COMPARISON OF AN *IN SITU* DNA PROBE HYBRIDIZATION ASSAY AND A RAPID ENZYME IMMUNOASSAY WITH STANDARD CELL CULTURE FOR THE DETECTION OF HERPES SIMPLEX VIRUS INFECTIONS

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ABSTRACT

COMPARISON OF AN *IN SITU* DNA PROBE HYBRIDIZATION ASSAY AND A RAPID ENZYME IMMUNOASSAY WITH CELL CULTURE FOR THE DETECTION OF HERPES SIMPLEX VIRUS INFECTIONS

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Rapid diagnosis of HSV is needed for better management of patients with HSV infections. HSV isolation by cell culture remains the standard method for diagnosis, despite the drawbacks associated with this test. The performance of an *in situ* DNA probe hybridization assay (HSVDISK) and a rapid enzyme immunoassay (Surecell) was evaluated in comparison to cell culture, which was considered to be 100% accurate in the detection of HSV in clinical specimens. Of 154 specimens from both asymptomatic and symptomatic patients, 42 were tested by Surecell and cell culture, and 152 were tested by HSVDISK and cell culture. Based on cell culture results, incidence of herpetic infection in the patient population was 23/154 (14.9%). The sensitivity and specificity of the Surecell assay were 88.9% and 87.9%, and those of the HSVDISK assay were 90.9% and 99.2%, respectively. The Surecell assay was an easy test to perform and was an excellent screening test for positives, especially since virus present in the patient

specimen did not need to be active for detection. However, the Surecell assay did produce results that were difficult to read with respect to intensity of color for 5/42 specimens. The HSVDISK assay required minimal prior experience with cell culture and was more compact and easier to work with than cell culture tubes. However, this assay may not detect HSV in specimens with low titer. For rapid diagnosis of an HSV infection, the 15 minute Surecell and 24 hour HSVDISK assays are an improvement on cell culture, but cell culture can detect HSV in specimens with low HSV titer, making it a more accurate detection method for HSV infection.

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INTRODUCTION

Herpes simplex virus (HSV) infections are among the most common viral infections affecting humans world wide. These infections are caused by two genetically distinct viruses belonging to the alphavirinae subfamily of herpes viruses, herpes simplex virus type-1 (HSV-1) and herpes simplex virus type-2 (HSV-2).

The anatomic site of HSV infection and the age and immune status of the host determine the duration, severity, and type of HSV infection caused (1). HSVs are capable of infecting most areas of the body (2, 3), however, in immunocompetent hosts these infections are most commonly localized to skin, mouth, pharynx, eyes, genitalia and brain. For immunosuppressed hosts HSV infection can result in extensive damage to dermal tissue and could even result in infection of visceral organs. The number of severe cases of HSV infection has increased during recent years due to the widespread use of immunosuppressive therapy in cancer and transplant patients and due to the increased number of persons with AIDS (4).

In order for a primary infection to be established, HSV must overcome host physical and chemical barriers (e.g intact skin and skin pH) as well as nonspecific defense mechanisms (e.g. phagocytic and natural killer (NK) cell

activity). HSV infection may not, however, always lead to productive replication of virus and appearance of vesicular lesions. In fact the primary infection with HSV typically results in symptoms that are so mild that the individual only experiences a little discomfort (1, 5). In persons with genital HSV-2 infection, asymptomatic primary episodes occur because childhood exposure to HSV-1 has instituted partial immunity to HSV-2 (2, 5). Despite whether or not clinically apparent lesions occur during a primary episode, neural tissue underlying the original infection site usually becomes infected, resulting in establishment of latent HSV which may later be reactivated from this neural tissue to cause recurrent infection (2).

Acquired immunity has a role in limiting the severity of the HSV infection but cannot prevent establishment of latent HSV and thus cannot prevent future symptomatic recurrences of infection (6). Progeny HSVs are able to avoid destruction by humoral immune mechanisms by spreading directly from cell to cell by fusion of cell membranes, i.e., formation of syncytia (7). In this manner, HSVs may never contact the extracellular environment, where anti-HSV antibodies can bind to and neutralize the HSV directly or can coat the HSV so that it may be opsonized by a phagocyte.

HSVs can also evade some cellular immune mechanisms. Cytotoxic T-lymphocytes are able to bind to and kill infected cells that express HSV antigens

and major histocompatibility complex (MHC) antigen on their cell surface (2). However, neuronal cells do not express the MHC antigen that is required for the cytotoxic T-lymphocyte to bind (2). Thus, latently infected neuronal cells cannot be destroyed by these lymphocytes.

Both HSV-1 and HSV-2 are the cause of genital and orofacial infections, and infections caused by each type are usually clinically indistinguishable. Therefore both types of HSV are not exclusively associated with specific infection sites, presumably because HSV-1 and 2 share 50% DNA sequence homology (1). In the United States, most orofacial infections are caused by HSV-1 while approximately 85% of genital infections are caused by HSV-2 (8). In Japan, however, most genital herpes cases are caused by HSV-1 (9). Symptoms and duration of infection are similar for HSV-1 and 2 genital infections, though recurrences are more likely for type 2 infections (4, 6). Similarly, orofacial HSV-1 infections recur more frequently than orofacial HSV-2 infections (6). HSV-1 also causes most eye and brain HSV infections. Infection of the brain usually results in herpes simplex encephalitis (HSE), which is one of the rarest and most serious diseases caused by HSV-1.

Genital herpes is the most common disease caused by HSV-2. This disease is most often sexually transmitted, though it can be transmitted by self-inoculation of virus from an oral or finger infection in immunosuppressed patients (4).

Sexually transmitted HSV-2 infections may also be associated with rectal and perianal HSV infection, especially in immunosuppressed patients whose genital infection has spread and in people having rectal intercourse (4).

The most serious consequence of genital herpes infection in females is neonatal herpes. Infection in the neonate may occur before, during or after the time of delivery (10). Most infections are caused by contact with the mother's infected genital secretions as the baby passes through the genital tract, though transplacental transmission, ascending infection following rupture of membranes and postpartum infection resulting from contact with relatives or health care workers has been known to occur (10, 11).

A rapid and sensitive technique is needed to detect HSV infection, as early treatment can prevent fatality in people with herpes simplex encephalitis (12) as well as disseminated disease in infected neonates and immunosuppressed patients (13), and may ensure that effective treatment is being given to patients with less severe infections. Current techniques are either difficult to perform, lack sensitivity and/or specificity or take too long for an accurate diagnosis (14-17).

The objectives of this research were to evaluate two diagnostic kits for HSV: one, a cell culture system (HSVDISK) that uses centrifugation to speed up the infection process and an HSV specific DNA probe for detection of HSV DNA

in the cells; and the other, an enzyme immunoassay (Surecell) that uses HSV specific antibody to detect viral antigen in the patient specimen. To determine sensitivity, specificity, and the ability of the Surecell and HSVDISK tests to predict "true" HSV positives and negatives, the two tests were compared to viral isolation by cell culture, the "gold" standard method of detecting HSV (17). At the same time, each of the three tests were rated according to speed of HSV detection and the ease at which the test was performed.

LITERATURE REVIEW

General Properties of HSV

HSV-1 and HSV-2 are classified in the alpha subfamily of herpes viruses (alphaherpesvirinae), which also includes varicella-zoster virus (18). The members of the alphaherpesvirinae share a number of characteristics. First of all, alphaherpesvirinae have linear, double-stranded DNA genomes which are packaged in an icosahedral capsid. In HSV-1 and 2, this capsid is surrounded by a protein and phospholipid tegument and an outerlying envelope that is derived from the host cell nuclear membrane (19). Viral glycoproteins are embedded in this envelope which function in viral attachment to the host cell membrane during the infection process.

