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# Detection of a Transforming Fragment of Herpes Simplex Virus Type 2 in Clinical Specimens by PCR

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**A PCR assay for the sensitive detection of a transforming fragment of herpes simplex virus type 2 (HSV-2) was developed. Oligonucleotide primers were selected in *Xho*-2, a transforming region of the *Bgl*II N fragment of HSV-2. The assay reached a sensitivity endpoint of 10 copies of the *Xho*-2 subfragment and did not show cross-reactivity with other herpesviruses, including HSV-1. All 42 HSV-2 isolates scored positive in the assay. The *Xho*-2 PCR assay was evaluated with 216 clinical specimens and the results were compared with those of cell culture. The best protocol for processing specimens contained in viral transport medium included a centrifugation step followed by cell lysis. Of the 107 specimens positive for HSV-2 by culture, 105 were PCR positive (sensitivity, 98.1%). For one of the two falsely negative samples, β-globin as well as sequences from the HSV-2 DNA polymerase gene could not be amplified. The other sample scored positive in both of these reactions but was indeterminate in duplicate tests by *Xho*-2 PCR. Two of 109 HSV-2 culture-negative specimens tested positive in the PCR assay (specificity, 98.2%). The latter two samples tested positive in a PCR test for the HSV-2 DNA polymerase gene. This novel tool was shown to be sensitive and specific for HSV-2 sequences and should allow for the investigation of the role of HSV-2 in genital cancers.**

Nearly one-fifth of adults in the United States are infected with herpes simplex virus type 2 (HSV-2). Although HSV-2 is the most common cause of genital ulceration in developed countries, subclinical HSV-2 infection affects an important proportion of infected individuals. The diagnosis of HSV-2 infection is commonly performed by cell culture with appropriate clinical specimens (25). However, the ability to isolate HSV-2 in cell culture is reduced when specimens from old lesions are used, in the presence of a host immune response, and during episodes of reactivation.

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In recent years, the use of PCR has allowed for the detection of HSV-2 DNA in patients with diseases for which culture failed to establish a diagnosis, such as herpes simplex encephalitis (23). Since it does not require the presence of infectious viral particles, PCR has also demonstrated the presence of viral DNA in culture-negative specimens (5, 24, 30, 32). PCR could thus prove to be useful for the analysis of specimens sent to the laboratory from remote clinics. PCR could also detect asymptomatic shedding of HSV-2 (18), a clinical situation in which the number of virions released is low (6).

Inactivated HSV-2 can transform rodent or human cells (21). Because of its importance as a sexually transmitted agent and because of its transforming abilities in vitro (21), HSV-2 had been proposed initially as a causative agent of genital cancer in women (17). Conflicting in vitro and in vivo experiments have shed doubts on the role of this viral agent in cancer of the uterine cervix (17). However, a transforming region of the HSV-2 genome, the 7.6-kb *Bgl*II N (map units 0.58 to 0.63) segment (15, 21), has been described. Initially, the transforming ability of HSV-2 was thought to be located on the left end (*Xho*-3 fragment) of the *Bgl*II N segment (15, 16). However, neither the presence of a viral protein (13, 20, 36) nor the persistence or integration (15, 31, 36) of specific HSV sequences seemed to be required for the maintenance of the transformed state (31). The transforming ability of HSV-2 has been left unexplained.

Transfection of NIH 3T3 cells with the right end (22, 31, 33) of the *Bgl*II N fragment (the *Xho*-1 and *Xho*-2 subfragments) showed an increase in the number of transformed foci, and HSV-2 sequences were retained more efficiently in transformed cells (31). Preliminary results also showed that cells

transfected with *Xho*-2 and *ras* generated tumors in nude mice and that *Xho*-2 sequences were retained in tumors (21a). *Bgl*II-N sequences can also cooperate with oncogenic human papillomaviruses to transform cells (10).

