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Improved detection of HSV by electron microscopy in clinical specimens using ultracentrifugation and colloidal gold immunoelectron microscopy: comparison with viral culture and cytodiagnosis

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Summary

Three tests were compared to diagnose herpes virus infection: electron microscopy (EM), viral culture (VC) and cytodiagnosis (Tzanck smear). The study comprised 67 patients with skin or mucous membrane lesions suggestive of herpes simplex virus (HSV) infection. The sensitivity of EM increased 25% after virus concentration by ultracentrifugation. Herpes virus infection was confirmed in 55 of the 67 cases by EM or VC or both. EM detected 53 herpes virus-positive lesion samples of which 14 were not detected by VC; only two lesion samples that were herpes virus-positive in VC were not detected by EM. The sensitivities of EM, VC, and Tzanck smear for the group of 55 herpes virus-positive cases were 96%, 75% and 76%, respectively. The specificity of the Tzanck smear was 83% (prevalence 82%). Colloidal gold immuno-EM was used to rapidly type HSV-1, HSV-2 and varicella zoster virus (VZV) present in skin and mucous membrane lesions in less than 4 h. Immuno-EM was able to detect antiviral antibodies on viral envelopes and viral cores in lesion samples with negative VC. Antiviral antibodies do not interfere with typing of herpes viruses by immuno-EM. It is suggested that formation of viral immune complexes and inactivation of virus particles by antibodies may have caused a

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negative VC. Improved EM is discussed for its applicability to special cases that cannot rely on VC and cytodiagnosis or when rapid diagnosis is required.

Rapid HSV diagnosis; Electron microscopy; Immunoelectron microscopy; Viral culture; Cytodiagnosis; Tzanck smear

Introduction

Most herpes simplex virus (HSV) infections can be diagnosed on the basis or characteristic clinical signs of the disease. However, because the characteristic vesiculobullous eruptions of HSV can resemble those of other viral and non-viral skin infections, patients can be misdiagnosed (Azon-Masoliver et al., 1990; Bhawan et al., 1984; Halal et al., 1978; Kalman et al., 1986; Oxman, 1986). The facial lesions of HSV and varicella zoster virus (VZV) infections, for example, can be easily confused, probably far more often than is generally acknowledged (Folkers et al., 1989; Kalman et al., 1986).

It is desirable to confirm clinical HSV and VZV diagnosis before treating the infection. The rapidity of obtaining a conclusive diagnosis of these herpes virus infections can be important, especially in immunocompromised patients, for prompt initiation of adequate antiviral therapy to prevent serious complications of the infection. Although viral culture (VC) by the demonstration of characteristic cytopathic effect (CPE) is still the 'gold standard' for the detection of HSV, this method is not completely specific or sensitive (Solomon, 1988). Virus isolation requires at least one day, but the CPE often does not become visible for several days (Gleaves et al., 1990). Because the herpes virus, especially in case of VZV, can be inactivated during transportation or storage, the samples must thus be inoculated as soon as possible, which is usually difficult in non-university hospitals, in out-patient clinics, and in general medical practice.

Colloidal gold immunoelectron microscopy (immuno-EM) was recently introduced as a technique to diagnose rapidly VZV infection (Folkers et al., 1989; Vreeswijk et al., 1988). This test can be used for detection of HSV as well as VZV infection.

The Tzanck smear is rapid, inexpensive, and is still frequently used in office practice. Expertise is required in interpreting virus induced cellular changes in light microscopy. The Tzanck test cannot differentiate between HSV-1, HSV-2 or VZV (Oranje et al., 1986; Solomon, 1986). Besides herpes infections, cytodiagnosis can also be used to detect non-viral agents like *Cryptococcus* species. Azon-Masoliver et al. (1990) reported that *Cryptococcus* species was

demonstrated by cytodiagnosis in a patient suffering from acquired immunodeficiency disease (AIDS) who showed herpes-like skin lesions.

