Guidelines for the laboratory diagnosis of genital herpes in eastern European countries

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These guidelines aim to provide comprehensive information about sexually transmitted herpes simplex virus (HSV) infection and its laboratory diagnosis in eastern European countries. They are primarily intended for professionals testing specimens from patients at a sexual healthcare clinic but may also be helpful for community-based screening programmes. In particular, the guidelines recommend: (i) either viral culture or validated and approved nucleic acid amplification tests (NAATs) as the tests of choice for symptomatic patients, which should be promoted for laboratory confirmation of HSV infection; (ii) if culture or NAATs are not available, antigen detection – a direct immunofluorescence test or enzyme immunoassay from samples from symptomatic patients – could be employed, but HSV type determination is of importance; (iii) only type-specific serology should be used for detecting asymptomatic individuals, testing pregnant women at risk of acquiring HSV infection close to delivery, men who have sex with men and people who are HIV positive; (iv) widespread screening for HSV antibodies should be discouraged; and (v) any non-validated diagnostic tests should be validated against a recommended, approved gold standard.

Introduction

During the past 20 years, genital herpes has emerged as one of the most prevalent sexually transmitted infections (STIs). However, data on morbidity due to genital herpes infections in eastern European countries is scarce and their reliability doubtful owing to the lack of validation studies for the diagnostic tests used. The World Health Organization (WHO) has estimated a prevalence of herpes simplex virus type 2 (HSV-2) infection of 29 million cases in men and 12.3 million cases in women in eastern Europe and central Asia in 2003 [1]. The international classification of diseases caused by herpes virus (anogenital) [2] is presented in Table 1.

Human herpes simplex virus infections can be caused by HSV-1 or by HSV-2. In general, infections caused by HSV-1 manifest above the neck and are acquired as a result of close contact with infected persons, usually in childhood. In contrast, the lesions of infections caused by HSV-2 are usually located below the waist and are usually acquired as a result of sexual contact with infected persons later in life. Unfortunately, the differentiation of HSV-1 from HSV-2 based on anatomical site of infection is not absolute, since genital herpes may frequently be caused by HSV-1 as a result of oro-genital sexual practices and *vice versa*. The lesions and natural history of the resulting illnesses are very similar. However, because HSV-2 is almost always associated with genital disease, whereas HSV-1 is associated with both oro-pharyngeal and genital disease, there is often considerable stigma associated with HSV-2 infection. Acquisition of HSV-1 usually results in lesions of the oro-pharynx and around the mouth and on the lips and chin. Occasionally the eyes are affected. Sexual transmission of HSV most often produces infection of the genital mucosa, genital skin (penile and labial) and the perigenital region. Virus from genital secretions

Table 1

International classification of diseases: anogenital herpes virus infection

Classification code	Description
A60	Anogenital herpesviral [herpes simplex] infection
A60.0	Herpesviral infection of genitalia and urogenital tract
A60.1	Herpesviral infection of perianal skin and rectum
A60.9	Anogenital herpesviral infection, unspecified

Source: [2].

can also infect other areas, including the eyes and oropharynx and rectal mucosa [3,4].

Primary herpetic infection, i.e. when an HSV-seronegative person acquires HSV-1 or HSV-2, is usually the most severe manifestation of infection. Children may develop severe oro-pharyngitis following primary exposure to HSV-1. This episode resolves spontaneously, but recurrences may occur as a result of reactivation of the infection that has become latent but persists in the cervical ganglia. Similarly, if an individual has not been exposed to HSV-1 in childhood, he or she may develop severe genital lesions following sexual exposure to HSV-2 later in life. As with HSV-1 infections, primary HSV-2 infections resolve spontaneously but recurrences are likely to occur as a result of reactivation of latent infection that has been established in the sacral ganglia. In cases of initial, non-primary infection, i.e. when a person with antibodies to HSV-1 subsequently acquires HSV-2, the genital infection is less severe, but is also associated with recurrences. In most cases of genital herpes (80–90%) the disease progresses subclinically, but may become symptomatic at any time [5,6]. The incubation period of both HSV-1 and HSV-2 is usually from two to 10 days (up to four weeks). Therefore, the first episode may indicate either recent or long-lasting infection [7].

Recurrent herpetic infection is associated with reactivation of the virus. The recurrences arise with different frequency: from once every few years to several times per month. The localisation of the primary and recurrent lesions usually coincides. Both oral and genital herpes are manifested by acute recurrences followed by varying periods of latency, when the virus remains in a non-multiplying episomal form in the nuclei of the neurons in the ganglia. Classically, each episode or recurrence is characterised by a patch of redness at the site of the recurrence, followed by a localised papular then vesicular rash. The vesicles contain a clear fluid that contains many thousands of infectious viral particles. These vesicles burst, forming shallow ulcers or erosions that eventually crust and heal spontaneously without leaving scars. These episodes usually last less than 10 days, but may be prolonged as a result of secondary bacterial infection or immunosuppression.