PCR assays amplifying sequences from the left end of *Bgl*II-N have been described (27, 37). Those studies demonstrated that, in contrast to normal tissue, precancerous or cancerous lesions of the uterine cervix contained specific HSV-2 DNA sequences. We have cloned and sequenced the *Xho*-2 subfragment of *Bgl*II-N. The goal of the present work was to develop and optimize a PCR assay for the specific detection in clinical specimens of transforming sequences of HSV-2 from the right end of *Bgl*II-N. Various clinical isolates of herpesviruses were evaluated in vitro in this PCR assay. The optimized PCR assay was then compared with cell culture for their abilities to detect HSV-2 infections in specimens collected from various sites and submitted to the diagnostic laboratory for viral culture.

(This work was presented in part at the Canadian Association for Clinical Microbiology and Infectious Diseases meeting, Montréal, Québec, Canada, November 1994.)

## MATERIALS AND METHODS

**Viral isolates.** Undiluted cell culture supernatants of human cytomegalovirus (CMV), varicella-zoster virus (VZV), HSV-1, and HSV-2 isolated from clinical specimens were kept frozen in liquid nitrogen for at most 1 year. The Raji cell line (which contains the Epstein-Barr virus [EBV] genome) and the carcinoma cell line HeLa (which contains 40 copies of human papillomavirus type 18 [HPV-18] DNA per cell) were obtained from the American Type Culture Collection (Rockville, Md.). The plasmid designation p*Xho*-1+2 in this report refers to the *Xho*-1 and *Xho*-2 subfragments of *Bgl*II-N cloned into a pBR322 vector as described by Saavedra and Kessous-Elbaz (33). Uninfected human MRC-5 diploid fibroblasts were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum.

**Clinical samples.** Between January and October 1994, 216 clinical specimens were consecutively obtained from 151 individuals (48 men, 91 women, and 12 individuals whose gender was unknown). A total of 137 genital specimens, 71 nongenital specimens, and 8 specimens from unknown sites were obtained. Samples were obtained from the following sites: genital lesions ($n$ = 128), noncutaneous and extragenital sites ($n$ = 32), cutaneous vesicles or ulcers ($n$ = 26), perioral and oral lesions ($n$ = 13), perianal lesions ($n$ = 9), and unidentified origin ($n$ = 8). The noncutaneous and extragenital specimens included 27 bronchoalveolar lavage specimens, 3 urine specimens, 1 eye scraping, and 1 esophageal brushing. They were selected from stored aliquots kept at −20°C on the basis of growth in cell culture of herpesviruses other than HSV-2 to evaluate the specificity of the HSV-2 PCR assay. Cutaneous and mucosal specimens were collected on Dacron swabs from lesions in the throat, mouth, genital tract, or skin. Swabs were placed immediately into 2 ml of viral transport medium (Hank's medium supplemented with 1% HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 2% fetal calf serum, 25 μg of vancomycin, 10 μg of gentamicin, and 1.25 μg of amphotericin B) and were transported to the laboratory in less than 4 h. The project had the approval of the Ethics Committee of l'Hôpital Notre-Dame.

**Viral culture.** For the preparation of each cell line monolayer, 0.2 ml of a suspension of 500,000 cells per ml of MEM containing 10% fetal calf serum was added to each well of a polystyrene microplate (Falcon 3072 Microtest III 96-well plate with flat-bottom wells; Becton Dickinson, Lincoln Park, N.J.). The plates were incubated at 37°C in 5% $CO_2$, and confluence generally occurred within 3 days after inoculation.

For each specimen, 50 μl of viral transport medium was inoculated immediately into each of six wells of a 96-well microtiter plate (4, 25) containing the following monolayer cell lines in duplicate wells: Vero, Mink lung, and MRC-5 (diploid human embryonic lung fibroblasts) cell lines. The microplates were centrifuged at 2,800 rpm (IEC-centra-8R centrifuge; International Equipment Company) at 37°C for 30 min. Each of two shell vials with MRC-5 cells was also inoculated with 200 μl of viral transport medium (6, 25). If culture could not be immediately performed, the specimen was kept at 4°C for at most 18 h. A portion of the viral transport medium (1,500 μl) was routinely kept at −20°C, until completion of the cell culture, for reinoculation onto cell lines if necessary.