The second common characteristic is that viruses belonging to this subfamily have short replication cycles (HSV replicates in approximately 18 hours) and the site of replication is the nucleus of the infected cell (20). HSV infection commences with attachment of the virus to receptors on the host cell followed by fusion of the viral envelope with the host cell membrane (1). This process allows the viral nucleocapsid to gain entry into the cell cytoplasm. The nucleocapsid is then transported to the cell nucleus and disassembled to release viral DNA. Subsequent expression of the viral genes needed for replication (alpha, beta, and

gamma genes) then occurs in a sequential manner which is necessary for the formation of an infectious viral particle (20, 21). Alpha genes are expressed earliest, producing proteins that are used primarily to regulate expression of beta and gamma genes. Subsequently, the beta genes are expressed which code for regulatory proteins of gamma genes and for enzymes needed in replication of viral DNA. The structural proteins that make up the viral particle are then coded for by the gamma genes, which are expressed last in the replication cycle. The replicated viral DNA is then packaged in the structural proteins coded for by the gamma genes and the resulting nucleocapsid acquires an envelope by budding through the host cell nuclear membrane (22). Progeny virions can then infect neighboring cells after they are transported via the endoplasmic reticulum and Golgi apparatus to the surface of the infected cell (23). Release of the progeny virions may or may not result in the lysis of the infected cell.

The life cycles of the various members of the alphaherpesvirinae subfamily differ in some aspects, but the common factor is that all these viruses have the ability to replicate in mucous membranes, skin, and neural tissues, enabling them to infect virtually any area of the body. In order to establish infection, it is important that the virus contact mucosal surfaces or abraded skin of the host and also survive host immune defenses (6). Replication of the virus in infected cells and spread of progeny virus to neighboring cells may then result in a primary symptomatic infection, which for members of alphaherpesvirinae classically

manifests as vesicular lesions on the skin or mucosal surface surrounding the infected area. Primary infections with HSV, however, are most commonly asymptomatic or so mild that symptoms are barely noticeable (1, 5).

Another characteristic of the alphaherpesvirinae subfamily is that primary infection generally results in the establishment of latent virus in neural and dermal tissues which surround the site of primary infection. During latency, normal cellular activity continues to occur even in the presence of virus because the viral genome remains in a dormant phase of growth (non-replicating) in the nucleus of the infected cell (24-26). The virus may remain latent in an infected cell throughout an individual's lifetime and may become spontaneously active in conditions of physical and emotional stress (6). Stressful factors such as excessive sunlight, hormonal changes, and trauma can trigger reactivations by derepressing latent viral genes (2), so that viral DNA replication and production of progeny viruses ensues. This reactivation may result in lytic infection in which progeny viruses are released by lysis of the infected cell or it may result in viral shedding in which viruses are released from the cell in a slow, controlled manner without lysing the infected cell (27). The released progeny can then infect neighboring cells or can be transported along neuronal axons to a new site where latent or active infection may be established (2). Symptoms produced during active recurrent infection at the same or a new site are generally less severe, more localized to the reinfection site, and are shorter in duration than the symptomatic

primary episode (4, 5, 28). This is presumably due to immunity acquired as a result of primary infection (2). In most cases, reactivation of virus from latently infected cells does not lead to a symptomatic recurrence of infection, as these viruses are usually eliminated by host immune defense (29, 30). However if host immune defense is suppressed, as is the case for many cancer and AIDS patients or other persons on immunosuppressive therapy, these symptomatic recurrences are typically more frequent and severe (31). Thus host immune status primarily determines the frequency and severity of symptomatic recurrences (31). Unfortunately the physical and emotional stresses that trigger reactivations also suppress host defense mechanisms against the virus (2), so that during times of stress, even immunocompetent hosts are more likely to experience a symptomatic recurrence.

Host Response to HSV

The various types of responses elicited by the host during an HSV infection include local, cellular, and humoral immune defense. HSV cannot normally penetrate intact skin, however, injured skin, mucous membranes, and conjunctiva are more susceptible (6). Nonspecific effectors such as macrophages, monocytes, and polymorphonuclear leukocytes (PMNs) are important in both the innate and acquired immune response to HSV infection (32). These cells can eliminate both extracellular and intracellular virus by phagocytosis, which can occur with or

without the help of anti-HSV antibodies (33). Macrophages also act as antigen presenting cells (APCs) by processing HSV and presenting HSV antigen to a B- or T-lymphocyte so that the antigen's effectiveness as an immunologic stimulus may be increased (34). APCs thus serve to prime the immune system so that immune response is much faster upon subsequent exposure to the antigen.

Initial encounter with HSV causes the proliferation and differentiation of specific B- and T-cell clones. Some of the progeny lymphocytes become the effector cells that produce the primary immune response, while others become memory cells which serve in eliciting a faster immune response during a secondary HSV exposure. B-cell derived effector cells are plasma cells which produce and secrete specific anti-HSV antibodies that can neutralize extracellular virus and bind to infected cells so that the infected cells may be opsonized (33). T-cell derived effector cells (T cytotoxic, helper, or suppressor cells) help eliminate extracellular HSV and HSV infected cells through the production of soluble factors called cytokines (35, 36). Contact of a primed T effector cell with HSV antigen stimulates the release of cytokines which can have several effects. The released cytokines can act to 1) neutralize extracellular HSV directly, 2) lyse an HSV infected cell by causing perforations in the host cell membrane, or 3) cause proliferation and migration of other effector cells (such as macrophages) at/to the site of infection (32). Various in vitro studies have shown that the production of the cytokines, interleukin-2 (35, 36), alpha-interferon (37), and interleukin-7 (38)

by antigen activated T helper cells is necessary for cytotoxic T-lymphocyte lysing of HSV infected cells. Cytokines can also be produced by other immune cells such as macrophages, PMNs, and NK cells. The cytokines produced by these cells may act on the same type of cell or on other immune cells to ultimately produce an anti-HSV response. Interferon, for example, whether produced by macrophages or T-lymphocytes, causes pleiotropic effects on macrophages (32). In addition, exogenous alpha- and gamma- interferon appears to inhibit HSV-1 genes encoding early gene products in spleen macrophages of mice (39, 40). Thus interferon not only causes proliferation and migration of macrophages to the infected site but also is able to prevent replication of HSV-1 in infected spleen macrophages of mice. Indeed the role of cytokines in immune defense against herpes simplex viruses has not been fully elucidated.

In addition to effector cells, clones of memory B- and T-cells are produced as a result of the initial encounter with HSV. Upon subsequent exposure to HSV antigen, such as in the case of reactivated virus or exposure to another viral strain, these memory B- and T-cells produce a faster and more efficient immune response. This is accomplished by the immediate transformation of memory B cells into plasma cells that produce an increased amount of antibody and proliferate forming other identical plasma cells and/or other memory B-cells. A similar process occurs with the memory T-cells, with these cells producing T-helper, suppressor and cytotoxic cell clones.

Host defense mechanisms against HSV are not capable of completely eliminating HSV from an infected body, making HSV infection a lifelong threat. Certain characteristics in the HSV life cycle enable HSVs to avoid destruction by certain humoral and cell-mediated immune activities. First, progeny HSVs may spread directly from cell to cell during the infection process, bypassing host defense mechanisms that are only able to eliminate extracellular virus (including destruction via antibody neutralization, and by opsonization and other phagocytic mechanism requiring extracellular virus) (7). Secondly, HSVs can avoid destruction by the complement cascade, a series of enzymes (C1-C9) found in blood serum that can sequentially bind to and lyse an infected cell by making a hole in the cell membrane. HSVs produce a C3-binding molecule, glycoprotein C1, which can cause decay of the C3 convertases of the classical and alternative pathways of the complement cascade (41). Thus the complement cascade does not provide protection against HSVs that express surface glycoprotein C1. HSV strains and HSV infected cells that do not express the glycoprotein C1, however, are susceptible to complement mediated cell lysis and viral neutralization (42). Thirdly, HSVs remain latent in neural cells which are not able to be killed by cytotoxic mechanisms due to the inability of cytotoxic T-lymphocytes to recognize and bind to cells that don't express MHC antigen (43). Despite the viruses ability to evade destruction by these methods, both humoral and cell-mediated immune mechanisms help to keep HSV infection localized and shorten the duration of infection (1). Thus viral replication is inhibited by humoral and cellular immunity,

but these immune mechanisms cannot prevent establishment of latency or viral reactivation which may lead to recurrent infection.