Genital and oral herpes are life-long infections. Neonatal herpes (including neonatal encephalitis) and increased risk for acquiring and shedding human immunodeficiency virus (HIV) are the most serious

Table 2

Main clinical symptoms, manifestations and complications of genital herpes infections

Patients	Clinical symptoms	Clinical manifestations	Complications
Females	• Papular and/or vesicular rash on genitals or thighs • Genital ulceration • Dysuria • Vaginal and/or cervical discharge • Dyspareunia • Inguinal discomfort	• Papular and vesicular rash on vulva, perineum, thighs • Urethritis • Vaginal discharge, Dysuria • Dyspareunia • Hyperaemia of the mucous membranes of vulva and vagina • Cervicitis	• Viral meningitis • Radiculomyelopathy with the involvement of sacral nerves • Extensive vesicular skin rash • Increased risk for acquiring and shedding human immunodeficiency virus (HIV)
Males	• Papular and/or vesicular rash on genitals or thighs • Genital ulceration • Perineal pain • Dysuria • Inguinal discomfort	• Papular and vesicular rash on thighs, penis, perineum • Urethral discharge • Dysuria	
Newborns (and/or infants)	• Vesicular skin rash • Keratoconjunctivitis • Mild pyrexia • Lethargy • Convulsions	• Vesicular skin rash • Keratoconjunctivitis • Mild pyrexia • Irritability • Convulsions	• Generalized skin rash • Encephalitis • Infant death

Table 3

Patient type and main indications for testing for genital herpes

Patients	Indications for testing for genital herpes
Males	• Presence of vesicular and/or ulcerative lesions on penis, buttocks or perineum • Symptoms of dysuria following treatment for gonorrhoea and/or nongonococcal urethritis • History of recurrent vesicular and ulcerative genital skin lesions
Females	• Presence of vesicular and/or ulcerative lesions on the genitals, buttocks or thighs • Presence of a mucous or purulent vaginal discharge • History of recurrent vesicular and/or ulcerative genital skin lesions on the genitals, thighs, buttocks, perineum
Newborns	• Born to mothers who had genital herpes during pregnancy • Vesicles, vesicular rash or crusts on skin
Other	• Had sexual contact with a proven case of genital herpes • Being examined for other sexually transmitted infections • Sex workers

consequences of genital herpes infection [8,9]. The main clinical symptoms, manifestations and complications of genital herpes infections are summarized in Table 2.

Importance of laboratory diagnosis of genital herpes

The clinical differentiation of genital HSV infection from other infectious and non-infectious aetiologies of genital ulceration is difficult and laboratory confirmation of the infection should always be sought [5,9]. Accordingly, exclusive reliance on clinical diagnosis could lead both to false positive and false negative diagnosis of the condition [6,9]. HSV is the most common cause of sexually acquired genital ulceration, however, the role of causative agents of other STIs, such as *Treponema pallidum* and *Haemophilus ducreyi* should not be forgotten. Occasionally HSV and *T. pallidum* can be recovered from the same lesion [9,10]. Non-infectious causes of genital ulceration, such as inflammatory bowel disease (Crohn disease), mucosal ulcerations associated with Behcet syndrome or fixed drug eruption, may also be confused with genital herpes [9]. The types of persons who are recommended to be tested for genital herpes infections are listed in Table 3.

Table 4

Recommendations for sample collection for the diagnosis of genital herpes infections

