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Dilution Assessment of Cervicovaginal Secretions Collected by Vaginal Washing To Evaluate Mucosal Shedding of Free Human Immunodeficiency Virus

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A 10 mM concentration of lithium does not interfere with reverse transcription (RT) or PCR. Sampling of cervicovaginal fluid by vaginal washing, with lithium (10 mM) in the washing buffer as a marker of dilution, may be utilized to accurately determine in HIV-infected women, by quantitative RT-PCR, the genital shedding of acellular HIV RNA at the level of the mucosa itself.

Heterosexual intercourse is the predominant mode of transmission of human immunodeficiency virus (HIV) worldwide. Cervicovaginal secretions (CVS) are believed to represent an important vehicle for HIV heterosexual transmission. HIV has been cultured from this fluid, and HIV antigens and HIV genome have been detected in the CVS of HIV-infected women. Although the parameters influencing the HIV transmission rate by infectious CVS are not well understood, the virus load, or the quantity of HIV, in the cervicovaginal fluid must be considered to be one of the major determinants affecting the infectivity of the genital inoculum (6). Indeed, longitudinal studies have shown that the risk of heterosexual transmission increases with advanced stages of HIV infection (3).

Attempts to evaluate the cervicovaginal shedding of HIV have been based upon the detection of HIV provirus by DNA PCR amplification assay of endocervical or vaginal wall swab specimens eluted in a fixed volume of culture medium (2, 8) or upon direct quantitation of HIV RNA in the acellular fraction of a vaginal washing by domestic competition PCR with an internal control (5). One obvious methodological problem occurring with these sampling methods is the difficulty of accurately quantifying the HIV load at the level of the cervicovaginal mucosa itself. Indeed, precise knowledge about the quantity of CVS collected by the swab, or about the dilution factor introduced by the vaginal washing, is lacking.

We have adapted a simple method to permit quantitative evaluation of the dilution factor introduced into CVS collected by a vaginal washing (1). This method consists of adding an inert substance, lithium chloride (LiCl), into the washing buffer used to carry out the vaginal washing for collecting CVS to measure its concentration with a flame absorption spectrophotometer before ($[Li]_1$) and after ($[Li]_2$) the specimen is sampled. Lithium at a concentration of 10 mM gives the most accurate measure. Determination of the dilution factor (dx) of a soluble component introduced by the washing is approximated by the formula $dx = [Li]_1/[Li]_1 - [Li]_2$. By definition, free HIV in female genital secretions is part of the acellular fraction of CVS. The RNA genome may then be considered as belonging to the soluble components of the CVS. Consequently, the dilution factor of free HIV in CVS collected by vaginal washing may be approximated by the ratio dx.

We had previously verified that 10 mM lithium has no effect on immunoassay results. In this study we tested whether LiCl in the sample could affect the efficiency of reverse transcription (RT) and PCR amplification. Indeed, cations may be inhibitory to DNA polymerases such as reverse transcriptase or *Taq* polymerase (4).

In a first step, the effect of LiCl on PCR was evaluated on two DNA targets: a clinical isolate of varicella-zoster virus (VZV) and an internal control containing 310 bp of the gp220-encoding gene of Epstein-Barr virus (EBV) constructed in our laboratory (PCR Mimic Construction Kit; Clontech Laboratories, Palo Alto, Calif.). Five microliters of the supernatant of VZV culture in MRC5 fibroblasts and 1,000 copies of the EBV internal control were subjected to PCR for VZV (7) or for EBV (9) respectively, as previously reported. The composition of the mix for both PCRs (2 IU of *Taq* DNA polymerase [Pharmacia Biotech, Uppsala, Sweden], buffer, nucleotides, and $MgCl_2$) was identical. Increasing concentrations of LiCl were added to the PCR mix, at final concentrations of 1, 10, 25, 50, and 100 mM; a mix without LiCl served as positive control. PCR products were visualized on a 2% agarose gel, stained with ethidium bromide (5 μg/ml), under UV illumination. Band intensity was graded as follows: −, absent (undetectable); 1, faint (better visualized on UV light than on picture); 2, moderate; and 3, strong. VZV PCR and EBV PCR gave the same patterns. PCR products appeared as marked bands at 1, 10, and 25 mM LiCl, a faint intensity of amplicons was observed at 50 mM LiCl, and PCR products were undetectable at 100 mM LiCl. The control without LiCl gave a strong amplification. Increasing concentrations of targeted EBV DNA control (10, 100, 1,000, and 10,000 copies of the EBV internal control) spiked in distilled water with increasing concentrations of LiCl (1, 10, 25, 50, and 100 mM) were further subjected to EBV PCR, and the PCR products were visually read after migration on a 2% agarose gel. For each EBV DNA concentration, PCR inhibition was only observed with LiCl concentrations of 50 mM and higher. These findings show that the cation Li is inhibitory for PCR amplification only at high concentrations, i.e., more than 50 mM. We also evaluated the effect of increasing LiCl concentrations (1, 10, 25, 50, and 100 mM) on EBV PCR analysis of CVS spiked with either 100 or 1,000 copies of the EBV DNA internal control; a control ex-