Antibody-dependent cellular cytotoxic (ADCC) activities which use both humoral and cellular immune mechanisms have also been shown to slow down viral replication. ADCC involves the cytotoxic activity of natural killer (NK) cells or some other leukocytes. During this process, the infected cell is coated with anti-HSV antibodies which bind to the viral antigens expressed on the cell's surface. NK cells have receptors that can bind the Fc portion of the antibodies, so that the NK cell may contact the infected cell. This contact triggers the release of proteins called perforins which destroy the infected cell by making holes in the cell membrane (44, 45).

Because of the numerous activities involved in controlling HSV infection, it is hard to determine the relative contribution of humoral and cell-mediated immune mechanisms (46). Most of what is known about cell mediated immunity (CMI), for instance, comes from animal and human tissue culture studies which may not accurately portray CMI in vivo (46). CMI does seems to be of chief importance in limiting infection and maintaining latency, since patients with depressed CMI, such as people with AIDS, chronic eczema, and cancer, have more frequent and severe infections (1, 13, 47). Reactivated virus is not removed as effectively in these patients as it is in immunocompetent or

agammaglobulinemic individuals (1, 6).

Clinical Scope of HSV infection

Based on seroepidemiologic data, HSV-1 infections have been shown to occur in 50-100 percent of adults, depending largely on socioeconomic status (4). HSV-1 infections in persons of lower socioeconomic status are more prevalent and generally occur at an earlier age (6). Since HSV-2 is usually acquired as a sexually transmitted disease, antibodies against HSV-2 don't usually appear until after adolescence. Studies have shown anywhere from 0.3 to 22% of adults in the United States have anti-HSV-2 antibodies (6, 30, 48, 49). Prevalence of seropositivity depends on age, sex, race, socioeconomic status, and sexual history of the individual (30, 48, 49).

Most primary infections with HSV-1 are asymptomatic, but may be followed by recurring clinically active infections (1, 5). Gingivostomatitis and pharyngitis are the most common symptomatic primary HSV-1 infections, while herpes labialis, or cold sores, is the most common recurrent HSV-1 infection (1). Clinical symptoms of gingivostomatitis and pharyngitis include vesicular or ulcerative lesions of the oropharynx and face accompanied by fever, malaise, and irritability. Recurrent infections of this type are referred to as cold sores or fever blisters, and are typically milder and more localized. The primary infection most

often occurs before the age of 5 (5), but since most of these infections are asymptomatic most people experience the recurrent infection (cold sores/fever blisters) without ever having gingivostomatitis.

Infection of the eye with HSV-1 is a common cause of corneal blindness (50, 51). Primary and recurrent eye infection typically result in keratoconjunctivitis which is characterized by lesions surrounding the eye. However, recurring infections may also result in the formation of dendritic ulcers. Recurrences involving corneal stroma can lead to loss or impairment of vision (50, 51).

HSV-1 is responsible for most skin infections. Two common HSV skin infections include herpetic whitlow and eczema herpeticum. Herpetic whitlow is caused by inoculation of HSV into a cut or sore on the hand (52) which results in vesicular lesions on the fingers. This disease occurs primarily in health care workers and thumb sucking children (53, 54). Eczema herpeticum occurs most frequently in people with chronic skin disorders. This disease results from inoculation of virus into skin lesions associated with eczema, which produces large vesicular lesions at the site of infection. These lesions are often fragile and burst, leaving the skin unprotected (4). Infection in these patients may rapidly spread covering extensive areas of the skin and may even disseminate to visceral organs.

Herpes simplex encephalitis (HSE) is the rarest and most serious disease caused by HSV-1. This disease occurs predominantly in patients aged 5-30 and over 50 and may be the result of primary infection or recurrent infection (55). HSE results from transmission of virus from the periphery up the olfactory bulb to the brain or from reactivation of latent virus in nerve root ganglia with spread of infection to the brain (56). Fever, headache, neurological problems associated with temporal lobe, and other non-specific clinical symptoms make diagnosis of HSE difficult. Untreated mortality rate is over 70%, and even with antiviral therapy, neurologic sequelae usually occurs (4, 57).

The most common disease caused by HSV-2 is genital herpes. Over 500,000 new cases of symptomatic primary genital herpes and over 10,000,000 symptomatic recurrences occur yearly in the United States (30). Primary symptomatic episodes of genital herpes last an average of three weeks and are characterized by painful vesicular lesions covering the external genitalia, buttocks, cervix and urethra. These lesions may accompany dysuria, fever, headache, malaise, genital itching and discharge, and tender lymph nodes (1). Prior HSV-1 infection seems to lend partial immunity to HSV-2 genital infections, as these patients have less frequent symptoms and faster healing with the primary episode (5). The highest incidence of primary symptomatic genital infection occurs in people age 20-30 (6).

Aseptic meningitis may be associated with primary genital HSV infection. Though rare, HSV may ascend to the spinal cord, where further replication can lead to aseptic meningitis (58). The disease course is self-limiting, but since latent infection may be established, recurrences may occur (59).

The most serious consequence of genital herpes infection is neonatal herpes. Infection in the neonate may occur before, during, or after the time of delivery (10). Currently, cesarean section is recommended if active maternal infection is present, but most women who deliver infants who get HSV infections are asymptomatic at the time of delivery (60, 61). Not all infants who are exposed to the virus around the time of delivery will acquire a herpes infection (62, 63). Being born to a mother with primary genital infection poses the greatest risk to the infant (64). Half the infants born to a mother with primary infection will develop an HSV infection, while only 4% born to mothers with recurrent infection will develop infection (64). Moreover, infants born to a mother with a primary genital infection more often develop disseminated infection, while infants born to a mother with a recurrent infection typically only develop skin, eye, or mouth lesions or localized encephalitis (63, 65). These differences are probably due to immunity acquired transplacentally from the mother in infants born to mothers with recurrent infections (65, 66). Disseminated herpes usually presents as a sepsis-like illness with fever and perhaps even the appearance of skin, eye, or mouth lesions (62). Other complications such as necrosis of the liver and adrenal

glands, thrombocytopenia, meningoencephalitis or pneumonia may develop (67). Incidence of infection has been estimated to range from 1 in 7500 to 1 in 30,000 births (68). Of these infected neonates, approximately 65% will die (4, 57) and those that live will most likely develop severe neurologic sequelae (67). Unfortunately, reactivation of HSV-2 infection is more common in pregnant women than in other women (69) and at least 20% of pregnant women have had prior HSV-2 infection (10, 70).

Due to the increased number of AIDS cases and the widespread administration of immunosuppressive therapy for transplants, cancer, and other diseases, the number of severe cases of HSV infection has increased during recent years (4). Patients undergoing such therapy can be subjected to frequent and severe reactivations of HSV infection. These recurrences of HSV infection may be associated with prolonged viral excretion and chronic lesions in these patients. These lesions can result in extensive tissue necrosis and though rare, may even lead to viremia and dissemination through multiple organs (28). Diseases such as meningoencephalitis, pneumonitis, hepatitis, coagulopathy, esophagitis, and proctitis may occur as a result of dissemination (1).