Specimen type or collection site	Tools for sample collection	Collection method
Male skin or mucous membrane lesions	• Sterile needles • Sterile cotton-wool or Dacron swab on a wooden, plastic or aluminium shaft • Microscope slides	• Open the vesicles with a sterile needle. • Collect the content of the vesicles with a swab and: ○ apply to a microscope slide (for immunofluorescence staining) or ○ introduce into transport media for viral culture or NAATs.
Male urethra	Sterile cotton-wool or Dacron swab on a wooden, plastic or aluminium shaft	• Clean the external urethral opening region with a swab moistened in saline. • Draw back the prepuce to avoid contamination when sampling. • Insert a cotton-wool or Dacron swab carefully into the external urethral meatus (to a depth of 0.5–2 cm) and collect urethral exudates for testing.
Female skin or mucous membrane lesions	• Gauze and cotton swabs • Microscope slides	As for male skin or mucous membrane lesions.
Female urethra	• Sterile gauze swab (to remove excess discharge) • Sterile cotton-wool or Dacron swab on an aluminium shaft	• Clean the introitus using a sterile gauze swab. • Carefully insert a cotton-wool or Dacron swab on an aluminium shaft into the urethra (to a depth of 0.5 cm) to collect exudates for testing.
Cervix	• Vaginal speculum • Sterile gauze swab • Sterile cotton-wool or Dacron swab on a wooden or plastic shaft	• Insert the vaginal speculum, which may be moistened in advance with warm water and clean the cervical canal opening thoroughly with a sterile gauze swab. • Insert a cotton-wool or Dacron swab carefully into the cervical canal (to a depth of 2 cm) and collect the material from lesions.
Vagina (of prepubertal girls)	Cotton-wool or Dacron swab on an aluminium shaft	Insert a cotton-wool or Dacron swab on an aluminium shaft carefully through the hymen into the vagina and collect the material from the back wall of the vagina.
Urine	Sterile container for urine	Ask the patient to collect the first 10–20 ml of voided urine (first catch). Note: patients should avoid urinating for least two hours before sampling.
Conjunctiva	• Sterile cotton-wool or Dacron swab on wooden, plastic or aluminium shaft • Kimura platinum conjunctival scraper • Topical ophthalmic local anaesthetic	• If there is purulent discharge, it must be removed with a cotton-wool swab. • Move a swab over the conjunctiva of the inferior eyelid towards the interior angle of the eye (use a thin swab on an aluminium shaft for newborns). • The Kimura scraper is used to sample the bases of lesions (either ulcers or the bases of burst vesicles). Before collecting the sample, the spatula is sterilised by heating in a flame and allowed to cool.
Rectum[a]	• Rectal speculum or proctoscope • Sterile cotton-wool or Dacron swab on a wooden or plastic shaft	• Rectal material is taken under direct vision, with the aid of a proctoscope or rectal speculum. Use of a blind technique results in considerable loss of sensitivity. • Insert a swab on a wooden or plastic shaft to a depth of 3 cm and collect the material from all rectal walls by circular motions for 10 seconds. Note: if faecal material is impacted, the swab should be discarded and the sampling procedure repeated.

NAAT: nucleic acid amplification test.

[a] Material from the rectum is collected when the patient has had anal sexual contact, there are inflammatory changes, or if perianal skin or anal folds are thickened.

The guidelines presented here represent the first attempt to introduce an evidence-based approach to the laboratory diagnosis of genital herpes infections in eastern Europe. It is recognised that national adjustments to these guidelines may be needed in some eastern European countries to meet local laws and health strategies and according to the availability of kits and reagents. They are a consensus document of the Eastern European Sexual and Reproductive Health (EE SRH) Network [11,12] and comprise one element of a series of guidelines aimed at optimising, standardising and providing guidance on quality assurance of laboratory testing for reproductive tract infections [13-16]. They are primarily intended for professionals testing specimens from patients at sexual healthcare clinics but may also be helpful for community-based screening programmes.

Methods for laboratory diagnosis of genital herpes

Laboratory confirmation of the clinical diagnosis is necessary for estimating the potential infectivity during episodes of lesions, identifying persons at risk of transmitting infection subclinically, selecting women at future risk of transmitting the infection to the neonate and confirming the clinical diagnosis in those for whom antiviral therapy for HIV infection should be prescribed [8].

Methods used for the diagnosis of HSV could be divided into direct detection of virus in material from lesions and serological diagnosis. Both virological detection and type-specific serological tests for HSV should be available in clinical settings that provide care for patients with STIs or those at risk for STIs.

The recommended sampling sites and type of sample and methods to be used for the diagnosis of genital herpes infection are presented in Table 4.

The recommendations for sample transportation for testing using microscopy, culture and NAATs are presented in Table 5.

The recommended sites and methods to be used for the diagnosis of genital herpes infection are presented in Table 6.

Microscopy

General

Microscopic examination of lesion materials using Romanovsky staining is used by a number of laboratories in Eastern Europe [17]. This method, however,