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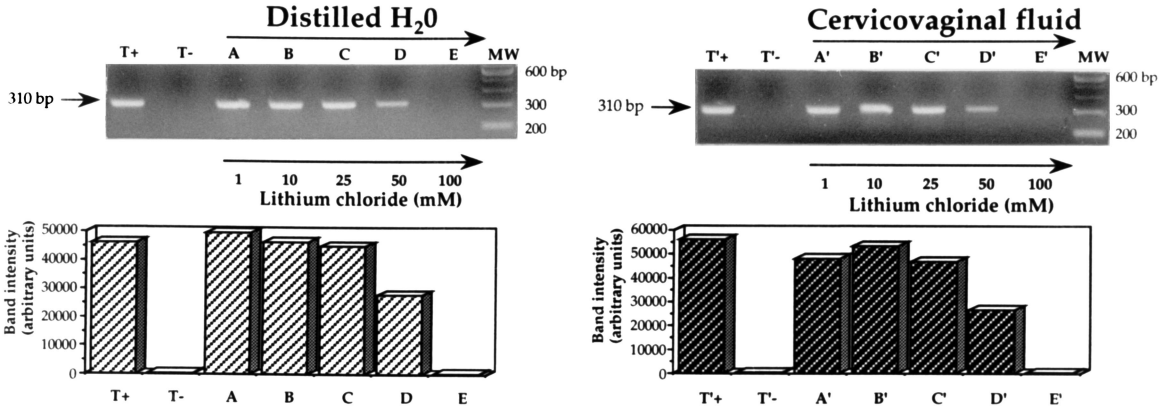

FIG. 1. Visualization of EBV PCR-amplified products by electrophoresis on a 2% agarose-ethidium bromide gel (top) and quantitation of PCR products with a densitometer (bottom). A fixed amount of 1,000 copies of an internal control containing 310 bp of the gp220-encoding gene of EBV was spiked in a pool of EBV-negative cervicovaginal fluids (right), in the presence of increasing LiCl concentrations as indicated, and subjected to EBV PCR after DNA extraction. A control experiment (left) utilized distilled water instead of cervicovaginal secretions. A mix without LiCl served as a positive control (T+ and T′+). A master mix with distilled water instead of DNA served as a negative PCR control (T− and T′−). Molecular weight markers (100-bp DNA ladder; Gibco BRL) are indicated on the right of each gel.

periment utilized distilled water instead of CVS. In this latter experiment, a densitometer (Herolab EASY RH; Fisher Scientific, Hampton, N.H.) was used to read the gels to more accurately determine the intensity of the PCR products. At each concentration of LiCl, the same intensity of PCR products was for the same concentration of EBV DNA spiked either in cervicovaginal fluid or in distilled water (Fig. 1). The inhibitory effect of LiCl was observed with final concentrations of LiCl of 50 mM or above, confirming the previous results assessed by visual reading. Finally, these observations indicate that the possible occurrence of combinations of cations existing in CVS and LiCl does not have an inhibitory effect on PCR. We further confirmed that 1,000, 100, and 10 copies of an EBV DNA internal control in 5 μl of distilled H_2O with or without 10 mM LiCl added to a volume of 45 μl of PCR mix gave exactly the same level of EBV PCR amplification. Taken together, these findings indicate that the amplification of DNA from a microorganism present in the acellular part of CVS, collected by vaginal washing containing 10 mM LiCl, can be easily detected by conventional PCR.

In a second step, the effect of LiCl on RT was evaluated on a pool of plasmas from 10 HIV type 1 (HIV-1)-subtype B-infected individuals, employed as an RNA target, with a RT-PCR conceived for amplification of the *pol* gene of HIV-1, as previously described (10). The HIV RNA load of the pool was estimated to be about 200,000 copies of RNA per ml, as assessed by quantitative PCR (HIV-1 Monitor test; Roche Diagnostic Systems, Inc., Branchburg, N.J.). Three concentrations of HIV-1 RNA (200, 2,000, and 20,000 copies per ml, obtained by dilution of the pool in distilled water) were used for the experiment. Increasing amounts of LiCl were added to the normal RT mix, which contained 200 IU of Moloney murine leukemia virus reverse transcriptase (Superscript II RNase H reverse transcriptase; Gibco BRL, Gaithersburg, Md.), at final concentrations of 1, 10, 25, 50, and 100 mM; a mix without LiCl served as positive control. Final RT-PCR products constantly gave a strong intensity on agarose gels, whatever the concentration of LiCl present during the RT step and for each initial level of HIV-1 RNA. These findings indicate that LiCl has no significant inhibitory effect on reverse transcriptase, even at the highest tested concentrations.

In a third step, we evaluated the possible effect of LiCl on the efficiency of a commercially available RT-PCR designed to quantitate HIV-1 RNA. A total of 100 μl of the previously used pool of plasmas, at 200,000 copies of HIV-1 RNA per ml, was mixed with increasing amounts of LiCl in distilled water (final concentrations of 1, 10, 50, and 100 mM). The viral RNAs within the mixtures of diluted plasma, LiCl, and lysis buffer were then precipitated and extracted, and quantitative evaluation was performed with the HIV-1 Monitor test as recommended by the manufacturer. A control sample without LiCl was processed in parallel. Differences between the measured level and the theoretical quantity of viral RNA (20,000 copies/ml) in each tested sample never exceeded the usual intra-assay variation of the test, i.e., 0.3 log units, indicating that the cation Li, under these experimental conditions, does not significantly inhibit this quantitative PCR, even at its highest concentration in the initial mixture.

We conclude that a 10 mM concentration of lithium does not interfere with enzyme immunoassay (1) or with RT or PCR and that cervicovaginal fluid sampling by vaginal washing, using lithium (10 mM) in the washing buffer as a marker of dilution, may be utilized to accurately determine in HIV-infected women, by quantitative RT-PCR, the genital shedding of HIV RNA at the level of the mucosa itself. Such a procedure could also be used to evaluate by quantitative techniques of molecular biology the mucosal genital shedding of other microorganisms mainly present in the acellular part of CVS.

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