Therapy for HSV infections

Acyclovir, an antiviral therapeutic agent, is typically administered for both

immunocompetent and immunosuppressed patients (13, 71). Treatment of immunosuppressed patients is usually started at first sign of HSV infection, even without culture confirmation of infection (13). Early treatment is imperative in these patients as HSV infection can be life threatening. Persons with AIDS however may develop resistance to acyclovir (13, 72). Vidarabine is an effective alternative to acyclovir therapy, however, this drug is not as efficient as acyclovir in fighting mucocutaneous HSV infection (73).

Laboratory Detection of HSV infections

The discovery of effective anti-HSV drugs has prompted the need for more rapid and sensitive HSV diagnostic tests in recent years. Early diagnosis and treatment ensures better patient prognosis, puts the patient at ease to receive reliable information on management and prognosis, ensures the doctor that proper treatment is being given, and decreases cost of health care by limiting patient hospital stay and eliminating the expense associated with unnecessary testing and treatment. HSV tests currently available to clinical laboratories either lack sensitivity or specificity, are tedious to perform, or take too long for an accurate diagnosis.

The most common methods for diagnosing herpes presently include viral isolation by cell culture, detection of HSV antigens by a variety of immunologic

techniques such as enzyme-linked immunosorbent assays, enzyme immunoassays, immunoperoxidase staining, and immunofluorescence staining, and demonstration of amplified or non-amplified HSV DNA using various DNA hybridization methods (14, 16, 17).

Virus isolation by cell culture used with another method for confirmation of HSV-caused cytopathic effect (CPE) is the most sensitive, specific and widely used technique to date (74). Immunofluorescence staining is most commonly used to confirm HSV-caused CPE, however other confirmation techniques such as immunoperoxidase staining, ELISAs, EIAs, or DNA probe hybridization may be used. Even though cell culture is still considered the "gold standard" for HSV detection, this method often takes too long to detect infection, requires expertise, and may be affected by toxic components in the patient specimen. CPE takes an average of three days and may take as long as eight days to appear if low numbers of the virus are present (14, 17). A more rapid diagnosis is needed to test pregnant women near delivery for asymptomatic shedding of the virus (10), to ensure rapid treatment of neonates, immunocompromised patients, and persons with HSE for whom dissemination of HSV infection may be fatal (31, 60, 75), and to ensure that proper treatment is being given in cases where treatment has been initiated without clinical confirmation of the infection as being caused by HSV. Cell culture also requires technical expertise for recognition of CPE and maintenance of the culture (74, 76). The expert must be trained in distinguishing

CPE caused by HSV and CPE caused by other viruses in order to perform the appropriate confirmation test. As a result of this, many hospitals and clinics send the clinical specimens to a virological laboratory for expert recognition of CPE, further delaying detection of the disease because of transport time. Another problem with cell culture is that toxic components in the specimen may also cause cell deterioration after a three to ten day incubation period (14, 76), making microscopic examination of the cells for CPE caused by HSV hard to interpret. Corey reported four cases in which toxic components in the rectal specimens of infants caused false positives in cell culture (14). The infants were immediately hospitalized and treated for HSV infection costing the hospital 10,000 dollars per infant, only to find out that repeat specimens taken from the infants were negative (14). Confirmation of HSV CPE by using a technique such as immunofluorescence staining of cells has reduced the chance of this occurring, however some labs still rely on cell culture alone to determine infection by the virus. Though cell culture used in conjunction with an immunologic confirmation technique is currently the most accurate and widely used technique today, a more rapid, inexpensive, and more easily managed technique would be beneficial to the patient and hospital or clinic. Moreover, cell culture detection of HSV is not practical for small community hospitals or clinics that receive only a small number of patient specimens to be tested for herpes, as cell culture can be expensive and laborious to maintain. Many of these hospitals or clinics only receive a few specimens per week if any and staffing is not appropriate to perform such testing.

Immunofluorescence, immunoperoxidase staining, immunoassays such as ELISA and EIA, and DNA hybridization techniques can be used alone (without cell culture) for detecting HSV in clinical specimens (14, 16). The main advantage of these techniques is that they are generally much faster and easier than HSV isolation by cell culture, but when used alone, these tests have generally shown lower sensitivities and specificities than cell culture (14, 15). Studies involving direct immunoperoxidase staining of specimens scraped from lesions have shown sensitivities of about 80% in comparison to cell culture used without a confirmation test (77-80). Because of the low sensitivity associated with direct immunoperoxidase staining of specimens, immunoperoxidase staining is generally only used to verify HSV isolates from cell culture (77, 80, 81). Another problem with this technique being used alone to directly detect HSV in the patient specimen is that a negative result is reliable only if the sample specimen contains intact cells. Schmidt and coworkers reported that out of 180 specimens, 23 contained insufficient cells to permit valid interpretation of results (77). Thus the laboratory must confirm the adequacy of the specimen before processing it and specimens must be reacquired if they are unsatisfactory (77). Kits that combine cell culture with immunoperoxidase staining for the detection of HSV have found staining of cells at 48 hours to be less sensitive than standard cell culturing confirmed by immunofluorescence staining (82-85). Immunofluorescence and immunoperoxidase staining have, however, exhibited similar sensitivities in confirming an HSV isolate after CPE was observed in cell culture (77, 82).

Sensitivities of ELISAs and EIAs range from 35 to 100% of that obtained by cell culture, depending on whether the CPE in cell culture is confirmed by another immunologic method (86-95). Specificities of immunoassays have been nearly equivalent however to those of cell culture (86-89, 94-96). The fact that results can be obtained in a few minutes to up to six hours using immunoassays warrants the use of these techniques for emergency diagnosis in case of suspected neonatal infection or infection in the immunocompromised (14, 86). However since this technique involves detection of viral antigen, a positive result is not necessarily synonymous with active infection of HSV (88), and false positives can sometimes occur due to non-specific binding of antibody (88, 94). At the present time, EIAs and ELISAs are most commonly used in combination with viral isolation by cell culture to verify a positive or negative result (89, 94, 97), or as a screening test for positives (89, 95) since specimens producing negative ELISA/EIA results are usually tested by cell culture as a backup.

A number of reports have shown immunofluorescence staining to be a less sensitive method than viral isolation by cell culture (98-103), although this method seems to be ideal for confirmation of HSV isolated by cell culture (104). In a long term study comparing immunofluorescence staining with monoclonal antibodies to viral isolation by cell culture in patients with recurrent genital herpes, Lafferty and coworkers (100) reported an overall sensitivity and specificity of 74 and 83%, respectively, in comparison to when both cell culture and

immunofluorescence staining were used. However viral isolation by cell culture and the immunofluorescence staining technique when used alone had similar sensitivities (53 and 51%, respectively) for the initial sample received from the patient (100). Thus to maximize laboratory confirmation of HSV infection on a single visit by a patient, Lafferty and coworkers suggest that using just immunofluorescence staining or viral isolation by cell culture is not sufficient (100). In a similar experiment by Pouletty and coworkers (101), immunofluorescence assays using monoclonal antibodies were performed on 652 specimens directly. This method showed a sensitivity of 84.6% in comparison to viral isolation by cell culture, however, for one patient, only one of three specimens gave a positive result (101). Numerous investigators have suggested that these false negatives may be due to the quality of the specimen sample (98, 100-102). Nerurkar reported problems in distinguishing negatives from positives when a specimen smear was dried or stored too long before staining and when nonspecific intense staining of debris in the specimen cells occurred (102). Thus in addition to the decreased sensitivity of this method in comparison to cell culture with confirmatory immunofluorescence staining, specimen quality and preparation may be a hindrance in obtaining a correct positive or negative result. Although immunologic methods used in conjunction with virus isolation by cell culture seems to be the most sensitive and rapid way of obtaining an accurate result, the ultimate goal for HSV diagnosis is to have a single test that is rapid, sensitive, reliable, simple and inexpensive.