The present study was undertaken to demonstrate that recently developed electron microscopy (EM) techniques can be applied to clinical specimens of skin and mucous membrane lesions for rapid diagnosis of HSV infection.

Materials and Methods

Specimens

The study was conducted at the out-patient section of the Department of Dermatology and Venereology of Hospital De Heel in Zaandam. Samples were collected from skin, orolabial and genital lesions of 67 patients (46 women and 21 men) with characteristic symptoms of HSV infection. The median age of the population was 35 years, with a range from 2 to 80 years. The herpetic lesions of the skin ($n=26$) and mucous membranes ($n=41$) were classified as being maculopapular, vesicular, pustular, ulcerous, or crusted. Thirteen lesions were from genital, 7 from oral, 25 from orolabial, and 22 from other body sites. Of all genital samples, 4 were collected from skin lesions, 7 from penile and 2 from vulval mucous membrane lesions.

Viral culture

Samples were collected with dry cotton swabs by firmly rubbing the base of the lesion. The swabs were immediately transferred into a transport medium (3 ml Hank's balanced salt solution with 0.5% gelatin, sodium bicarbonate to adjust the pH to 7.2, and antibiotics), and transported within 24 h to the Department of Virology, GG&GD (City Health Services), Amsterdam. If transportation of the specimen could not be done within 24 h, the sample was stored at $-80°C$ within 0.5 h after collection, and then transported afterwards frozen to the laboratory. Virus isolation and virus typing by immunofluorescence were carried out according to standard virological procedures with efforts to cultivate HSV as well as VZV.

Tzanck smear

Samples were generally collected from the same lesion as was used for VC. Scrapings from the edge and base of the lesion were smeared onto a glass slide, air dried, and stained with Hemacolor (Merck) (Folkers et al., 1985). The preparations were examined first by the author (EF) and then double blind by two other investigators (APO and JND). Infected epithelial cells with characteristic nuclear changes were identified by light microscopy (Barr, 1984).

Electron microscopy

Sample processing

The sample for EM diagnosis was collected from one single lesion, present at the same site as those used for VC and Tzanck smear. Infected cells were scraped from the base and the edges of the lesion with the curved side of a vaccinostyle, fixed with the scarification side in the cap of a plastic sampling tube. After the sample was collected, the cap with the vaccinostyle was placed on an empty tube with a small moistened gauze on the bottom, to prevent freeze drying of the sample. The tube was immediately placed in a refrigerator at 4°C. Within 0.5 h it was moved to a freezer at −25°C. Transportation of the sample to the laboratory of EM was arranged within 2 weeks after sampling. During transportation the temperature was carefully kept below zero. If storage for a longer period was necessary, the samples were frozen at −80°C. Virus suspensions used for EM detection were prepared by two different methods denoted as *collection of viruses from smears,* and *collection of viruses by ultracentrifugation.* Virus collection from smears was used in one experiment that compared the virus particle detection levels of both methods.

Collection of viruses from smears

Lesion samples were transferred to a glass slide followed by rupture of the tissue by firmly rolling a glass rod over the slide. Viruses were collected in approximately 30 μl (1 drop) distilled water that was spread all over the smear. The virus suspension was carefully removed with a Gilson micro pipette and transferred to Parafilm. Viruses were adsorbed to carbon coated collodion-nickel grids for 10 min. The grids were placed on 1% glutaraldehyde in 10 mM Tris-HCl buffer (pH 7.3) for several seconds, washed on two drops of Tris- HCl buffer and stained with 2% PTA (phosphotungstic acid, pH 6.8). Equal parts of the virus suspension and PTA were mixed and some grids were floated on this PTA diluted virus suspension and dried. Magnifications for screening in the electron microscope (Phillips EM300) were routinely set around 10 000 ×.

Collection of viruses by ultracentrifugation

The lesion sample was removed from the vaccinostyle with 0.4 ml Tris-HCl buffer, transferred to a mortar and homogenized with a minimum amount of ultra-fine sterile quartz sand. The sand was pelleted by low-speed centrifugation. Virus particles, present in the supernatant, were adsorbed to carbon coated collodion-nickel grids for 10 min and fixed and stained as described above.

