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Shedding of Epstein-Barr Virus and Cytomegalovirus from the Genital Tract of Women in a Periurban Community in Andhra Pradesh, India▽

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We found a large number of false-positive readings by visual inspection with acetic acid (VIA) in a study of cervical cancer screening strategies (VIA, human papillomavirus HPV DNA testing, and Pap cytology) in a periurban community in Andhra Pradesh, India. We evaluated whether these false-positive readings might be occurring as a result of infections with Epstein-Barr virus (EBV) or cytomegalovirus (CMV), prevalent latent herpesviruses known to be shed from the female genital tract. While we found that there was no association between VIA results and the presence of EBV or CMV in the cervix, we did find a high prevalence of both viruses: 20% for EBV and 26% for CMV. In multivariate analyses, CMV prevalence was associated with younger age, lack of running water in the home, and visually apparent cervical inflammation. EBV prevalence was associated with older age and a diagnosis of cervical intraepithelial neoplasia grade 1 or greater. The biological and clinical implications of these viruses at the cervix remain to be determined. The strong association between the presence of EBV and cervical disease warrants future exploration to determine whether EBV plays a causal role in disease development or if it is merely a bystander in the process.

We conducted a study of cervical cancer screening strategies in a periurban community near Hyderabad in the state of Andhra Pradesh in southern India. Cervical cells were collected from women for cytological and virological investigation, and the cervix was examined with naked eye visual inspection after application of acetic acid (VIA). We found that a positive VIA reading (presence of an acetowhite lesion in the cervical transformation zone) had a surprisingly low sensitivity (26.3%) and moderate specificity (76.4%) for the detection of cervical neoplasia (9, 28).

Given the large number of false-positive VIA readings, we inquired if these lesions might be occurring as a result of infections with Epstein-Barr virus (EBV) or cytomegalovirus (CMV), prevalent latent herpesviruses which are known to be shed from the female genital tract (2, 6, 14, 21). We also examined whether cervical EBV and CMV detection correlated with either cervical HPV detection or other markers of cervical pathology, since these viruses have been postulated to have a possible cocarcinogenic role with human papillomavirus (HPV) for cervical neoplasia (19, 23).

MATERIALS AND METHODS

Study participants. The study subjects were a subpopulation of the CATCH (Community Access to Cervical Health) project, a population-based screening study in a periurban community near Hyderabad, in the state of Andhra Pradesh, India. The CATCH project aimed to enroll all eligible women 25 years of age and older who were not pregnant and who had not had a hysterectomy. A total of 2,331 women, or approximately 40% of those eligible, were enrolled and consented to an interviewer-administered questionnaire to collect data on demographics, reproductive health, cervical cancer screening history, and smoking status. Participants also provided a self-collected vaginal swab specimen and serum and were screened by Pap smear, HPV DNA testing of a physician-collected cervical swab specimen, and VIA. Physicians noted the visual appearance of the lower genital tract, including presence of cervicitis, on a standardized case report form. The study protocol was approved by the institutional review boards in India (SHARE Research/Mediciti Institute of Medical Sciences, Ghanpur, Andhra Pradesh) and in Baltimore, Maryland (Johns Hopkins Bloomberg School of Public Health).

The sample used in this analysis is a subset of the first 892 women enrolled in the study enriched for the presence of women who were positive in the cervical screen marker analyses, as previously described (22). This subset of 470 women included (i) all 220 women who screened positive on at least one of the tests (Pap smear, HPV DNA, or VIA), (ii) all women who were randomized to colposcopy but who screened negative by all three tests ($n = 122$), and (iii) a random sample ($n = 128$) of women negative by all three screening tests. From this group, 6 were excluded because of an insufficient sample for virology, resulting in a final sample size of 464 women. The proportion of women who were positive on at least one screening test was 46.8% (217/464) in this sample, compared to 25.3% for the 892 registered women. The high-risk HPV prevalence was 10.7% in the 892 women and 20.0% in the 464 women.