HSV infection may also be detected by demonstration of nonamplified HSV DNA using DNA probe hybridization. Probes can be labeled with enzyme or radioisotope and are usually added to the specimen sample directly (105-107). Using DNA probe hybridization has proven to be very rapid and convenient, however, the sensitivity and specificity of the probes used thus far to detect HSV DNA in a specimen seem to vary greatly and thus produce unpredictable results (105-107). In one experiment by Langerberg and coworkers (105), the sensitivity and specificity of a test using a biotinylated HSV DNA probe was compared to viral isolation by cell culture with immunofluorescence staining confirmation. The sensitivity and specificity of the test for detecting HSV was determined to be 92% and 63% to that of cell culture, respectively. Similarly, Fung and coworkers (106) reported a sensitivity of only 71.4% and a specificity of 90.6% for a biotinylated probe in comparison with cell culture used with immunofluorescence staining confirmation. The disparity in these results may be indicative of the unpredictability of this method. Furthermore Langenberg and coworkers (105) reported that the sensitivity of this method dropped to 57% that of cell culture with IF staining if a specimen of less than 20 cells was evaluated. Apparently, the sensitivity of the HSV DNA probe decreases dramatically at low virus concentrations (106). Another drawback to this method is that some DNA probes exhibit non-specific cytoplasmic binding (106, 107) when added directly to patient specimens, and thus may give a false positive reading. Fung and coworkers found that 27.2% of the specimens tested exhibited non-specific cytoplasmic staining with

an HSV DNA probe (106). Because of the unpredictable sensitivity and specificity of these DNA hybridization tests, they have thus far only been used in conjunction with viral isolation by cell culture in determining if a patient is infected with HSV.

More recently, probes have been used to detect amplified sequences of HSV DNA in the patient specimen directly using polymerase chain reaction (PCR), however, current problems with PCR such as carry-over of amplified products and requirement of specimen purity (16) make it hard to predict when this test will be able to be performed routinely in the clinical laboratory (15). Studies that have used this technique to determine the presence of HSV in clinical specimens generally report an equal or higher sensitivity for PCR as compared to cell culture (108-112), however, some of the results regarding patients whose specimens were positive by PCR but negative by cell culture weren't conclusive as to whether the patient was experiencing an active infection (108, 111). Methods that use cell culture for the detection of HSV have an advantage in that only actively infecting virus will be detected. At the present time there is no clinically available PCR technique that can distinguish between a latent and an active HSV infection (16); previous studies using PCR to detect HSV have made diagnosis of active or latent infection based on the clinical status of the patient (16, 108, 110).

Study Method

In this research two currently available kits, Diagnostic Hybrids HSVDISK™ and Kodak's Surecell, were evaluated and compared to results with cell culture for detection of HSV in clinical specimens. Diagnostic Hybrid's HSVDISK is a test kit for the culture and detection of HSV by *in situ* DNA probe hybridization. This method combines amplification of viruses by cell culture and detection of the viruses using *in situ* DNA probe hybridization after only 24 hours as compared to a 1-8 day diagnosis when using standard cell culture. This method has one advantage over other DNA probe hybridization methods in that viral numbers are amplified before detection, so clinical specimens containing only a few intact cells are not a concern. The 24-well cell culture trays are easier to handle than individual cell culture shell vials. The number of wells containing cell monolayers can also be varied according to the needs of the hospital or clinic. Thus as many as 11 patient specimens can be tested on one tray. Kodak's Surecell is a monoclonal antibody-based enzyme immunoassay that detects HSV antigen from patient specimen in under fifteen minutes, making it an appealing rapid test in cases where quick confirmation of HSV infection is needed. Since the Surecell assay detects HSV antigen, active HSV need not be present in patient specimen in order for a positive result to be obtained. The Surecell assay would thus be particularly useful in detecting HSV in late stage lesions which characteristically contain low titers of HSV. The Surecell test tray comes with

built-in positive and negative control wells and accommodates only one patient specimen per tray. Thus the Surecell assay would be most appealing to hospitals, clinics, and doctor's offices that rarely receive clinical specimens to be tested for HSV.

MATERIALS AND METHODS

Specimen Collection

Specimens were obtained from both asymptomatic and symptomatic patients from St. Elizabeth's Medical Center, Dayton, Ohio. It was not known if the patients had ever experienced a prior HSV infection. All specimens from asymptomatic patients were collected from the hospital's obstetrical-gynecological clinic. These patients were either pregnant or in their childbearing years.

A total of 154 specimens were analyzed, including 142 genital specimens and 12 specimens from other sites such as skin, esophagus, and abdomen. Of the 154 patient specimens collected for the study, 40 were tested by standard cell culture, HSVDISK, and Surecell, 2 were tested by cell culture and Surecell only, and 112 were tested by cell culture and HSVDISK only. When available, age, sex, and clinical presentation of each patient was also recorded.

Specimens from active lesions were collected with sterile cotton swabs, and those from asymptomatic patients were collected by swabbing the cervix and/or vagina. All swabs collected were placed into viral transport medium consisting of approximately 1.8 mls of minimum essential medium with 10% fetal bovine serum, and 10 μg/ml gentamicin. A portion of each specimen was sent to a virological

laboratory (Diagnostic Virology Services, Inc., Centerville, Ohio) to be tested by cell culture, while the other portion was reserved to perform the HSVDISK™ and/or the Surecell test(s) at St. Elizabeth microbiology lab. The swab from each specimen was reserved for use in the Surecell test. The HSVDISK™ test was performed within 48 hours of specimen receipt or the specimen was frozen at -70°C and tested at a later time. Surecell and cell culture tests were performed within 24 hours of specimen receipt.

Cell Culture Method

Cell culturing at the reference laboratory was performed by inoculation of the patient specimen onto triplicate African Green Monkey Kidney cells. The cells were then centrifuged to allow absorption of virus present in the patient specimen onto the cell monolayer and incubated at 37°C in 5% CO_2. Cultures were observed daily for 10 days for CPE. If CPE did not occur within this time period, cultures were considered negative. Immunofluorescence staining of the cells was used to confirm CPE caused by HSV.

HSVDISK™ method

HSVDISK kits were obtained from Diagnostic Hybrids, Inc., Athens, Ohio. The HSVDISK kit includes 24-well culture plates containing African Green

Monkey Kidney Cells (CV-1), replacement medium, blocking solution, fixative solution, wash solution, chromogenic substrate solution, and probe hybridization solution. Positive controls were not provided. The probe hybridization solution contains a single stranded HSV-1 and -2 specific DNA probe linked to alkaline phosphatase in a buffer solution containing 25% formamide. The probe is 2-kb in length and includes 800- and 1,200-bp *Pst*I discontiguous regions of DNA from HSV strain 1F that are cloned in the M13mp19 bacteriophage (113).

Cell culture plates containing African Green Monkey Kidney Cells (CV-1) were maintained using kit replacement medium consisting of minimum essential medium supplemented with 10% fetal bovine serum and 10 μg/ml gentamicin. Cell monolayers were inoculated with 0.2 ml of patient specimen eluate per well, centrifuged at 700 x g for 10 minutes at room temperature to allow absorption of virus to cell monolayers, and incubated at 35-37°C in a humidified 5% CO_2 atmosphere for 24 hours. One positive and one negative control well were included on each plate. HSV-2 strain G and HSV-1 strain F obtained from American Type Culture Collection (Rockville, Md.) were used as positive controls.