Table 5

Recommendations for sample transportation, by type of test

Test method	Conditions	Comments
Microscopy	• If there is a need to save the material for more than 24 hours, the smear should be fixed with 96% ethyl alcohol for three minutes. • Each smear on a microscope slide should be placed in the transportation container and transported to the laboratory accompanied by the relevant documentation including the investigation method requested.	• If the rules of sampling and conditions of transportation of the biological material are not followed (e.g. slides are broken, unmarked or stuck together or there is no material on the slide), microscopy should not be carried out.
Viral culture	• Immediately after sampling the material must be placed in relevant transport medium, such as Eagle's medium with addition of antibiotics, or the medium validated for this purpose. • The material should preferably be transported to the laboratory on ice. • Material should not be kept for more than 24 hours at room temperature. • Accurately marked test tubes must be placed in a hermetic reservoir and transported to the laboratory accompanied by the relevant documentation including the investigation method requested.	• Herpes simplex virus is sensitive to both the temperature and to drying out, so failure to observe the transportation rules may influence the success of viral culture considerably, i.e. it is unlikely that the virus will be isolated or identified.
Antigen detection and nucleic acid amplification tests (NAATs)	Transport medium is usually provided by the manufacturer of the diagnostic system. If the sample transportation procedure is not described in the manufacturer's instructions or in-house test systems are used, transportation is performed as follows. • Clinical material placed, for instance, in transport medium should be transported in the cold only (e.g. in a cool bag at 6 ± 2 °C). • Urine should be delivered to the laboratory within three hours of collection, at ambient temperature. Test tubes containing clinical material should be transported to the laboratory accompanied by the relevant documentation including the investigation method requested.	• The material is delivered in special test tubes with transport medium according to the manufacturer's instructions for each test. • Frozen (–70°C), specimens to be tested using NAATs may be kept for up to three months. However, storage conditions must be in line with the recommendations of the manufacturer of the NAAT.

NAAT: nucleic acid amplification test.

as well as cytological examination using Tzanckand Papanicolaou smears, have been found to have low sensitivity and specificity, and therefore should not be relied upon for diagnosis [5,9,18].

Antigen detection

General

Viral antigen from swab specimens can be detected using either direct immunofluorescence (DIF) or enzyme immunoassay (EIA). Commercial diagnostic tests produced in eastern European countries for the detection of herpes-specific virus antigen have not been validated against any international standard test; therefore the data presented below reflect characteristics of tests produced in western countries.

Direct immunofluorescence

DIF could be classified as a rapid diagnostic test allowing type differentiation of genital herpes viruses [19,20]. It can be valuable when testing high-prevalence populations [21], but when testing asymptomatic patients, the sensitivity may drop to less than 50% when compared with culture [19,21]. The disadvantages of DIF are that it is time consuming, labour intensive and, compared to NAATs, has a suboptimal sensitivity.

Antigen capture enzyme immunoassays

The sensitivity of commercially available EIAs, when compared with that of viral isolation, is greater than or equal to 95% and with specificities ranging from 62% to 100% for symptomatic patients [22-27]. The sensitivity of antigen capture EIAs may be higher than that of virus culture for typical presentations, but lower for cervical and urethral swabs [22-24,27]. Most commercially available assays, however, do not differentiate between serotypes.

Viral isolation in cell culture

General

Virus isolation in cell culture has been the cornerstone of HSV diagnosis over the past two decades in laboratories of western Europe [28,29] and the United States [30]. Although HSV can be isolated from over 90% of vesicular or pustular lesions, the isolation rate from ulcerative lesions is only 70% and falls to 27% at the crusting stage [4]. Delayed transport of samples to the laboratory and lack of refrigeration during transportation substantially affect the outcome of the testing [31]. The characteristic cytopathic effect of HSV in tissue culture generally appears within 24–72 hours, but may take up to five days.

Virus isolation in tissue culture roller tubes is slow and labour intensive, but has the advantage of demonstrating active infection within a clinical lesion and also allows virus typing and antiviral sensitivity testing [32]. More rapid culture of HSV can be achieved by using shell vials [33] or multiwell plates [34] and centrifuging the specimen onto cell monolayers on coverslips. Commonly used cells include primary human fibroblasts and cell lines such as MRC-5, Vero cells, baby hamster kidney and rabbit kidney cells [35,36].

Typing of HSV using cell culture can be performed directly on infected cell cultures using fluorescein isothiocyanate (FITC)- or immunoperoxidase-labelled type-specific monoclonal antibodies by DIF or by testing the cell supernatant by nucleic acid amplification tests (NAATs), with specifically designed primers.

Storage of HSV isolates

Isolates of HSV may be stored in 0.2 M sucrose in 0.02 M phosphate-buffered saline pH 7.2 (2SP medium) at –70 °C or in liquid nitrogen.