Quantitative PCR. Real-time TaqMan PCR was performed to quantify EBV and CMV DNA from the physician-collected cervical swabs using a 7300 real-time PCR thermocycler from Applied Biosystems. DNA was extracted from the physician-collected cervical swabs as previously described (22). In brief, 90 μl of sample was digested in a proteinase K–laureth-12 solution for 1 h at 65°C. Proteinase was heat inactivated at 95°C for 10 min, and DNA was precipitated in an ethanol-ammonium acetate solution at −20°C overnight. DNA was pelleted by centrifugation for 30 min at 4°C, and supernatant was removed with a disposable fine-tip transfer pipette. DNA was air dried and resuspended in 50 μl of loTE (20 mM Tris-HCl, 1 mM EDTA, pH 8.5). Overall, 5 μl or 10% of the extracted DNA was used for each assay.

For the EBV assay, a 50-μl reaction volume containing 5 μl extracted DNA, 25 μl ABI Universal Mastermix, 0.250 μM probe, and 0.4 μM each primer was added to each well of a 96-well PCR plate. The primers and probe targeted the BamHI-W region of the EBV genome as previously described (13). The reaction

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TABLE 1. EBV and CMV prevalence by screening test result

Screening test result	EBV		CMV	
	No. (%) positive	*P* value	No. (%) positive	*P* value
Overall (n = 464)	93 (20.04)		121 (26.08)	
VIA negative (n = 414)	83 (20.05)	0.994	110 (26.57)	0.487
VIA positive (n = 50)	10 (20.00)		11 (22.00)	
Pap smear negative (n = 346)	58 (16.76)	0.003	91 (26.30)	0.851
Pap smear positive (n = 118)	35 (29.66)		30 (25.42)	
HPV negative (n = 371)	66 (17.79)	0.015	94 (25.34)	0.468
HPV positive (n = 93)	27 (29.03)		27 (29.03)	
Screen negative (n = 247)	36 (14.57)	0.002	63 (25.51)	0.765
Screen positive[a] (n = 217)	57 (26.27)		58 (26.73)	

[a] Positive on at least one of the screening tests.

conditions were 2 min at 50°C, followed by 10 min at 95°C and then 50 cycles of 15 s at 95°C and 30 s at 60°C.

For the CMV assay, a 30-μl reaction volume containing 5 μl extracted DNA, 15 μl ABI Universal Mastermix, 0.10 μM probe, and 0.415 μM each primer was added to each well of a 96-well PCR plate. For this multiplex assay, two primer-probe sets targeting the gB and EX-4 regions of CMV were used as previously described (4). The reaction conditions were 2 min at 50°C, followed by 15 min at 95°C and then 45 cycles of 1 min at 94°C and 1 min at 60°C.

In our validation of these assays using 2-fold limiting dilutions of target DNA, both EBV and CMV were reproducibly detectable at levels below 2 copies per μl, with average coefficients of variation (CVs) of 2.1% and 1.5%, respectively. Quantification of samples was based on amplification to generate standard curves derived from 1:2 dilutions starting from 10,000 copies per PCR mixture down to 156 copies per PCR mixture, using quantified viral DNA (Applied Biosystems, Foster City, CA). All oligonucleotide primers and probes were manufactured by IDT DNA, Coralville, IA.

EBV and CMV assays were considered positive if any viral amplification was detected. Human genomic DNA amplification targeting a single-copy endogenous human retrovirus gene (*ERV3*) was used as a positive amplification control and for normalizing EBV and CMV loads as previously described (30). Assuming 2 *ERV3* copies per human cell, we normalized the EBV and CMV loads to numbers of copies per 10,000 cell equivalents (*ERV3* copy/2) and $\log_{10}$ transformed those values.