The plates were incubated at 37°C for 28 days in 5% $CO_2$ and were routinely observed for the presence of a cytopathic effect. When a cytopathic effect was observed, HSV was typed with fluorescein-labeled monoclonal antibodies against HSV-1- and HSV-2-specific antigens (Pantho Dx; Diagnostic Products Corporation, Los Angeles, Calif.). Identification was also done by immunofluorescence staining with monoclonal antibodies for VZV (Merifluor VZV; Meridian, Cincinnati, Ohio) and CMV (gift of clone E-13 from Y. Perrol, Hôpital Saint-Louis, Paris, France).

**Treatment of samples for PCR.** Aliquots of specimens in viral transport medium kept at −20°C were prepared as described in the Results section (see below). After processing, the pellet was resuspended in a solution of 20 mM Tris (pH 8.3). Samples were lysed by the addition of Tween and Nonidet P-40 each to final concentrations of 0.4% (vol/vol), and were digested for 2 h at 60°C with proteinase K at a final concentration of 250 μg/ml. Cell lysates were denatured at 95°C for 10 min and were stored at −70°C until they were tested by PCR.

**Oligonucleotide selection.** Primer sequences were selected from the sequence data of the *Xho*-2 subfragment of *Bgl*II-N (21b). Primers *Xho*-a and *Xho*-b were used to amplify *Xho*-2 sequences, and *Xho*-p was used as the internal probe for the detection of PCR-amplified products. Oligonucleotides were synthesized by the Regional DNA Laboratory Synthesis, Calgary, Alberta, Canada.

The sequences of primers and probe were as follows: *Xho*-a (upstream primer), 5′-AACACTGCCTCGGAGGGGATGAT-3′; *Xho*-b (downstream primer), 5′-AGGCCGGTGCTCCCGATCACGAA-3′; and *Xho*-p (internal probe), 5′-TTCGTGGGGTCTGTCGTTCTGT-3′.

**Genomic amplification for HSV-2 (*Xho*-2 PCR).** Samples were tested by PCR with no prior knowledge of the culture results. Ten microliters of each sample lysate was added to 90 μl of a PCR mixture containing (final concentrations) 10 mM Tris (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ (Gene Amp PCR buffer II; Perkin-Elmer Cetus, Montréal, Québec, Canada), 50 pmol each of *Xho*-a and *Xho*-b, 200 μM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP), and 2.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (Roche Molecular Diagnostics, Mississauga, Ontario, Canada). The samples were overlaid with 100 μl of mineral oil and were amplified in a 480 DNA thermal cycler heat block instrument (Perkin-Elmer Cetus) through 35 cycles of denaturation at 94°C for 1 min, primer reannealing at 60°C for 1 min, and primer extension at 72°C for 1 min. In each run, a buffer-negative control and 10 copies of the plasmid p*Xho*-1+2, were included. A run was discarded if one of the negative controls tested positive or if the 10-copy positive control scored negative. Discordant results between culture and the *Xho*-2 PCR were resolved by supplementary testing. Samples were retested in duplicate by *Xho*-2 PCR to control for primer efficiency, were amplified for β-globin with PCO4-GH20 primers (2) to control for DNA integrity and the presence of inhibitors, and were tested by a standard PCR test for the detection of sequences from the DNA polymerase gene of HSV-2 (30) with primers HCJ3-HCJ12 and probe HCJ6.

Measures taken to avoid false-positive reactions because of contamination included the use of disposable pipettors for the initial processing of clinical samples or isolates, performance of each step of the PCR in different areas of the laboratory, the use of filter pipette tips (USA Scientific Plastics Inc., Ocala, Fla.), the use of aliquoting of the PCR reagents, inclusion of multiple negative controls per run, and meticulous laboratory procedures. Pipettors for pre-PCR manipulations were used solely for this purpose.

