The laboratory diagnosis of herpes simplex virus infections

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A Singh, J Preiksaitis, B Romanowski. The laboratory diagnosis of herpes simplex virus infections. Can J Infect Dis Med Microbiol 2005;16(2):92-98.

Herpes simplex virus (HSV) types 1 and 2 cause genital herpes infections and are the most common cause of genital ulcer disease in industrialized nations. Although these infections are very common, the majority of them remain underdiagnosed because they are asymptomatic or unrecognized. A clinical diagnosis of genital herpes should always be confirmed by laboratory testing; this can be accomplished through the use of direct tests for viral isolation, the detection of antigen or, more recently, the detection of HSV DNA using molecular diagnostic techniques. Testing for serotypes is recommended because of the different prognostic and counselling implications. Type-specific HSV serology is becoming more readily available and will enhance the ability to make the diagnosis and guide clinical management in select patients.

Key Words: *Diagnostic; Genital herpes; Genital ulcer disease; Herpes simplex virus; STI*

Le diagnostic de l'herpès en laboratoire

Les herpès simplex virus (HSV) de types 1 et 2 provoquent des infections génitales et sont la cause la plus fréquente des ulcères affectant les parties génitales dans les pays industrialisés. Bien qu'elles soient très répandues, la majorité de ces infections passent inaperçues parce qu'elles sont soit asymptomatiques soit ignorées. Le diagnostic de l'herpès génital doit toujours être confirmé en laboratoire, soit par le biais de tests de dépistage du virus, de son antigène ou, plus récemment, de l'ADN du HSV par technique moléculaire. On recommande de confirmer les sérotypes en raison de leurs implications sur le pronostic et le counselling. La typologie du HSV est de plus en plus accessible et facilite l'établissement du diagnostic et permet d'orienter la prise en charge des patients.

Genital herpes simplex virus (HSV) infection is extremely common throughout the world, with epidemiological surveys demonstrating rising infection rates in most countries (1,2). HSV is the most common cause of genital ulcer disease in industrialized nations, and infections may be due to HSV types 1 or 2 (2). Although the majority of genital herpes is due to HSV-2, an increasing proportion is recognized as being due to HSV-1 (2). Although the clinical course of acute first episode genital herpes among patients with HSV-1 and HSV-2 infections is similar, the frequency and severity of recurrences is less with HSV-1 than with HSV-2 (3). In addition, the severity of clinically apparent first episodes and reactivation with HSV-2 infection are lower in those with prior HSV-1 (2). Despite increased awareness of these infections, they remain underdiagnosed because the majority of infections are asymptomatic or unrecognized (4). Symptomatic infections may present in unusual or atypical ways, increasing the diagnostic challenge (5). Most transmission to partners, or less commonly to the neonate, occurs while the infected person is asymptomatic (6,7). Infection with HSV has also been shown to increase the risk of acquisition or transmission of HIV infection (8). Antiviral therapy reduces subclinical shedding of HSV, thus significantly reducing transmission (9). Given the complex issues involved in the management of genital HSV infection, the challenge for the clinician is to determine when and how to test for genital herpes infection.

There have been many recent advances in diagnostic techniques for HSV infections, including new viral detection methods and serological tests. The clinical diagnosis of genital herpes should always be confirmed by laboratory testing, including serotyping, because the serotype influences both the prognosis and counselling. The definitive diagnosis of genital herpes relies on demonstrating the presence of HSV in the genital area, either by virus isolation or detection of antigen. In some laboratories, the detection of HSV DNA using molecular diagnostic techniques is replacing viral culture and antigen detection. Serological testing is sometimes useful in symptomatic patients when direct methods have yielded negative results or in asymptomatic patients to determine past or present infection. The value of any laboratory test for the diagnosis of HSV infection will depend on the type of test, the quality of the specimen obtained, the ability of the laboratory to perform the test accurately, and the interpretation of the test results by the requesting clinician.

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SPECIMEN CHOICE, COLLECTION AND TRANSPORT

Direct methods

Specimens obtained from vesicular lesions within the first three days after their appearance are the specimens of choice, but other lesion material from older lesions or swabs of genital secretions should be obtained if suspicion of HSV infection is high (10,11). Once crusting and healing have begun, the recovery rate of HSV drops sharply. The use of alcohol or iodophors to cleanse the lesions may inactivate the virus and should therefore be avoided. Calcium alginate swabs are toxic to HSV and therefore should not be used (12).