At 24 hours, cell monolayers were submerged in 95% ethanol for 5 minutes after which 0.25 ml of blocking solution consisting of neutralized triethanolamine containing 0.05% sodium azide was added to each well for 5 minutes to reduce nonspecific background staining. The cell monolayers were fixed and the DNA

denatured with 0.25 ml of fixative solution containing ethanol and sodium hydroxide. Alkaline phosphatase labeled HSV DNA probe hybridization solution (0.25 ml) was added to each well and the plate incubated in a 45°C water bath for 30 minutes. The plate was washed three times with wash solution made of phosphate buffered saline and incubated with wash solution for 10 minutes at 45°C. To develop color, 0.25 ml chromogenic substrate solution containing dimethylformamide, bromochloroindolyl, and nitrotetrazolium blue was added to each well and the plate placed in a 45°C water bath for 60 minutes. The wells were washed with distilled water and each well examined for stained cells at 40 and 100X using an inverted light microscope. A positive result was indicated by >10 cells in the monolayer whose nuclei are stained purple.

Surecell method

Surecell kits were obtained from Eastman Kodak Company, Rochester, New York. The Surecell kit includes a test cell with three wells (negative control, positive control, and patient specimen well), extraction buffer, three wash solutions, peroxide solution, negative control conjugate, antibody conjugate, leuco dye solution, extraction tubes and filter tips.

The Surecell test is able to detect HSV-1 or -2 directly from patient swabs or in patient swabs placed in viral transport medium. All specimens in this study

were tested after placement of the swab in viral transport medium. HSV-specific antigens, if present in the patient specimen, are extracted by placing the swab, 0.5 ml of the viral transport medium, and 0.5 ml of extraction buffer into an extraction tube. The swab was rotated for one minute in the extraction solution to release HSV-specific antigens and then discarded. A filter tip was attached to the tube and equal aliquots of solution were filtered into the three test cell wells. Each well contains one filter membrane and an underlying absorbent pad so as the filtrate is drained through the wells, any HSV-specific antigen present in the filtrate binds to the filter membrane present at the bottom of the well. To eliminate non-specific binding reactions, each of the three wells were rinsed with buffered wash solution followed by hydrogen peroxide solution. The negative control conjugate containing non-HSV specific monoclonal antibodies was then added to the negative control well and the HSV-specific monoclonal antibody conjugate was added to the positive control and patient wells. After five minutes, the wells were rinsed and a leuco dye solution was added to develop color. Presence of a uniform pink color in the patient and positive control wells, but not in the negative control well, indicated a positive result.

Statistical Analysis

Calculations of sensitivity, specificity, and positive and negative predictive values for the HSVDISK and Surecell assays were made in comparison to the

standard cell culture method, which was assumed to be 100% accurate in the determination of whether a patient specimen was positive or negative. The calculations were as follows:

$$\text{Sensitivity} = \frac{\text{true positives}}{\text{true positives} + \text{false negatives}} \times 100$$

$$\text{Specificity} = \frac{\text{true negatives}}{\text{true negatives} + \text{false positives}} \times 100$$

$$\text{Positive Predictive Value} = \frac{\text{true positives}}{\text{all positives}} \times 100$$

$$\text{Negative Predictive Value} = \frac{\text{true negatives}}{\text{all negatives}} \times 100$$

RESULTS

HSV Detection in Clinical Specimens

One hundred fifty four specimens collected from patients of St. Elizabeth Medical Center were analyzed for presence of HSV. Of these, 131 were cervical and/or vaginal cultures, 11 were penis cultures, and 12 cultures were from other sites such as skin, esophagus, stomach and abdomen. Sixty-five specimens were taken from patients experiencing symptoms suggestive of an HSV infection (symptomatic), while 89 were from patients showing no signs of HSV infection (asymptomatic). All specimens taken from asymptomatic patients were cervical and/or vaginal cultures.

Of the 154 patient specimens collected for the study, 40 were tested by standard cell culture, HSVDISK, and Surecell, 2 were tested by cell culture and Surecell only, and 112 were tested by cell culture and HSVDISK only. To assess their overall performance for detecting HSV in clinical specimens, the results from the Surecell and HSVDISK assays were compared to standard cell culture, the "gold standard" method of detecting HSV. Table 1 shows the comparison of results obtained for the 152 specimens tested by the HSVDISK and cell culture assays, while Table 2 shows the comparison of results obtained for the 42 specimens tested by the Surecell and cell culture assays. There was disagreement

in results for 3/152 specimens tested by the HSVDISK and cell culture assays (Table 1) and for 5/42 specimens tested by the Surecell and cell culture assays (Table 2). Thus there was concordance in results for 98% of the specimens subjected to the HSVDISK and standard cell culture methods, whereas 88.0% of the results obtained by the Surecell method agreed with the results determined by cell culture.

HSV was isolated by standard cell culture in 23 of the 154 specimens. Incidence of herpetic infection in the overall population based on standard cell culture analysis was thus 14.9%. Only one of the positives by cell culture came from an asymptomatic patient. The HSVDISK method detected herpes simplex virus in 21/152 and the Surecell method detected HSV in 12/42.

Performance of HSVDISK and Surecell

The sensitivity, specificity, positive and negative predictive values were calculated to assess the effectiveness of the HSVDISK and Surecell tests in detecting HSV in clinical specimens. Calculations of sensitivity, specificity, and positive and negative predictive values for the HSVDISK and Surecell assays were made in comparison to the standard cell culture method, which was assumed to be 100% accurate in the determination of whether a patient specimen was positive or negative. Table 3 shows the sensitivity, specificity, positive and negative predictive

values calculated for the HSVDISK and Surecell tests. Both the HSVDISK and the Surecell tests exhibited similar sensitivities of detecting HSV (90.9% and 88.9%, respectively), though the ability of the Surecell test to predict true positives was much lower (66.6%) than that for the HSVDISK test (95.2%). The HSVDISK test was 99.2% as specific as standard cell culture in detecting HSV in clinical specimens, whereas the specificity of the Surecell test was 87.9% that of cell culture.

Detection times of each of the tests studied were also compared. Results could be reported for the Surecell assay within 15 minutes and for the HSVDISK assay in one day. Positive results by standard cell culture were reported between 1 and 4 days. The average detection time for a positive result by standard cell culture was 2.2 days. Table 4 gives the times to detection for the positives by cell culture. 30.4% of cell culture positives were detected as positive in one day, 65.2% were detected positive in 2 days, 82.6% were detected in three days, and 100% were detected by the fourth day. Two specimens, a skin and an abdominal fluid specimen, were found positive by standard cell culture but negative by the HSVDISK assay. CPE was detected on day 2 for the skin specimen and on day 4 for the abdominal fluid specimen. The abdominal fluid specimen was also found negative by the Surecell assay.

Patient Population

Incidences of herpetic infection in the symptomatic and asymptomatic subpopulations were also calculated. Of the 65 patients (18 males, 52 females) who were known to be symptomatic, there was an overall incidence of active herpetic infection in the population of 22/65 (33.8%). This value is comparable to those found in similar symptomatic patient populations (96, 114). Only one of the 89 asymptomatic, obstetrical/ gynecological patients tested positive for HSV by cell culture. Incidence of active herpetic infection in the asymptomatic population was thus 1.1%.

Since most of the specimens obtained in this study came from genital sites, an assessment of the incidence of genital herpes infection and the ages at which the patients experienced the genital infection was also made. Of the 142 genital specimens, twenty were found positive by cell culture. The other three non-genital cell culture positives included a wound, an abdominal fluid, and a gastric fluid specimen. Eighteen of the 20 positive genital cultures were taken from females. All positive female genital specimens came from women between the ages of 14 and 30, while the two positive male specimens came from 18 and 28 year old males. The mean age of persons experiencing a herpes genitalis infection as confirmed by cell culture was 20.6 ± 2.96 with an age range of 14-30. Two of these patients were known to have a history of herpes infection.

Four of the genital cultures found positive were taken from symptomatic pregnant women. Symptoms in two of the women included hyperemesis gravidarium (excessive morning sickness) for the woman who was 21 weeks pregnant and vaginal drainage for the women who was 24 weeks pregnant. Herpetic lesions were observed at delivery in the two other cases. One of the women, who had a herpetic lesion on her right thigh, delivered prematurely because of ruptured membranes at 24 weeks. It was unknown if any of the infants were affected by neonatal herpes infection.