**Detection of HSV-2 DNA-amplified products.** Confirmation of the specific amplification of *Xho*-2 sequences was obtained by hybridization of the amplified products with an internal oligonucleotide in a dot blot assay. Ten microliters of amplified products was mixed with 100 μl of denaturing solution composed of 0.4 N sodium hydroxide and 25 mmol of EDTA per liter, and the mixture was applied to replicate nylon membranes. Filters were prehybridized at 62°C for 30 min in 6× SSC (1× SSC is 0.15 sodium chloride and 0.015 M sodium citrate [pH 7.0]), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 100 mg of denatured salmon sperm DNA per ml. The internal probe *Xho*-p, which was end labelled with [$^{32}$P]ATP, was added to the hybridization buffer at 150,000 cpm/ml. Hybridization was performed for 90 min at 62°C. Blots were washed twice for 15 min at 62°C in 2× SSC–0.1% SDS. Autoradiograph exposures were obtained 16 h after hybridization.

**Statistical analysis.** The sensitivity and specificity of the *Xho*-2 PCR were first calculated by considering culture to be the "gold standard." The crude percentage agreement between both detection methods was the percentage of samples for which the results were identical. The kappa statistic was calculated to adjust for chance agreement between detection methods (11). In general, a kappa value above 0.75 represents excellent agreement beyond chance. Because PCR is more sensitive than culture (4, 24, 32), the sensitivity and specificity of PCR were also calculated against an expanded gold standard (26), which was the presence of a positive culture result or reactivity in the *Xho*-2 PCR test confirmed by the standard HSV-2 DNA polymerase PCR test (30) for culture-negative samples.

## RESULTS

**Optimization of HSV-2 PCR.** *Xho*-2 is a 2.479-kb fragment with a G+C content of 71.4% (21b). Primer sequences were selected within a potential open reading frame of *Xho*-2 located between nucleotides 179 and 598 (21b). We avoided GC stretches and nucleotide positions within a palindromic sequence located between base pairs 1401 and 1500 of the sequenced fragment and forming a stem loop structure (21b). The primers selected, *Xho*-a and *Xho*-b, did not form primer

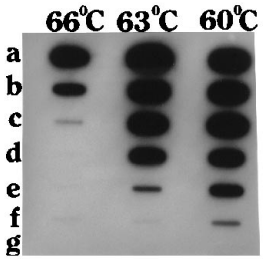


FIG. 1. Influence of the primer reannealing temperature on the efficiency of *Xho*-2 PCR for the detection of HSV-2 DNA. Serial fivefold dilutions of p*Xho*-1+2 (row a, 15,625 copies; row b, 3,125 copies; row c, 625 copies; row d, 25 copies; row e, 5 copies; row f, 1 copy; row g, no copies) were amplified by *Xho*-2 PCR under different primer reannealing temperatures (66, 63, and 60°C) and were hybridized with radiolabeled *Xho*-p.

dimers, had similar G+C contents, and did not contain repetitive sequences. The primer and probe sequences were unique to HSV-2 by comparison with all human, viral, and bacterial sequences in the GenBank database (Los Alamos National Laboratories) and by alignment by using the BLAST sequence analysis software (1) from the Genetic Computer Group. The *Xho*-a and *Xho*-b primers define a 189-bp DNA fragment from HSV-2.

The various parameters of the PCR were first optimized by amplifying 10,000 copies of p*Xho*-1+2. These amplification products were migrated on an agarose gel and stained with ethidium bromide. The intensities of the bands generated through the different PCR conditions were compared. A HotStart PCR technique at 75°C did not alter the specificity or sensitivity of detecting the HSV-2 DNA template (data not shown). While the efficiency of amplification was reduced for concentrations of $MgCl_2$ below 2 mM, no difference was recorded for concentrations ranging from 2 to 4 mM (data not shown). The $MgCl_2$ concentration was adjusted to 2.5 mM for the remaining experiments.

Because of the high G+C content of *Xho*-2, we tried to improve the sensitivity of the *Xho*-2 PCR test by increasing the length of the denaturation step from 1 to 2 min. Four clinical specimens contained in viral transport medium and 10,000 and 3,300 copies of p*Xho*-1+2 were amplified with various denaturation times. The increased denaturation time resulted in increased signals for HSV-2 DNA detection for all specimens (data not shown).