The vesicle should be unroofed with a sterile needle or scalpel, and a sterile Dacron or rayon swab with a plastic shaft should be rotated firmly in the base of the lesion to allow epithelial cells to be collected onto the swab. Ideally, more than one lesion should be sampled. Similarly for ulcerative lesions, a swab should be firmly rotated in the base of one or more lesions. The swab(s) should be immediately inserted into viral transport medium such as M5 transport medium. The swab's shaft should be broken before the cap is replaced so that the shaft will not interfere with closure and leakage will be prevented. The specimen should be held at 4°C and transported to the laboratory for further processing within 48 h. During transportation, the specimen should be protected from heat by including a cold pack or ice cubes in a sealable plastic bag in the package. Virus specimen collection swabs with matching transport tubes are commercially available.

A cytospin preparation of the original viral transport medium is the best way to prepare a slide for direct fluorescence assay (DFA) because of the quality of the resulting slides. At the bedside, slides may be prepared by the clinician by rolling the swab, collected as above, firmly over one or more discrete areas on a microscope slide. Alternatively, the base of the lesion may be scraped with a spatula or similar instrument without causing the lesion to bleed, and the material should be applied to a glass slide over one or more 5 mm to 10 mm diameter areas. The glass slide should then be allowed to air dry. When a slide is made for DFA, multiple smears may be made to allow for staining with specific HSV-1 and HSV-2 antisera. Teflon-coated slides with circumscribed wells are available commercially for this application.

The Tzanck test is rarely used now for diagnosis. However, material for this procedure can be collected by scraping the base of the lesion with the edge of a scalpel blade; the material on the blade is then touched to a microscope slide and allowed to air dry (13). Alternatively, material can be collected by firmly swabbing the base of the lesion with a cotton or Dacron swab. A smear is then prepared by rolling the swab on a microscope slide.

Electron microscopy on lesion fluid may yield positive results in some instances. This procedure, although rapid, is relatively insensitive and usually yields positive results only on external lesions such as those occurring on the buttocks or thighs. The positivity rate on mucous membranes is lower. Fluid is collected, preferably from an unbroken vesicle, using a tuberculin or similar syringe and needle, using only enough suction to bring the fluid into the needle but not into the syringe. The drop of fluid is placed on a microscope slide and allowed to air dry. Alternatively, the vesicle is broken and a microscope slide is touched onto the exposed drop of fluid. The slide is allowed to air dry and is then transported to the laboratory in the usual way. If the laboratory is nearby and the specimen is transported there immediately, the syringe can be used to inoculate a cell culture tube by drawing the cell culture fluid up into the needle and expelling it back into the tube (13).

Molecular approaches for HSV detection and typing have been implemented in some laboratories. In general, samples taken for isolation or antigen detection are also suitable for DNA detection methods. The enhanced sensitivity of methods based on nucleic acid amplification above other direct methods (culture or antigen detection) ensures that even lesion samples containing minimal cells can be analyzed with good sensitivity.

Indirect serological methods

Approximately 8 mL to 10 mL of blood is usually collected in tubes without anticoagulant or preservatives. After the blood has clotted at room temperature, the serum is separated by centrifugation and removed to another vial. If it is necessary to store the serum, it can be refrigerated at 4°C for several weeks or frozen at or below –20°C. Whole blood should not be frozen because the cells will hemolyze, making the specimen unsuitable for serological testing. In general, a single specimen is preferred, but acute and convalescent sera collected six to eight weeks apart may be preferred in select situations (14).

DIAGNOSTIC TESTS

Direct methods

Direct tests endeavour to demonstrate the presence of HSV in a suspicious lesion or in genital secretions. Ideally, the sample should be taken from a vesicular lesion that has been present for less than 24 h because once the lesion has begun to crust, the test sensitivity will decline. If multiple vesicles are present, more than one lesion should be sampled. In addition, test sensitivity is lower in patients with recurrent lesions than in those with first episodes (15).

Viral isolation

Standard viral culture: Tube culture isolation is the traditional gold standard for HSV detection and the reference method against which all other tests are measured (16,17). While the test has 100% specificity for HSV-1 or HSV-2, the sensitivity depends on the stage of the lesion at the time of specimen collection. The sensitivity also varies from 75% for first episodes to 50% for recurrences (18,19).