Study outcomes. A positive VIA outcome was defined as sharp, distinct, well-defined, dense (opaque, dull, or oyster white) acetowhite areas with or without raised margins abutting the squamocolumnar junction in the transformation zone, strikingly dense acetowhite areas in the columnar epithelium, or condyloma and leukoplakia occurring close to the squamocolumnar junction and turning intensely white 1 min after the application of a 5% acetic acid solution. A Pap smear result of atypical squamous cells of unknown significance (ASCUS) or greater was considered positive. Presence of high-risk HPV was determined using the Hybrid Capture 2 test (Qiagen, Gaithersburg, MD); any sample with a relative light unit per control sample (RLU/CO) value of ≥1.0 was considered positive.

Data analysis. Univariate analyses for demographic and clinical exam variables were conducted using chi-square tests for EBV and CMV positivity and Poisson regression to estimate prevalence ratios and 95% confidence intervals (CIs). Multivariate Poisson regression was used to estimate the independent association of each covariate with the respective outcome by adjusting for potential confounding variables. Tests for colinearity and goodness of fit were also performed. A P value of ≤0.05 was considered statistically significant. All analyses were conducted using the STATA (version 11.0) program.

RESULTS

The prevalence of EBV in the genital tract of these rural Indian women was 20.0%, and the prevalence of CMV was 26.1% (Table 1). For both EBV and CMV, the viral loads varied over a wide range (Fig. 1), with median values of 32 copies per 10,000 cells for EBV and 525 copies per 10,000 cells for CMV.

There was a marked difference between the two viruses in their prevalence in women with positive versus negative cervical cancer screening results (Table 1). When using a composite positive screening result (i.e., VIA, Pap smear, or HPV posi-

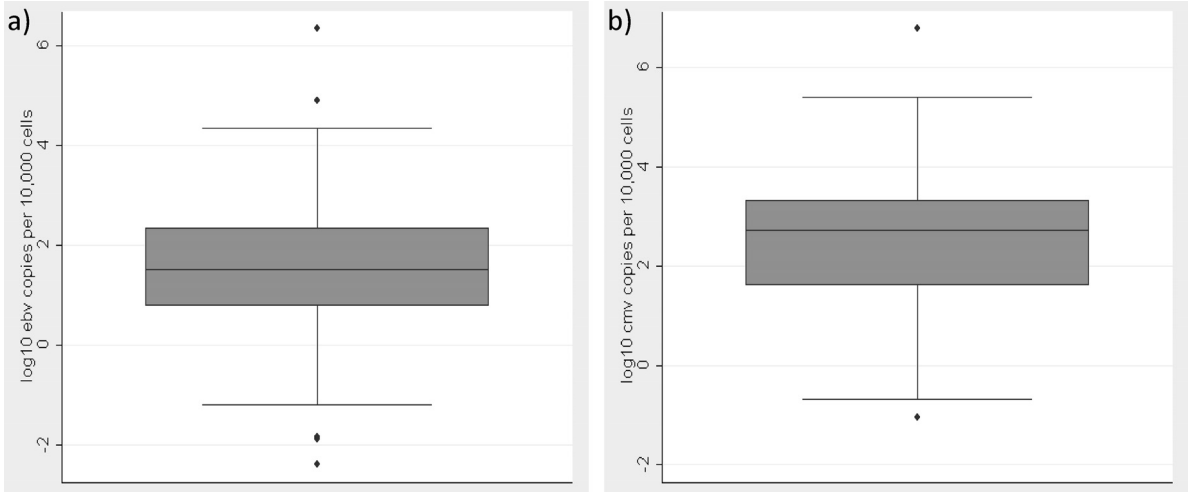

FIG. 1. Viral load (in $\log_{10}$ copies per 10,000 cells). Box plots of the distribution of viral load (in log10 copies per 10,000 cells) at the cervix for EBV (a) and CMV (b).