DISCUSSION

The HSVDISK DNA probe assay exhibited a number of visually-distinct types of positive reactions depending on the extent of infection of the cell monolayer. In the majority of the clinical isolates, a positive reaction was determined by the observance of a focal cluster of cells with nuclei stained purple. These foci would most often be accompanied by signs of CPE (e.g., syncytia formation and holes in the cell monolayer). Some patient wells possessed as few as two purple foci, though results from specimens containing a high titer of virus showed considerable cell destruction and detachment. There was no problem in determining whether a patient result was positive or negative, despite the fact that some positives looked different than others. In addition, no background staining due to non-specificity of the probe or loss of purple stain due to high cellular toxicity occurred, as was previously reported (115).

In the Surecell EIA test, there were difficulties in interpreting a result when there was a low amount of or non-specific deposition of pink dye on the filter membrane. Instructions provided by Kodak suggest that a patient specimen is positive for the presence of HSV antigen if a uniform pink color in the patient well is greater than the color found in the negative control well. For 4 of the total 42 specimens analyzed by this method, it was difficult to determine if the patient well a)was uniformly colored with the pink dye or b)was more pink than the

negative control well. Two of the four were most likely false positive results, as the color in each patient well was very light pink and the cell culture assay and HSVDISK assay results were negative. The remaining two results agreed with the results obtained by the HSVDISK assay and the cell culture assay, despite the difficulty that was encountered in interpreting these results.

The cell culture and HSVDISK methods reported different results for three specimens, all of which came from symptomatic patients. One abdominal fluid specimen and one skin specimen were found to be negative by the HSVDISK assay but positive by standard cell culture. The abdominal fluid specimen found negative by HSVDISK was positive by cell culture on day 4. The four day detection period required by cell culture suggests that the HSVDISK result was most likely a false negative due to a low titer of infectious virus in the patient's specimen. The Surecell result for this particular specimen, however, was also negative. Thus there is the possibility that the cell culture may have been contaminated by carryover of HSV, but such contaminations are not likely. Nevertheless, the HSVDISK assay was successful in detecting HSV in three other specimens that took 4 days for cell culture to detect.

The HSVDISK result for the skin specimen was most likely a false negative result also, as this specimen had been frozen for 17 days prior to HSVDISK testing and freezing specimens has been shown to decrease the number of

infective viruses in a sample (116). Ten other cell culture-positive patient specimens were frozen for 2-30 days prior to HSVDISK testing. Despite the possible decrease in titer, all ten of these specimens were found positive by HSVDISK.

The HSVDISK method detected one positive result which was not detected by cell culture or by the Surecell assay. A total of five foci with stained cells were found in the two HSVDISK wells, indicating that the patient specimen probably contained a low HSV titer. This patient was experiencing genital blisters at the time the viral culture was taken. The attending physician suspected the infection was caused by yeast or HSV, however, no follow up information on the physician's final diagnosis of the patient was obtained. Since both wells contained foci with stained cells, it is not likely that the patient wells were contaminated by carryover from the positive control, especially since the patient wells were not adjacent to the positive control well and no other wells on the plate were positive for HSV. The low titer of HSV present in the specimen may have been inactivated during transport to the virological lab, or the specimen may have produced such a low amount of CPE that it was not detected during screening.

Overall, the sensitivity, specificity, positive and negative predictive values for the HSVDISK system were close to that of cell culture. This result was expected as the only differences between these two assays are that 1)the

HSVDISK assay uses DNA probe hybridization instead of immunofluorescence staining to confirm an HSV isolate and that 2)the HSVDISK assay does not screen for CPE prior to performing a confirmatory test for HSV presence. The use of an HSV-specific DNA probe gives the HSVDISK assay the ability to detect HSV in the cell monolayers before the appearance of CPE. Thus all results can be reported in one day, since it is not necessary to wait for the appearance of HSV-caused CPE before the *in situ* DNA probe hybridization test is performed.

The Surecell and cell culture tests produced different results for five specimens. One of these results was the aforementioned abdominal specimen which yielded a negative result by Surecell and a positive result by standard cell culture. The other four discrepancies occurred with genital specimens from symptomatic patients. These specimens were found to be negative by standard cell culture and the HSVDISK assay but positive by the Surecell assay. Several reasons could account for the discrepancies. First, these four specimens may have been false positives in which case the antibody used in the Surecell test to detect viral antigen may have non-specifically bound to the filter membrane or to some other substance present in the patient specimen. Non-specific binding of antibody has been suspected to occur in other immunoassays (88, 94), nevertheless, the Surecell test formerly demonstrated a high specificity (98.9%) and positive predictive value (96.7%) in comparison to cell culture (89). Another reason for the four discrepancies may be that active virus originally present in the patient

specimen was inactivated during transport, so that active infection of the cell monolayer was never established in the cell culture and HSVDISK tests. In such a case, viral antigen in the patient specimen would still have been detected by the Surecell assay. Loss of infectious HSV may have occurred as a result of improper collection or mishandling of the specimen prior to receipt in the hospital laboratory. If this occurred, the 66.6% positive predictive value calculated for the Surecell test would be falsely low. Loss of infectious HSV during transport most likely occurred in two of the four patient specimens producing discrepant HSVDISK and cell culture results, since both of these patients had a history of genital herpes infection and both were experiencing genital pain, drainage and lesions at the time the specimens were taken.

The Surecell test is only recommended for use either for confirmation of cell culture or screening for positives before cell culture is performed. For 37/42 specimens, this assay was able to duplicate cell culture results, making it a rapid, easy, and accurate screening test for clinics, doctor's offices and hospitals that normally send patient specimens to virological labs for cell culturing. The Surecell assay by itself, however, is not a reliable detector of HSV infection because patient results are sometimes hard to interpret and because false positive results may be produced by the assay. On the other hand, HSV present in the patient specimen need not be viable to be detected by the Surecell assay.

Though cell culture with confirmation of HSV infection by immunofluorescence staining, EIA, ELISA, or immunoperoxidase staining is the most sensitive technique to date, cell culture still takes an average of 3 days before positive results can be reported to the physician (14, 17), and can take even longer (up to 14 days) to report an accurate negative result (74). The average time for isolating HSV in cell culture was 2.2 days and only 30.4% could be detected at 24 hours. The HSVDISK result could be reported in one day and the Surecell result could be reported in approximately 15 minutes from specimen receipt. For hospitals such as St. Elizabeth Medical Center which do not have the proper equipment and/or personnel to perform cell culture, transport time of the specimen from the hospital lab to the virological lab where cell culturing will be performed delays reporting of results (usually by one day). Thus rapid and accurate tests such as the Surecell and HSVDISK assays could be attractive alternatives to cell culture testing.

One genital culture from an asymptomatic patient was found to be positive by the HSVDISK and cell culture assays. The patient specimen was found positive by cell culture at 4 days post-inoculation and only produced two foci of stained cells per well when tested by the HSVDISK assay. Thus the HSV titer in the patient specimen was most likely low, as would be expected from a person who was asymptomatically shedding HSV. Cell culturing and related techniques have in the past been shown to be poor predictors of asymptomatic genital

shedding (10). The detection of HSV in the patient specimen was therefore an unanticipated occurrence. No information on whether the patient had a prior history of genital HSV infection was available.