Once received in the laboratory, the specimen should be vortexed. The swab should then be removed from the transport medium and firmly rolled against the inside of the tube to express as much fluid as possible.

Some laboratories may add an antibiotic preparation to the primary samples before inoculation into cell culture. The specimen may be inoculated into the culture medium or may first be adsorbed onto the cell monolayer after removal of the medium (11). Adsorption facilitates more direct contact of viral particles with the cells and enhances infectivity, increasing both the number of isolates and the speed with which they are recovered. After adsorption of the inoculum onto the monolayer for 30 min to 60 min at 37°C, the medium is replaced and incubation is continued. Any remaining specimen should be refrigerated at 4°C or frozen at –70°C in case the inoculation must be repeated due to toxicity, bacterial contamination or other reasons.

HSV grows readily in a wide variety of cell lines including human foreskin fibroblasts, MRC-5, A549, rhabdomyosarcoma, mink lung, primary rabbit kidney, CV-1, Vero and HEp-2 cells. The first two are used most often because of their increased sensitivity compared with the other cell lines (20,21). Although HSV isolation times vary depending on the condition and sensitivity of the cell lines used for isolation and the amount of infectious virus present, most isolates will show visible cytopathic effect (CPE) after two to three days of cultivation. The cell culture monolayers should be examined daily for evidence of CPE. Cultures should be held for seven to 10 days, depending on the cell line used. Provisional identification of HSV can be made based on the development of the characteristic CPE. The CPE due to HSV typically develops as enlarged, refractile, rounded cells (11). The CPE starts focally but spreads rapidly to affect other parts of the monolayer. Occasionally, multinucleated giant cells may be present. Some laboratories include a DFA procedure using monoclonal antibodies in their virus isolation algorithm to confirm and type the isolate in a single step.

Shell vial or centrifugation-enhanced culture: Many laboratories now use centrifugation-enhanced (shell vial) culture methods to reduce viral isolation times (17). The same specimens used for traditional viral culture methods may be used for shell vial cultures. Shell vial culture can reduce viral isolation times from one to seven days to a duration of 16 h to 48 h. However, although these methods are rapid and specific, they are slightly less sensitive than traditional tube cultures and are more expensive (22).

Although a number of cell lines may be used, MRC-5 cells are used most often. Because of the reduced sensitivity of the shell vial method, it has been suggested that an additional standard tube culture should be inoculated in parallel for each specimen. Staining of the coverslips with type-specific HSV antibodies is used to identify HSV in shell vials.

Genetically engineered cell lines, also available commercially, allow for the early detection of HSV-1 and HSV-2 using the Enzyme Linked Virus Inducible System (ELVIS, Diagnostic Hybrids, Inc, USA). Replication of HSV in these cells induces galactosidase production, and infected cells stain blue when overlaid with an appropriate substrate. Typing can then be performed using type-specific antisera on any monolayers showing blue cells.

Typing of HSV isolates: As discussed previously, the serotype of HSV responsible for infection can have prognostic implications. Therefore, if typing is not done routinely, the isolate should be saved until it is determined whether typing is required or not.

Once CPE forms, the cultures should be stained with HSV type-specific monoclonal antibody reagents (eg, commercial products from Trinity Biotech, Ireland, or Chemicon International, USA) before reporting a positive result (23). The reporting of type-specific HSV will aid the clinician in counselling and management of the patient.

Antigen detection

Viral antigen detection may be a suitable alternative to culture for smaller laboratories in which the expense of maintaining cell lines is unwarranted. Antigen detection is also an alternative where specimen handling and transportation conditions could inactivate any virus present. This could occur, for example, in laboratories serving remote locations with prolonged specimen transportation times under uncertain conditions. For detecting HSV in lesions, the sensitivity of antigen detection tests may be the same as or greater than that of culture (24,25).

Detection of HSV antigens has been achieved in fixed cells by DFA tests or immunoperoxidase tests on fixed, solubilized cell specimens (24-26). These methods can give a useful result even in the absence of cultivable virus.

Antigen detection by DFA: The demonstration of the presence of HSV antigen by DFA staining of smears can provide a rapid adjunct to cell culture. It is essential that a high-quality specimen is obtained for this test; in this setting, test sensitivity may be as high as 90%, particularly in initial infections (17).