A virus concentration procedure was carried out to increase further the sensitivity of virus detection (Vreeswijk et al., 1988). The virus suspension obtained after sand homogenization was centrifuged in a Beckman polypropylene tube (n° PAT22) through a 70 μl 30% potassium tartrate cushion in a Beckman SW 50 rotor (32 000 rpm, 30 min). The pellet was suspended in 20 μl Tris-HCl buffer and sonicated in closed microtubes by a Branson disrupter

with a cuphorn attachment (No. 627-003-020). In an additional experiment this sonicated pellet suspension was clarified by low-speed centrifugation in a desk centrifuge.

Virus particles were adsorbed to carbon coated grids for screening in standard EM, for immuno-EM to detect immune complexes (anti-herpes virus antibodies), and for virus typing (detection of HSV-1, HSV-2 and VZV) (Vreeswijk et al., 1988).

Solid phase immunoelectron microscopy

In solid phase immuno-EM (SPIEM) the virus is picked up by a bilayer of protein A and capture antibody absorbed to the EM grid. Nickel grids (400 mesh) supported by a carbon coated collodion film were floated for five minutes on a drop of protein A (25 μg/ml) in PBS. The grids were washed three times with PBS and transferred to a drop of rabbit IgG anti-HSV (National Institute of Public Health and Environmental Protection, RIVM, Bilthoven, the Netherlands), diluted 1:100 in PBS, for 10 min. The optimum concentration of protein A and the optimum serum dilution for HSV trapping were assessed essentially as described by Van Nieuwstadt et al. (1988). Grids were washed with PBS-1% BSA and incubated overnight on a drop of virus suspension. The grids were rinsed with PBS, fixed with 1% glutaraldehyde for a few seconds and stained with PTA.

Immunogold labelling

Only virus suspensions obtained after ultracentrifugation were used for colloidal gold labelling because soluble proteins present in unpurified suspensions interfere with labelling.

HSV was identified in an indirect labelling test using a monoclonal antibody against a common HSV-1 and HSV-2 antigen (RIVM, Bilthoven, the Netherlands) in the first labelling step and gold-tagged rabbit-anti-mouse antibodies in the second labelling step (Fig. 2, obtained from specimen number T5-1745/Table 3). HSV-1 and HSV-2 were differentiated in another indirect test; for the antibody used in first step a monoclonal antibody directed against HSV-1 (RIVM, Bilthoven, the Netherlands) was used. Virus typing of VZV was carried out with gold-tagged highly specific polyclonal human antibodies against VZV. Identification of antiviral immune complexes was carried out with gold tagged, affinity chromatography purified rabbit anti-human IgG antibodies against IgA, IgM and IgG (Dakopatts A190, Vreeswijk et al., 1988). Virus identification by EM and virus typing by immuno-EM was carried out without prior knowledge of the clinical appearance of the lesion and the results of the other laboratory tests.

Statistical analysis

Sensitivities and Tzanck smear specificity were calculated according to standard methods (Weinstock, 1989). For the difference in sensitivity between

two diagnostic tests exact 95% confidence intervals (CI) were calculated (Gardner et al., 1989) and the McNemar test (Siegel, 1956) was applied. McNemar's test was also used to compare the percentages of positive and negative scores obtained from the Tzanck smears by different investigators (EF, APO and JND).

Results

The sensitivities of VC, EM, and Tzanck smear were calculated for the total number of patients with clinically obvious HSV infection ($n = 67$; Table 2) and for the number of samples that were herpes virus positive confirmed by either VC, EM or both ($n = 55$; Table 4).