In order to study the influence of the reannealing temperature on the performance of PCR for the detection of HSV-2 DNA, fivefold dilutions of p*Xho*-1+2 were amplified under various reannealing temperatures, analyzed by gel electrophoresis, and reacted with the internal probe at 62°C. Temperatures above 60°C generated less intense nonspecific bands on ethidium bromide-stained gels from non-HSV-2 herpesviruses (data not shown) and decreased sensitivity (Fig. 1). At 60°C, a very low level of background reactivity was inconsistently observed. This background reactivity was similar to that of the negative control containing the usual master mixture but without *Taq* polymerase (data not shown). When present, the background reactivity was always weak and much lower than that of the 10-copy HSV-2 DNA control (data not shown).

**In vitro sensitivity of *Xho*-2 PCR for detection of HSV-2 DNA.** To estimate the sensitivity of the PCR assay for the detection of *Xho*-2 DNA, serial fivefold dilutions of p*Xho*-1+2 were prepared, and each dilution was tested for the presence of *Xho*-2 DNA by PCR and hybridization with the internal probe. The results are presented in Fig. 1. The assay could detect one copy of p*Xho*-1+2 DNA. Using DNA extracted from HSV-2, we reached a similar endpoint (data not shown).

**Specificity of *Xho*-2 PCR.** To determine if primer and probe sequences were conserved among HSV-2 isolates, 43 HSV-2 isolates from undiluted cell culture lysates kept frozen in liquid nitrogen were lysed and tested by PCR. Viral isolates came from different individuals. All yielded a specific 189-bp band and reacted with the internal probe in the dot blot assay (Fig. 2).

To confirm that the primers and probe selected were specific for HSV-2 sequences, cell culture lysates from non-HSV-2 herpesviruses isolated from different individuals were treated as described above and analyzed by PCR. Test supernatants included 10 CMV isolates, 11 VZV isolates, 18 HSV-1 isolates, and 1 μg of DNA extracted from Raji cells (Fig. 3). We also analyzed 1 ng of HIV-1 proviral DNA contained in pSP64, human DNA from 100,000 lysed peripheral blood mononuclear cells, and 1-ng plasmid vectors containing HPV DNA inserts of types 6, 11, 16, 18, 31, 33, 35, and 45. After amplification of these various templates with *Xho*-a and *Xho*-b primers, no specific DNA product could be identified by hybridization with *Xho*-p.

**Treatment of clinical samples.** The optimal protocol for processing clinical specimens contained in viral transport medium for PCR was determined by comparing three sample preparation methods. Three samples contained in viral transport medium that had been positive by culture for HSV-2 were freeze-thawed, pooled, and treated as follows. In the first method, 300 μl of the pooled specimens in viral transport medium was centrifuged at 13,000 rpm for 10 min in a microcentrifuge (Eppendorf minifuge). The pellet was resuspended in 100 μl of 10 mM Tris (pH 7.0)–0.1 mM EDTA and was lysed with detergents and proteinase K at 60°C for 2 h as described in Materials and Methods. After denaturation at 95°C for 10 min, 10 μl of lysate was added to the PCR master mixture, and amplification was done by the standard procedure. In the second specimen treatment protocol, 300 μl of specimen in viral transport medium was lysed with detergents and proteinase K at 60°C for 2 h and denatured at 95°C for 10 min. DNA was then purified with two phenol-chloroform extractions and was precipitated with ethanol. The pellet was resuspended in 30 μl of TE, and 10 μl was amplified by PCR. In the third protocol, 300 μl of specimen in viral transport medium was lysed as

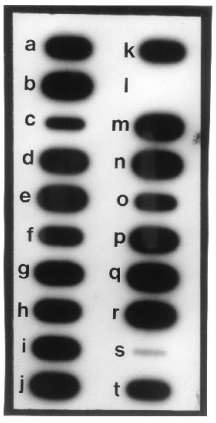


FIG. 2. Detection of various HSV-2 isolates by *Xho*-2 PCR. Various cell culture supernatants from which HSV-2 had been grown (dots a to k and m to r) were treated and amplified as described in Materials and Methods. Dot t, a positive control from a cell culture supernatant containing HSV-2; dot s, the 10-copy control of p*Xho*-1+2; 1, the blank negative control (buffer control).