TABLE 2. Univariate association of demographic and clinical risk factors for EBV and CMV infection[a]

Variable	Overall (n = 464)		EBV (n = 93)			CMV (n = 121)		
	No. positive	%	No. positive	%	PR (95% CI)	No. positive	%	PR (95% CI)
Age (yr)								
25–29	155	34.29	26	16.77	1	40	25.81	1
30–34	98	21.68	12	12.24	0.73 (0.39–1.38)	33	33.67	1.30 (0.89–1.92)
35–39	55	12.17	8	14.55	0.87 (0.42–1.80)	19	34.55	1.34 (0.85–2.10)
40–44	41	9.07	14	34.15	2.04 (1.17–3.53)	8	19.51	0.76 (0.85–2.10)
45–49	33	7.30	7	21.21	1.26 (0.60–2.67)	7	21.21	0.82 (0.40–1.67)
50–54	33	7.30	12	36.36	2.17 (1.22–3.84)	5	15.15	0.59 (0.25–1.38)
55–85	37	8.19	13	35.14	2.09 (1.19–3.67)	4	10.81	0.42 (0.16–1.10)
Education								
None	312	69.03	69	22.12	1	74	23.72	1
Any	140	30.97	22	15.71	0.71 (0.46–1.10)	43	30.71	1.29 (0.94–1.78)
Marital status								
Married	405	88.62	77	19.01	1	111	27.41	1
Divorced/widowed/separated	52	11.38	16	30.77	1.62 (1.03–2.55)	9	17.31	0.63 (0.34–1.17)
Age at 1st pregnancy (yr)								
Under 15	66	15.83	10	15.15	1	14	21.21	1
15–17	172	41.25	47	27.33	1.80 (0.97–3.36)	46	26.74	1.26 (0.74–2.14)
18–20	119	28.54	22	18.49	1.22 (0.61–2.42)	35	29.41	1.39 (0.81–2.39)
21+	60	14.39	6	10.00	0.66 (0.26–1.71)	16	26.67	1.26 (0.67–2.35)
Running water								
No	131	28.23	24	18.32	1	44	33.59	1
Yes	333	71.77	69	20.72	1.13 (0.74–1.72)	77	23.12	0.69 (0.50–.94)
Pap smear inflammation								
Absent	273	58.84	49	17.95	1	67	24.54	1
Present	191	41.16	44	23.04	1.28 (0.89–1.85)	54	28.27	1.15 (0.85–1.57)
Clinical inflammation								
Absent	382	82.33	72	18.85	1	89	23.30	1
Present	82	17.67	21	25.61	1.36 (0.89–2.08)	32	39.02	1.67 (1.21–2.32)
Screening outcome								
Negative	246	53.02	36	14.63	1	62	25.20	1
Positive	205	44.18	48	23.41	1.60 (1.08–2.37)	56	27.32	1.08 (0.79–1.48)
CIN positive	13	2.80	9	69.23	4.73 (2.95–7.59)	3	23.08	0.92 (0.33–2.53)
CMV								
Negative	343	73.92	70	20.41	1			
Positive	121	26.08	23	19.01	0.93 (0.61–1.42)			

[a] Missing data: age, n = 12; education, n = 12; marital status, n = 7; age at 1st pregnancy, n = 47.

tivity), the EBV prevalence in screen-positive women was 1.6-fold higher than that in screen-negative women (23.4% versus 14.6%). Among the screen-positive women, EBV prevalence varied from 20% in VIA-positive women to 29% in Pap-positive and HPV-positive women and to 69% in women who had cervical neoplasia (cervical intraepithelial neoplasia grade 1 [CIN1] or greater [CIN1+]). There was no difference in EBV prevalence among women on the basis of their VIA results; however, EBV prevalence was significantly higher in women who had a positive Pap smear result (29.7% versus 16.8% in those who were negative) or a positive HPV test (29.0% versus 17.8% in those who were negative). In contrast, CMV prevalence was essentially constant at about 25% in screen-positive and screen-negative women overall and for each of the screening tests. Viral load was not correlated with screening test results, but an increase in the median viral load from 25.7 copies per 10,000 cells to 181.2 copies per 10,000 cells was found only for EBV in the cervical neoplasia specimens, although this was not statistically significant (P = 0.09).

