, as well as suppression of BV, should be tested as strategies to prevent the acquisition of multiple STIs, not just HIV infection.

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