Although the slide may be prepared by the clinician, it is ideally prepared by the laboratory using a cytospin method and a swab specimen collected as described earlier. Staining of the slide is as directed by the manufacturer of the fluorescein-labelled antibody. The slide is examined using a fluorescence microscope, with a positive test indicated by the presence of a characteristic pattern of apple-green fluorescence in the nucleus and cytoplasm of the basal and parabasal cells. Only intact cells should be examined. An inconclusive result may be obtained if fewer than 50 intact cells are present on each well.

Tzanck smears

HSV infection causes typical cytopathic changes in genital epithelial cells (3). The cells become enlarged, with intranuclear inclusions, often with the formation of multinucleated cells. Prepared slides are stained with a Wright-Giemsa stain and then examined under light microscopy. Hematoxylin and eosin or the Papanicolaou stains may also be used. However, this method has low sensitivity and does not distinguish between HSV-1 and HSV-2, nor between HSV and varicella zoster virus infection. This test can be performed when an urgent result is needed and no alternative test is immediately available, but it does not negate the need for follow-up testing of all negatives with a more sensitive test.

Electron microscopy

Direct examination of vesicle fluid or other clinical material by electron microscopy for the diagnosis of HSV is limited by the fact that viral morphology cannot be used to distinguish HSV from other herpes viruses (eg, varicella zoster virus) (13). This traditional method has been largely replaced by DFA staining of smears that can provide type-specific differentiation of HSV-1 and HSV-2.

Virus DNA detection

Viral DNA may be detected by hybridization techniques using radiolabelled or biotinylated probes (27,28). These methods have largely been superceded by more sensitive and less laborious procedures which utilize amplification of the target HSV DNA by polymerase chain reaction (PCR). Specificity of the amplification method is assured by either undertaking a second PCR with target-specific primers (nested PCR) or by HSV-specific probe hybridization of amplified products. The majority of laboratories have confined their use of methods such as PCR to the investigation of suspected HSV encephalitis (29). In this situation, the enhanced sensitivity over culture- or antigen-based procedures is well-recognized, and the clinical value of positive results is clearly demonstrable. In the case of possible genital herpes, PCR detects viral DNA for several days after lesions do not contain demonstrable infectious virus (30).

TABLE 1
Potential uses of herpes simplex virus (HSV) type-specific antibody assays

Seroepidemiological studies	Seroprevalence studies
	Seroincidence studies
	Sexual transmission studies
Current and potential clinical uses	Patients with apparent first episode and recurrent genital herpes, especially pregnant women
	Clinically discordant couples, particularly where the man is positive and the woman is negative and of child-bearing potential
	Women of child-bearing potential with a history of lesions suspicious for genital herpes where repeated direct testing for HSV has been negative
	Sexually transmitted infection screening, especially those at risk of acquiring HIV infection
	Diagnosis of genital herpes when lesions tested using direct tests are negative on at least two occasions
	Screening of all HIV-infected individuals at the time of initial diagnosis with HIV, with a view to providing suppressive HSV antiviral therapy in those found to be HSV-2 antibody-positive

TABLE 2
Clinical, virological and serological classification of infection with genital herpes simplex virus (HSV)

	Type of	Detection of HSV antibodies		
Clinical designation	virus isolated	Acute phase serum	Convalescent phase serum	Classification of infection
First episode	HSV-2	None	HSV-2	Primary HSV-2
	HSV-1	None	HSV-1	Primary HSV-1
	HSV-2	HSV-1	HSV-1 and HSV-2	Nonprimary HSV-2
	HSV-1	HSV-2	HSV-1 and HSV-2	Nonprimary HSV-1
	HSV-2	HSV-2 with or without HSV-1	HSV-2 with or without HSV-1	First symptoms of prior HSV-2 infection; recurrent HSV-2
Recurrent	HSV-2	HSV-2 with or without HSV-1	HSV-2 with or without HSV-1	Recurrent HSV-2
	HSV-1	HSV-1 with or without HSV-2	HSV-1 with or without HSV-2	Recurrent HSV-1

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This may mean that a laboratory switching to sensitive procedures based on nucleic acid amplification may have an increased number of positive results on lesion samples with possible clinical dilemmas regarding the relevance of positive results obtained after treatment. Although PCR can detect HSV DNA from later stages of lesions than virus culture, there is a theoretical risk of false-positive results occurring due to sample contamination before amplification. Laboratories undertaking PCR-based procedures need to have separate areas and equipment for pre- and postamplification handling of specimens to minimize this kind of problem. Samples giving discordant results (eg, positive by PCR and negative on culture) are usually confirmed by a second PCR directed to a different gene to ensure assay specificity.