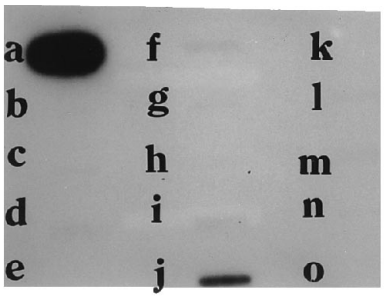


FIG. 3. Specificity of the *Xho*-2 PCR test for HSV-2 detection. Cell culture supernatants were treated and amplified as described in Materials and Methods. The isolates tested were CMV (dots b, c, and d), HSV-1 (dots e, f, g, and h), and VZV (dots i and k). One microgram of extracted DNA from Raji cells was also tested (dot l), as was 1 ng of HPV-16 (dot m), and 100,000 lysed peripheral blood mononuclear cells (dot n). Dot a, the strong positive control from a cell culture supernatant of an HSV-2-positive sample; dot j, the 10-copy control of p*Xho*-1+2; and dot o, the blank negative control (buffer control).

described above at 60°C for 2 h and denatured at 95°C for 10 min. Ten microliters of the lysate was then added directly to the PCR mixture and amplified. Products of amplification from each protocol were then migrated on a 2% agarose gel stained with ethidium bromide. The specimen preparation by the first protocol was more efficient than that by the other protocols (data not shown) and was selected for use in the comparison of cell culture and *Xho*-2 PCR with clinical specimens.

**Performance of PCR with clinical samples.** A total of 176 frozen specimens sampled from cutaneous and mucosal sites that had been cultivated for HSV before freezing were randomly retrieved and processed for PCR by using the first specimen treatment protocol described above. Thirty-two specimens from noncutaneous extragenital sites were also included to evaluate the cross-reactivity of *Xho*-2 PCR with other herpesviruses isolated from those clinical specimens. Eight specimens from unknown sites were also obtained. All samples were prospectively collected in viral transport medium and were kept frozen at −20°C after inoculation for cell culture. The presence of HSV-2 DNA was determined by dot blot analysis of PCR-amplified products hybridized with the radiolabeled *Xho*-p probe. Since 50 μl of viral transport medium was inoculated into each of six wells containing various cell lines, a total of 300 μl was tested in cell culture. The same quantity of viral transport medium was processed for PCR, but only 1/10 the amount was introduced in each PCR run. Of the 151 participating individuals, 127 patients each provided one specimen and 24 patients each provided more than one specimen. An average of 1.43 ± 1.62 specimens per patient was tested (range of the number of specimens tested per patient, 1 to 15; median, 1.00 specimen per patient).

Overall, viral sequences were detected in 107 (49.5%) of 216 specimens (Fig. 4A). *Xho*-2 DNA sequences were detected in 105 (sensitivity, 98.1%) of 107 specimens with culture-proven HSV-2 infections. Both tests were negative for HSV-2 in 107 specimens tested and both were positive in 105 specimens tested, for a concordance between these methods of 98.1% (212 of 216 specimens) and a near perfect kappa value of 0.96. Cell culture was negative for HSV-2 for 109 specimens, of which CMV was isolated in 15 specimens, VZV was isolated in 5 specimens, and HSV-1 was isolated in 22 specimens. Two of the culture-negative specimens contained HSV-2 DNA sequences by the *Xho*-2 PCR assay, for a specificity of 98.3% (Fig. 4B).