No follow-up information was available on the status of the infants of the pregnant women who tested positive for herpes during pregnancy. Only two of these women had herpetic lesions at the time the culture was taken. Women will often times first discover they have a genital HSV-2 infection during pregnancy (117), because the fluctuations in hormone levels reactivate an HSV-2 infection contracted prior to pregnancy. Reactivations from the cervix or vagina are especially a problem when they occur at the time of delivery. If the patient experiences lesions at this time, it is recommended that the baby be taken by caesarean section. However, these reactivations of latent HSV do not always accompany symptoms that are noticeable to the doctor or that would lead the doctor to perform a caesarean section. During these times of asymptomatic shedding, the baby may inadvertently be exposed to the virus. Fifty percent of newborns exposed to HSV at or around the time of delivery will die, and the majority of those that live will develop sequelae such as mental retardation, seizures, microcephaly, retinal dysplasia, encephalitis, or meningitis.

Because there are currently no clinically available tests that are sufficiently rapid and accurate to predict asymptomatic shedding of HSV at the time of

delivery, future research in the development of tests sensitive enough to detect such viral shedding is essential. PCR seems to be the most promising testing technique for the near future, though widespread use of PCR in clinical labs has not yet been instituted because of problems associated with purifying patient specimens (16). Past PCR studies have claimed the detection of asymptomatic viral shedding in women at delivery (109, 110). These studies have based their findings on the assumption that viral shedding can only originate from neuronal cells, and not from non-neuronal cells (such as the cervical or vaginal epithelium). Other researchers claim that these PCR studies do not distinguish between the amplification and detection of latent viral DNA and the DNA of viruses that have been shed from host cells (16). Whether HSV is capable of establishing and maintaining latency in non-neuronal tissue has long been a matter of debate (118, 119). Most researchers, nevertheless, seem to support that latency can only be maintained in neuronal cells (1, 2, 109, 110), where latent HSV DNA can be harbored without being destroyed by immune cells.

Despite the controversy of whether current PCR techniques can detect asymptomatic viral shedding in pregnant women at delivery, PCR has proven to be as sensitive and more rapid than cell culture in determining if neonates are infected with HSV (110), in detecting HSV in the cerebrospinal fluid (CSF) of persons with HSE (112), and in screening patients for HSV infection (108, 111). For neonates with HSE, HSV is demonstrable in CSF by cell culture in an

estimated 25-30% with the infection (56). Brain biopsy has thus far been the most accurate predictor of HSE infection (56). In a 1991 study performed on seven neonates with HSV infection, PCR was able to detect HSV DNA in multiple serum samples of one of the neonates in which cell cultures were repeatedly negative (110). The high sensitivity and rapidity of the PCR assay for detecting HSV DNA may in the future make it a preferable technique to cell culturing. In addition, diagnosis of HSE by performing PCR on serum or CSF samples is a safer alternative than diagnosis made by brain biopsy.

Development of more sensitive, rapid and accurate HSV diagnostic techniques would not be necessary if effective vaccines were available. A number of points must be considered in the development of HSV vaccines (120). First, the vaccine should provide protection against viral replication in the epithelial cells of mucous membranes, otherwise the HSV could establish latency in surrounding neurons. Preventing infection of these epithelial cells by exogenous HSV could be accomplished by creating a vaccine that will stimulate a strong IgA response in the recipient.

Secondly, the immunity provided by the vaccine should be long lasting. Development of attenuated vaccines which can be reactivated from latently infected cells would be ideal for generating long lasting immunity. The safety of the vaccine for human usage must also be considered. Since HSVs have been

implicated in oncogenesis, the use of live or inactivated HSV vaccines is highly undesirable. If not properly attenuated, these live vaccines could be the basis for symptomatic infections. Currently, however, vaccine research has been focused on the development of vaccines based on synthetic viral polypeptides, selective viral genes cloned into non-HSV vectors, and live genetically engineered HSV, since these vaccines have no transforming potential. None of the HSV vaccines that have been tested for therapeutic efficacy in humans have thus far been successful (121). However, some vaccines tested in animal models have been successful in providing protection against subsequent HSV exposures (122, 123). The course of HSV disease in animals and susceptibility of the animals to HSV infection differs in comparison to humans. Therefore success of vaccines in animal models does not necessarily indicate that these vaccines will be efficacious for human usage.

CONCLUSION

Presently there is no single diagnostic assay that is rapid or sensitive enough to detect HSV in pregnant women near delivery, in immunocompromised patients, or in persons with suspected HSE. PCR appears to be the most promising rapid diagnostic technique for the future. Further refinements with this method could produce an HSV diagnostic test that is less cumbersome and more rapid than cell culture. Based on data acquired in this research, assays such as the Surecell and HSVDISK assays can, however, provide dependable alternatives to standard cell culture. The HSVDISK assay is a cell culture method that is compact, easy to work with and requires minimal prior experience with cell culture. The *in situ* DNA probe eliminates the need of daily cell culture screening for CPE and since cells are delivered weekly, there is no need for the maintenance of a cell culture line. In addition, results are easy to read and can be obtained in 24 hours. The HSVDISK assay, however, can only be used in a lab that has access to or is equipped with a laminar flow hood, centrifuge, humidified incubator, -70°C freezer (for freezing controls) and an inverted light microscope. Overall the HSVDISK assay produced results comparable to standard cell culture, making it a dependable alternative to the cell culture method.

The Surecell test is particularly appealing for hospitals, clinics, and doctor's offices that cannot afford to maintain expensive cell culturing equipment and

experienced staff. Since the test can be performed in less than 15 minutes, it is an excellent screening test for HSV, either directly from the swab or from swabs placed in viral transport medium. One of the drawbacks with the Surecell assay is that the interpretation of the result is not always clear, especially if the specimen swab contains a low titer of HSV. Because the positives are not always evident, at least one swab (or excess viral transport medium) should be reserved for cell culturing if a negative result is obtained by the Surecell assay. Despite this drawback, the Surecell assay is easy to perform and requires no specialized equipment. The Surecell assay could also be used as a confirmatory technique for viral isolates from cell culture, since the assay only takes a few minutes to perform.

In conclusion the continual evaluation of HSV diagnostic tests provides a service to hospitals, clinics, and doctor's offices that do not have the funding or the means to randomly try out various tests to see which is the most appropriate for their purposes. Moreover this research as well as other HSV research concerning cures, therapy, and prevention helps to a)provide more efficient patient care b)limit the prevalance of HSV infections and the significant morbidity and mortality associated with HSV infections and c)ease the psychosocial impact on persons with genital herpes.

TABLE 1

Comparison of HSV detection by HSVDISK *in situ* DNA probe assay to HSV isolation by standard cell culture

HSVDISK result	No.	Standard Cell Culture	
		No. positive	No. negative
Positive	21	20	1
Negative	131	2	129

TABLE 2

Comparison of HSV detection by Surecell enzyme immunoassay to HSV isolation by standard cell culture

Surecell result	No.	Standard Cell Culture	
		No. positive	No. negative
Positive	12	8	4
Negative	30	1	29

TABLE 3

Performance assessment of Surecell and HSVDISK test in comparison to standard cell culture[a]

Test	Sensitivity	Specificity	Predictive Value Positive	Predictive Value Negative
HSVDISK[b]	20/22 (90.9%)[d]	129/130 (99.2%)	20/21 (95.2%)	129/131 (98.5%)
Surecell[c]	8/9 (88.9%)	23/33 (87.9%)	8/12 (66.6%)	29/30 (96.7%)

[a] Sensitivity, specificity, and positive and negative predictive values were calculated according to equations found in the Materials and Methods

[b] Total specimens analyzed: 152

[c] Total specimens analyzed: 42

[d] Percentages were in relation to cell culture which was considered to be 100% accurate in the determination of whether a patient specimen was positive or negative

TABLE 4

Detection of HSV by cell culture at various times post-inoculation

Cell Culture Incubation[a]	No. HSV Positive (%)[b]
1	7 (30.4%)
2	8 (65.2%)
3	4 (82.6%)
4	4 (100.0%)

[a] Times listed do not take into account transport time from the hospital laboratory to the virological laboratory where cell culture testing was performed

[b] A total of 23 patient specimens were positive by cell culture

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