With the recent advances in automation and kit developments for HSV detection and typing by PCR (eg, Real Art HSV1/2 kit from Artus-Biotech USA), it is likely that this methodology will become more widely used for routine diagnostic purposes. As with other molecular diagnostic tests, the sensitivity of PCR is much greater than the gold standard of culture (31-38). The advent of real-time PCR systems, where products are detected in a closed-tube system without any post-amplification handling, has minimized the risk of false-positive results by PCR. While the equipment to undertake real-time PCR is still relatively expensive, the small reaction volumes and minimal technical hands-on time (particularly when kit-based reagents are used) make these methods very cost effective for many laboratories.

INDIRECT SEROLOGICAL TESTS

The detection of antibodies to HSV allows for diagnosis when other virological methods cannot be performed or yield negative results (39). It is particularly useful in identifying the asymptomatic carrier of infection because, as discussed above, the majority of transmission occurs while the person is asymptomatic. Thus far, the use of these tests has largely been confined to seroepidemiological studies and case management for HSV, while specific clinical uses for serological testing remains a much debated topic. Table 1 outlines some of the current and proposed uses for serological tests for HSV. Table 2 shows the interpretation of serological testing for herpes (2).

Although a number of tests can identify HSV antibodies, few available tests are able to differentiate between HSV-1 and HSV-2 (40). Serological assays that are not type-specific have limited clinical utility. In addition, no serological test is able to differentiate between oral and genital infection with HSV. Although there is a very close serological relationship between HSV-1 and HSV-2, they each encode a serologically distinct glycoprotein G (gG-1 and gG-2). This difference has been exploited in developing type-specific serological tests. A recent review describes the new HSV type-specific antibody tests (41). Finally, it appears that seroreversion or waning of immune response to gG-2 occurs with time, raising concerns about the long-term reliability of these tests (41).

Western blot

Western blot (WB) is the gold standard for the detection of antibodies to HSV (41). These tests have a high sensitivity and the ability to discriminate between HSV-1 and HSV-2 antibodies. Sera are reacted against separated, fixed protein arrays ('blots') from either HSV-1 or HSV-2 infected cell

lysates. The patterns of antibody binding bands are highly predictive of infection with either HSV-1 or HSV-2. This test is expensive, time consuming and requires skilled interpretation. When initial results are indeterminate or atypical, adsorption of sera with type-specific antigen and reblotting can sometimes 'clean up' the blot and improve interpretation. The WB for HSV is not currently commercially available.

Commercial gG-based type-specific tests

Although most of the available literature evaluating the performance of type-specific tests was based on kits developed by Gull Laboratories (USA), these tests have now been withdrawn from the market.

Presently, two companies produce four kits for the diagnosis of HSV type-specific antibodies. Focus Technologies (USA), formerly MRL Diagnostics, has three tests: HSV-1 and HSV-2 enzyme-linked immunosorbent assays, and an immunoblot test for both HSV-1 and HSV-2. The dual enzyme immunoassay test (HerpeSelect HSV-1 and HSV-2 enzyme-linked immunosorbent assay) has reported 97% to 100% sensitivity and 98% specificity for HSV-1 and HSV-2 (41). This test also reports a more rapid time to seroconversion as compared with WB, showing a median interval of 25 days from the onset of symptoms to seroconversion as determined by HerpeSelect HSV-1 versus 33 days by WB, and 21 days by HerpeSelect HSV-2 versus 40 days by WB in individuals not previously positive for HSV-1 (42).

ANTIVIRAL RESISTANCE TESTING

A number of antiviral agents have been developed for the management of HSV infections; of these, acyclovir is the most commonly used. Resistance of HSV to acyclovir has become increasingly common, with almost all clinically significant acyclovir-resistant strains seen in immunocompromised patients, especially those coinfected with HIV (43,44). The development of resistance usually results from mutations within the viral genome, and the presence of selective drug pressure usually results in the emergence of a resistant virus population. The isolation of HSV from persisting lesions despite adequate dosages and blood levels of acyclovir should raise the suspicion of acyclovir resistance.