Nucleic acid amplification tests

General

HSV detection using polymerase chain reaction (PCR) has been shown to be the test of choice in patients with genital herpes ulcers. The detection rates of the PCR assays were shown to be 11–71% superior to virus culture [30,31,37-39]. Furthermore, compared with traditional PCR, real-time PCR allows detection and typing of HSV in a single reaction tube, is faster (takes approximately two hours to perform), allows simplified conditions of performance and lowers the risk of

Table 6

Recommended sampling sites, type of sample and preferred diagnostic methods for genital herpes

Sampling site or type of sample	Preferred diagnostic method
Vesicular rash on skin and mucous membranes	Nucleic acid amplification test (NAAT) or antigen detection[a]
Urethra (male)	NAAT or antigen detection
Cervix/urethra (female)	NAAT or antigen detection
Conjunctiva	NAAT or antigen detection
Urine (men and women)	NAAT
Vulva/vagina (prepubertal girls), vagina (women after hysterectomy)	NAAT
Spinal cord fluid	NAAT
Venous blood	Serological assays[b], e.g. enzyme immunoassays (EIAs)[c]

[a] Viral culture is an additional method.

[b] For screening purposes, detecting newly acquired infections and diagnosis in persons who present without lesions or atypical lesions [31].

[c] For detection of type-specific herpes simplex virus type 2 (HSV-2) antibodies.

cross-contamination [37]. Use of NAATs for diagnosis of HSV also allows less strict sample transportation conditions, compared with those required for diagnosis by culture.

As in western Europe and the United States, there are no comprehensively validated and approved commercial NAATs available for detection of HSV in many eastern European countries. However, some NAATs for HSV detection have been developed and are available in eastern Europe, but have not been validated against their internationally acknowledged analogues.

Quality control

In each DNA extraction and subsequent analysis, an internal positive control – allowing detection of amplification-inhibited samples and controlling the quality of sample preparation – and a negative control are necessary.

Certified and registered reference panels comprising coded control specimens should ideally be used for intra- and inter-laboratory quality control. The use of specimen panels is standard for test system operation. These act as indicators of sensitivity, specificity and reproducibility, which are independent of the test systems used.

Serological tests

Serological tests detect antibodies to HSV in blood, which are indicative of ongoing latent infection. Both type- and non-type-specific antibodies to HSV develop during the first several weeks after infection and persist indefinitely. However, directly after infection there is a 'window' in which testing for antibodies will give a negative result. Serodiagnosis is useful for documenting newly acquired infections and for diagnosis in persons who present without lesions or with atypical lesions. Testing for HSV type-specific antibodies can also be used to diagnose HSV-2 infection in asymptomatic individuals [31,40], and other persons with undiagnosed HSV-2 infection. Whether genital herpes is caused by HSV-1 or HSV-2 influences prognosis and counselling. Up to 50% of first-episode cases of genital herpes are caused by HSV-1 [41], but recurrences and subclinical viral shedding are much less frequent for genital HSV-1 infection than genital HSV-2 infection [42,43].

Validation of diagnostic tests

General criteria for the validation of diagnostic tests have been published by the TDR diagnostics evaluation expert panel (TDR is a Special Programme for Research and Training in Tropical Diseases, sponsored by the United Nations Children's Fund (UNICEF), the United Nations Development Programme (UNDP), the World Bank and WHO [44]. The criteria are demanding and beyond the capacity of most individual groups. However, the minimum requirements for the validation of a new or modified test have also been published [45].

Summary

Older, classical tests can display cross-reactivity between HSV-1 and HSV-2 and even with varicella-zoster virus. During the past 20 years, a number of type-specific tests have been developed, the sensitivity and specificity of which have been evaluated to be approximately 97% and 98%, respectively [46]. Although the benefits of the serological assays (such as type-specific EISAs) include the possibility of automation and therefore simultaneous processing of a large number of samples at relatively low cost, they have a number of disadvantages that considerably limit their use in the diagnosis of genital herpes. Although the detection of HSV-specific IgM is theoretically useful to detect recent herpes infection in the absence of an IgG response, approximately a third of patients with recurrent genital herpes caused by HSV-2 have IgM responses; thus detection of IgM is a poor indicator of recent infection. Unfortunately, serological tests alone cannot inform the aetiology of a presenting genital lesion with any degree of certainty.

Recommendations

Where viral culture facilities exist, they should be maintained in order to detect the causative virus directly from skin and mucous membrane lesions. Where culture is not available, consideration should be given to the introduction of a NAAT for HSV. If NAATs are not available, antigen detection, namely DIF or EIA, could be employed, if high performance of those tests can be assured. HSV type determination is important to inform counselling and prognosis. Type-specific serology should be used for detecting asymptomatic individuals, testing pregnant women at risk of acquiring HSV infection close to delivery, men who have sex with men, and people who are HIV positive. Widespread screening for HSV antibodies should be discouraged. It is recommended that any non-validated diagnostic tests should be validated against a recommended, approved gold standard test.

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