In univariate analyses (Table 2), the demographic risk factors associated with EBV shedding were older age and being divorced/widowed/separated. The presence of cervical EBV was also strongly associated with a positive screening test and a diagnosis of CIN1 or greater. Unlike EBV, the women shedding CMV tended to be younger and lack running water in their homes. They were also more likely to have cervical inflammation, defined as visibly apparent erythema, edema, and/or bleeding on contact, as noted by the examining gynecologist. No association with CMV and screening results or disease diagnosis was seen. Additionally, the detection of EBV and CMV DNA in the cervix was not significantly correlated.

Most of these trends remained strong in the multivariate

TABLE 3. Multivariate association of risk factors for EBV infection[a]

Variable	No. of women	PR (95% CI)	Adj. PR[b] (95% CI)
Age (yr)			
25–39	308	1	1
40+	144	2.14 (1.50–3.06)	1.98 (1.39–2.82)
Screening outcome[c]			
Negative	239	1	1
Positive	201	1.59 (1.07–2.34)	1.43 (0.97–2.11)
CIN positive	12	4.43 (2.68–7.31)	3.92 (2.49–6.16)

[a] Data are for 452 women.
[b] Mutually adjusted (Adj.) for all variables in the table.
[c] Categories are mutually exclusive.

TABLE 4. Multivariate association of risk factors for CMV infection[a]

Variable	No. of women	PR (95% CI)	Adj. PR[b] (95% CI)
Age (yr)			
25–39	299	1	1
40+	141	0.56 (0.37–0.84)	0.57 (0.38–0.86)
Clinical inflammation			
Absent	361	1	1
Present	79	1.77 (1.28–2.46)	1.59 (1.14–2.22)
Running water			
No	125	1	1
Yes	315	0.69 (0.50–0.95)	0.70 (0.51–0.96)
Screening outcome[c]			
Negative	230	1	1
Positive	198	1.13 (0.82–1.55)	1.11 (0.81–1.52)
CIN positive	12	1.03 (0.38–2.82)	0.90 (0.33–2.47)

[a] Data are for 452 women.
[b] Mutually adjusted (Adj.) for all variables in the table.
[c] Categories are mutually exclusive.

analyses, as did the differences in risk factors for EBV and CMV (Tables 3 and 4). Women over age 40 years were more likely to have EBV detected at the cervix than women under age 40 years (prevalence ratio [PR] = 1.98; 95% CI = 1.39 to 2.82) but were less likely to have CMV detected (PR = 0.57; 95% CI = 0.38 to 0.86). A similar reduction in prevalence of CMV detection was seen in women with running water in their homes (PR = 0.70; 95% CI = 0.51 to 0.96). Women with clinically apparent cervical inflammation were more likely to have CMV detected in the cervix than women without inflammation (PR = 1.59; 95% CI = 1.14 to 2.22); however, no association between inflammation and detection of EBV was seen. Instead, EBV was more likely to be detected at the cervix of women found to have neoplasia (CIN or greater) than women who were negative on all 3 screening tests (PR = 3.92; 95% CI = 2.49 to 6.16). Women who screened positive on 1 or more tests but who did not have disease were also 40% more likely to be positive for EBV, although this difference was not statistically significant.

DISCUSSION

This study aimed to determine whether the presence of EBV or CMV at the cervix helped explain the large number of false-positive VIA results in the CATCH study. We found that there was no association between VIA results and the presence of EBV or CMV in the cervix.

Overall, 20% of the women in this study were positive for EBV at the cervix and 26% were positive for CMV at the cervix. The viral loads were lower than those generally found in the oral cavity; this is consistent with previous reports (17, 27, 29). Oral samples were not collected in this study, so we are unable to make direct comparisons between cervical and oral EBV and CMV shedding in this study. The considerable number of women who had any virus detected at the cervix is notable, given that this was a population-based sample, whereas most previous studies were performed in sexually transmitted disease (STD) clinics or other nonrepresentative populations. Although HIV testing was not performed on the women in this study, the HIV prevalence in the region was less than 1%. Large ranges of prevalence, from 7% to 66% for CMV and from 7% to 40% for EBV, have been reported in other studies, due at least in part to the differences in the populations studied and the methods used (1, 5, 7, 8, 15, 20, 24, 25, 27).