**Evaluation of discordant specimens.** *Xho*-2 PCR and cell culture provided discrepant results for four specimens. We investigated the discrepant test results by retesting duplicate specimen lysates by the *Xho*-2 PCR test, a standard HSV-2 PCR test for the DNA polymerase gene (30), and a β-globin PCR test. Both of the PCR-positive, culture-negative specimens scored positive in the HSV-2 DNA polymerase and *Xho*-2 PCR assays. One of these individuals had applied topical acyclovir on the lesion, while the other one was receiving oral acyclovir. For both individuals, previous samples were positive by culture and PCR for HSV-2 on the day before the PCR-positive, culture-negative specimen was obtained (Fig. 4B). They were thus considered true positives for HSV-2. One of the two falsely negative *Xho*-2 PCR specimens was indeterminate in duplicate tests by *Xho*-2 PCR (one positive and one negative reaction). Positive results were obtained for β-globin and HSV-2 DNA polymerase sequences for the latter specimen. The other *Xho*-2 PCR-negative sample tested negative for β-globin and HSV-2 DNA polymerase sequences, suggesting that this specimen may have been inadequate for PCR analysis. Dilution of the β-globin-negative sample did not improve the results obtained by the PCR assays. The HSV-2 isolates from cell culture supernatants from both patients were amplified by *Xho*-2 PCR.

The sensitivity and specificity of each technique were recalculated by using an extended gold standard definition of HSV-2 infection (see Materials and Methods). Overall, culture and PCR were each positive for 107 (98.3%) of 109 specimens from patients with HSV-2 infections. The specificity of PCR reached 100%.

## DISCUSSION

The present study demonstrated that HSV-2 *Xho*-2 DNA sequences could be detected in vitro and in clinical specimens by PCR, even in culture-negative samples. Type-specific PCR assays (24, 29, 32, 34) that use probes, restriction endonuclease cleavage (32), or nonisotopic hybridization (35) had already been described for the detection and typing of HSV isolates in clinical specimens. Most of these assays focused on the DNA polymerase gene. We detected and typed only HSV-2, since we wanted to develop and validate a method to evaluate the role of HSV-2 transforming sequences in cervical cancer of the uterus. In contrast to previous publications, our assay did not require extensive steps for DNA purification (16, 24, 32).

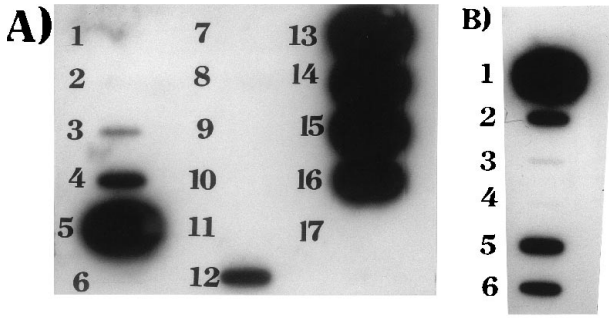


FIG. 4. Detection of *Xho*-2 DNA in clinical specimens. Samples were treated and amplified by *Xho*-2 PCR as described in Materials and Methods. (A) Specimens 1, 2, 7 to 11, and 17 were negative for HSV-2 by PCR and culture, while specimens 3, 4, 5, and 13 to 16 were positive by both tests. Specimen 12, the 10-copy control of p*Xho*-1+2; specimen 6, the negative control. (B) Serial specimens from the same individual were tested by PCR and culture. Specimens 1, 5, and 6 were positive by culture and PCR, while specimens 3 and 4 were negative by both tests. Specimen 2 was negative by culture but positive by PCR and was obtained 1 day after sample 1 was obtained.

The problems encountered in the optimization of the assay were related to the high G+C contents of the amplified segment, primers, and probe used. The PCR as well as hybridization conditions required high temperatures to avoid nonspecific signals. The prolongation of the denaturation step over 1 min increased the sensitivity of the assay by allowing a better denaturation of the template sequences and complete exposure of those sequences to primers. The assay under optimized conditions was shown to be specific for HSV-2 without cross-reactivity with other herpesviruses, human genomic sequences, or HPV DNA. The in vitro endpoint sensitivity of the assay reached one copy of HSV-2 DNA.

The overall sensitivities of cell culture and *Xho*-2 PCR for HSV-2 detection with an expanded gold standard were similar. In one publication report (25), the microtiter culture system was more sensitive than standard tube cultures using diploid fibroblast cells. An in-house comparison of the microtiter plate and standard tube culture methods showed that both systems were equivalent in terms of sensitivity and specificity (31a). The inclusion of a centrifugation step when using microplates and the use of multiple cell lines increase the sensitivity of detection of HSV-2 (31a).