Viral culture

Herpes virus was isolated from 41 of 67 lesion samples obtained from skin or mucous membrane lesions suggestive of HSV infection. (Table 1: all samples). This implies a sensitivity of 61% (95% confidence interval: 48–73%). VC was positive in 58% (15/26) of the skin lesion samples and in 63% of the mucous membrane lesion samples (26/41). Five out of 13 genital (38%), 18 out of 25 labial (72%) and 4 out of 7 oral lesion samples (57%) were VC positive. Of all vesicular, pustular, and ulcerous lesion samples studied, 100%, 63% and 42%, respectively, exhibited CPE in cell culture (Table 1) VC was positive in one single crusted lesion. The single maculopapular lesion showed a negative VC. HSV-1 infection was detected in 34, and HSV-2 in 6 lesion samples. In one patient VC suggested VZV isolation based on typical CPE, reason why HSV typing was omitted; afterwards, HSV-1 was demonstrated by immuno-EM.

TABLE 1

Results of viral culture (VC) in several lesion types of 67 patients suspected of HSV infection

Lesion type	Number of VC positive samples (%)	Virus type	
		HSV-1	HSV-2
Maculopapular ($n = 1$)	0		
Vesicle ($n = 11$)	11 (100)	11	
Pustule ($n = 30$)	19 (63)	15*	4
Ulcer ($n = 24$)	10 (42)	8	2
Crusted ($n = 1$)	1	1	
All samples ($n = 67$)	41 (61)	35	6

*In one patient, herpes infection was confirmed by the typical CPE in VC, suggestive for VZV. Afterwards, HSV-1 typing was done by colloidal gold immuno-EM.

Comparison of classical herpes virus detection by EM and ultracentrifugation-enhanced EM

Because this study covers different types of lesions, including ulcerous lesions, the method for sample processing had to be standardized for optimal recovery of virus particles. To find evidence for improvement of herpes virus detection after sand homogenization and ultracentrifugation treatment of the lesion samples, the following experiment was carried out. Duplo-samples of a vesiculopustular (T5-3625), a pustular (T5-2755) and an ulcerous lesion (T4-3594) with positive TC and positive EM were obtained from 3 different patients and used for comparison of the classical EM technique with the ultracentrifugation-enhanced EM method.

The 3 lesion samples were transferred to a glass slide and transformed into a single smear. Three drops of water, for each lesion 1 drop, were used to produce a mixed virus suspension of approximately 60 μl. One third of this suspension (20 μl) was mixed with an equal volume of PTA to 40 μl for herpes virus detection in smears by classical EM. One third was used for adsorption of virus without addition of PTA and fixed and stained afterwards. The third part was transferred to a prewetted mortar with sand. To this sample the ultracentrifugation-enhanced method was applied.

The results of herpes virus detection are shown in Fig. 1. The number of particles detected after ultracentrifugation increased with a factor 10. No difference in detection of virus particles was observed before and after clarification of the suspension made from the SW50 pellet, nor caused dilution of the virus suspension to 400 μl or use of sand for tissue homogenization a decrease of virus detection in comparison with herpes virus detection in cell smear suspension by classical EM. Experiments in our EM laboratory with different types of herpes viruses (such as equine herpes virus, unpublished data) also confirmed the usefulness of sand during processing of organ tissue samples. Therefore, tissue disruption by sand was used as a standard procedure for EM.

Solid phase immunoelectron microscopy

Solid phase immuno-EM (SPIEM) was applied to some specimens to test its usefullness in combining virus trapping with virus typing by colloidal gold immuno-EM. Virus trapping from either smears or sand homogenates could not be used for routine virus typing by colloidal gold immuno-EM because of high background labeling. However, SPIEM carried out on pellet suspensions obtained after ultracentrifugation showed a better virus/background-labelling ratio, but was still hampered by a higher background labelling, compared with colloidal gold labelling of viruses directly adsorbed to carbon coated collodion-nickel grids (compare Fig. 2 and Fig. 3). Therefore, SPIEM was not fully implemented in this study.