Both EBV and CMV infections are acquired in childhood, and virus may be shed chronically in adulthood. EBV and CMV are related viruses in the herpesvirus family, but the women had very different risk factors for their detection at the cervix. While EBV infection was more frequent in older women, CMV showed the opposite trend, with a lower prevalence at older ages. This decrease in CMV prevalence with age was also seen by Gradilone et al. (8). In that study, however, EBV prevalence remained constant over time (8).

CMV was also detected more frequently in women who lacked running water in their home and who showed clinical signs of cervical inflammation, suggesting an association between CMV infection and socioeconomic status and/or poor hygiene. From these data it is unclear whether the cervical inflammation is a result of the CMV infection or a risk factor for becoming infected. The association with CMV and cervical inflammation should be further evaluated, since CMV infection has been associated with adverse pregnancy outcomes, which are common in rural India (3, 10, 16). Similar to other studies, we did not see any association between CMV prevalence or viral load and cervical neoplasia (5), suggesting that CMV is unlikely to play a role in the development of cervical cancer.

Interestingly, EBV prevalence was most strongly associated with cervical cancer screening results and detection of disease. EBV prevalence was higher among women who screened positive and/or were diagnosed with CIN1+ than among women who were negative in all screening tests. In fact, women found to have CIN1 or greater were almost 4 times more likely to be EBV positive than women without disease. While the numbers of women with disease are small, the results are striking: 14% of normal women were EBV positive, while 2/2 (100%) women with CIN1, 2/5 (40%) women with CIN2, 2/2 (100%) women with CIN3, and 3/4 (75%) women with cancer were EBV positive. These results concur with those found in a recent

study by Santos et al., where cervical scrapings from women with normal colposcopy findings, women with high-grade CIN, and women with invasive cancer were tested for EBV (18). They found EBV DNA in 9.0% of normal women, 21.2% of women with high-grade CIN, and 64.3% of women with invasive cancers (18). A similar increase in prevalence of cervical EBV DNA shedding has been reported in other studies as well (11, 12, 26). Sasagawa et al. evaluated EBV mRNA expression at the cervix and found that the EBV genes *EBER-1*, *LMP-1*, and *EBNA-2* were expressed significantly more frequently in tissue samples from CIN and cancer patients than women with normal cervices (19). The strong association and high prevalence of EBV in women with disease in this and previous studies support more detailed investigation into the possible role for EBV as a cofactor in cervical carcinogenesis.

An important limitation of this analysis is that while the larger CATCH study involved population-based sampling, this substudy was not a random selection from those enrolled but rather was enriched for women who had previously tested positive by one of the three cervical cancer screening tests. As a result, the proportion of women screening positive is overrepresented: 46% in the subset compared to 31% in the total population. Thus, EBV prevalence may be overestimated in this subsample. The demographic characteristics of the women selected for the substudy were very similar to those of the total population; thus, the overall trends should still be consistent in the larger population. An additional area of concern is that only a single swab specimen was collected, and so we are unable to assess the effect of sampling variability by comparing the viral loads within an individual. Finally, while swab specimens were not collected during menses, there was a high level of cervical inflammation in the population, causing many swabs to be contaminated with blood. It is thus not possible to confirm whether the source of EBV or CMV DNA load was from epithelial shedding or from contaminating peripheral blood in the sample.

Despite these limitations, this study demonstrates a high prevalence of both Epstein-Barr virus and cytomegalovirus in the cervices of a population of women not considered to be high risk (older, married, low HIV prevalence), demonstrating that the presence of these viruses at the cervix is not restricted to those presenting at STD clinics or with HIV/AIDS. The biological and clinical implications of these viruses at the cervix remain to be determined. The strong association between the presence of EBV and cervical disease warrants future exploration to determine whether EBV plays a causal role in disease development or if it is merely a bystander in the process.

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