As in other PCR assays, some culture-negative samples were PCR positive (24, 32). The presence of sequences from another open reading frame of HSV-2 was documented in both culture-negative specimens, suggesting the presence of virus that could not be recovered by culture. Confirmation of reactivity in PCR-positive and culture-negative specimens by PCR assays targeted at other regions of the genome have been applied with success to other infectious agents (26). Consequently, we decided to confirm the presence of HSV-2 DNA in culture-negative yet *Xho*-2 PCR-positive specimens in order to exclude positive reactions caused by the carryover of PCR products. This second PCR test is a standard test which amplifies a segment of the DNA polymerase gene of HSV-2 (30), a gene that is essential for viral replication and that contains highly conserved sequences. False-positive PCRs as a result of contamination did not appear to be a problem during the present study, as evidenced by the repeatedly negative results for the negative controls. Amplification of β-globin sequences also permitted us to control for the integrity of cellular DNA and the quantity of cellular material treated in the sample (7).

PCR can also detect HSV DNA sequences for longer periods than viral culture (32). Culture reaches a sensitivity equivalent to those of sensitive direct detection assays if inoculation is performed immediately (8). In our study, a delay of up to 18 h sometimes occurred before inoculation into cell culture. This time frame represents more closely the usual clinical situation and could favor direct detection tests because of the loss of viability in delayed culture.

Various infectious agents have been investigated for their potential association with cervical cancer, including *Treponema pallidum*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, HPV, HSV-2, and CMV (12). Laboratory and epidemiological studies support the role of HPV in genital cancers (12). Compelling evidence suggests the interaction of multiple factors in cancer of the uterine cervix (12), and cofactors to HPV infection are thought to be required for progression to invasive cancer (19). In vitro, HPV often requires a second oncogene to reach tumorigenicity (9, 28).

Conflicting in vitro and in vivo results have shed doubts on the role of HSV-2 in cancer of the uterine cervix (17). Transformation of immortalized rodent cells to a malignant phenotype can be accomplished with HSV-2 subfragments (14, 16, 17, 21, 31). By analysis of specific HSV-2 fragments generated by restriction endonuclease digestion, two regions of the HSV-2 genome were shown to transform further immortalized cells. These regions of the HSV-2 genome were designated *Bgl*II-C and *Bgl*II-N (3, 14, 16, 21). The *Bgl*II-N fragment is capable of converting epithelial cells immortalized by HPV-16 into tumorigenic cells in nude mice (10).

There is an interest in detecting specifically transforming regions of HSV-2 in clinical samples, since other regions of the HSV-2 genome do not seem to be retained in tumor tissue even when PCR is used to detect them (37). One group used PCR for the detection in clinical samples of a BC 24 DNA fragment located at the left end of *Bgl*II-N (37). HSV-2 DNA sequences have been amplified from 14% of uterine cervix squamous cell carcinomas, 40% of cervical intraepithelial lesions, and 27% of cervical adenocarcinomas. Only 2 of the 15 samples in the latter study containing HSV-2 *Bgl*II-N sequences also contained HSV-2 DNA polymerase sequences (37). All of the HSV-2-positive samples also contained HPV-16 or -18 sequences. Thus, HSV-2 is not considered a major determinant of cervical cancer but could serve as a cofactor for a subset of women.

Our goal was not to develop a tool that could replace established methods for the diagnosis of HSV infection but to optimize and validate an assay that will allow for the appropriate investigation of the role of HSV-2 as a cofactor in genital cancers. This novel PCR assay will allow us to investigate, in cohorts of women with normal cervices or precancerous lesions of the uterine cervix, the value of detecting HSV-2-related sequences to predict the development of high-grade or cancerous lesions. In the near future, we will develop a nonisotopic assay to facilitate the mass screening of clinical samples. The assay described here is the first PCR assay which allows for the detection of HSV-2 DNA sequences from the right end of the fragment *Bgl*II-N, which is the most likely candidate responsible for the transforming ability of HSV-2 in vivo.

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