The antiviral activity of acyclovir requires an initial phosphorylation step by the viral enzyme thymidine kinase (TK) (45,46). Two subsequent phosphorylation steps are mediated by cellular kinases. The resulting triphosphorylated acyclovir then specifically inhibits herpesvirus DNA polymerases. Three different mechanisms of resistance of HSV to acyclovir have been identified. The most common is found in viruses that lack a functional TK (TK^- mutants) and, thus, are unable to monophosphorylate acyclovir. Less commonly, some resistant viruses produce a functional TK enzyme that is unable to phosphorylate acyclovir because of altered substrate specificity (TK^A mutants). Finally, resistance can be due to mutations resulting in altered DNA polymerase binding and utilization of acyclovir (DNA pol^A).

Foscarnet directly inhibits herpesvirus DNA polymerases and resistance develops because of altered viral DNA polymerases. TK^-- and TK^A-resistant HSV viruses remain sensitive to foscarnet, but those with polA mutations may be cross resistant.

Vidarabine resistance also occurs rarely. Vidarabine is phosphorylated by cellular enzymes and then inhibits virally encoded DNA polymerase. TK^- and TK^A mutants are still sensitive to vidarabine, while polA mutants are usually resistant.

Drug sensitivity assays

The complexity of drug sensitivity assays for antiviral resistance limits their availability. At the present time, they are only performed by specialized laboratories. Susceptibility testing of strains of HSV against various antiviral agents is usually performed in the laboratory using modifications of one of the following: plaque reduction assays, dye uptake assays or DNA hybridization assays (45,46). The plaque reduction assay was the first antiviral susceptibility testing method performed to determine the susceptibility of viruses to antiviral agents and is the standard against which other tests are compared. These tests are time consuming and may soon be replaced by genotypic tests that can be processed more quickly. The viral genes encoding the two targets of antiviral drugs (TK and DNA polymerase) are amplified by PCR; the PCR products are then sequenced.

PROFICIENCY AND QUALITY ASSURANCE

All laboratories providing diagnostic services for the detection of HSV in clinical samples or performing HSV serological assays must participate in the testing of proficiency panels provided by external agencies whenever possible for all tests performed. If proficiency testing for specific assays is not available (eg, HSV DNA detection in swab material or type-specific serological testing), then specimen exchange among laboratories performing such testing should be arranged as an alternative form of proficiency testing.

Culture

Subpassages of HSV clinical isolates should be inoculated with each batch of HSV roller tube or shell vial cultures to serve as positive controls. Uninfected tubes or shell vial cultures serve as negative controls. Both infected and uninfected cell monolayers should be observed for the presence or absence of HSV CPE and stained to observe for typical immunofluorescence with HSV monoclonal antibodies. Positive controls should exhibit characteristic CPE and immunofluorescence with type-specific antisera, while negative controls should not.

Variations in sensitivity may occur in cultured cell lines for various reasons. However, the routine use of two cell lines of acceptable sensitivities is unnecessary because the difference in recovery of HSV is less than 5% (11).

Direct smears

Positive and negative control slides should be included daily in each run to ensure that the antibody reagents are performing correctly. Typical immunofluorescence should be observed in the positive controls but not the negative controls.

Indirect serological methods

Positive and negative controls must be included with each batch of sera tested. When commercial kits are used, these controls are usually provided in the kit. Results obtained in serology assays should be discarded and not be reported if control samples are out of the expected range. In addition, the use of in-house positive and negative controls should be considered. Run-to-run variability in readings on the control samples should be tracked. Testing of new lots of kits should be performed before their use in the laboratory.

Molecular-based assays

Proficiency panels for HSV detection and typing are available (eg, College of American Pathologists), but these are geared largely towards the validation of PCR-based assays for the investigation of viral encephalitis and mimic cerebrospinal fluid rather than lesion/swab material. Most laboratories undertake regular tests to determine analytical sensitivity and specificity of any in-house procedures. Weak positive controls should be included in every PCR run to ensure consistent sensitivity of the assay along with negative extraction and amplification controls to assess any potential problems with contamination that could lead to false-positive results. Internal controls may be used to detect the presence of any amplification inhibitors that could lead to false-negative results, although this is rarely a problem.

ACKNOWLEDGEMENTS: The authors are indebted to Dr Bonita Lee and Barbara LeBlanc for their invaluable comments and review of the manuscript. The authors would like to dedicate this article to the memory of Dr Stephen Sacks, whose work contributed to significant advances in HSV infections.

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