Comparison of standard electron and immuno-electron microscopy with viral culture

The sensitivity of EM (36/67) increased approximately 25% (53/67) after virus concentration by ultracentrifugation. A sensitivity of 79% (95% CI: 67% to 88%) was calculated for the 67 patients with clinically overt HSV infection. The results of VC are compared with those of EM in Table 2. Positive VC with positive EM was obtained in 39 lesion samples. Viral culture detected herpes virus infection in 2 lesion samples which could not be confirmed by EM. In contrast, 14 lesion samples were herpes virus positive by EM but showed negative VC. The difference in sensitivity of VC and EM was statistically significant (McNemar test: $\chi^2 = 4.65$, $P_2 = 0.03$; 95% confidence interval: 6–23% difference in sensitivity).

In 31 of 53 specimens in which herpes virus was demonstrated by EM, herpes

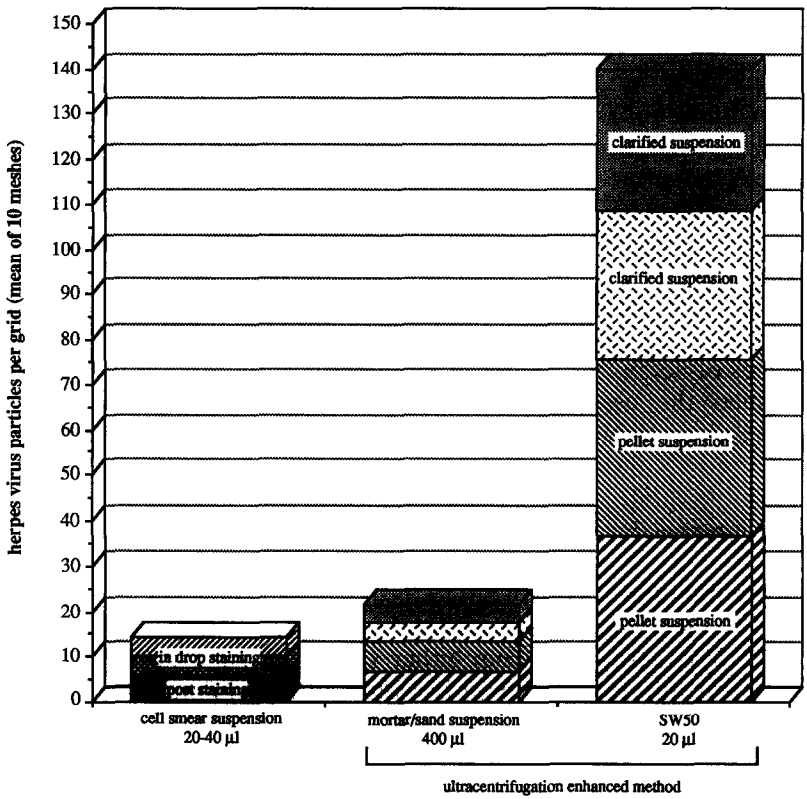
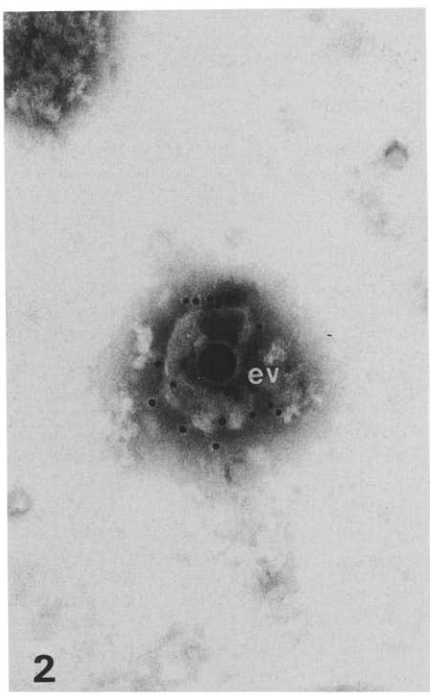


Fig. 1. Herpes virus particle counts obtained with classical direct EM on smears (left stack) and particle counts at 2 different stages of the ultracentrifugation-enhanced method (middle stack and right stack). The 4 layers of each stack represent the mean particle counts obtained with 4 different grids.



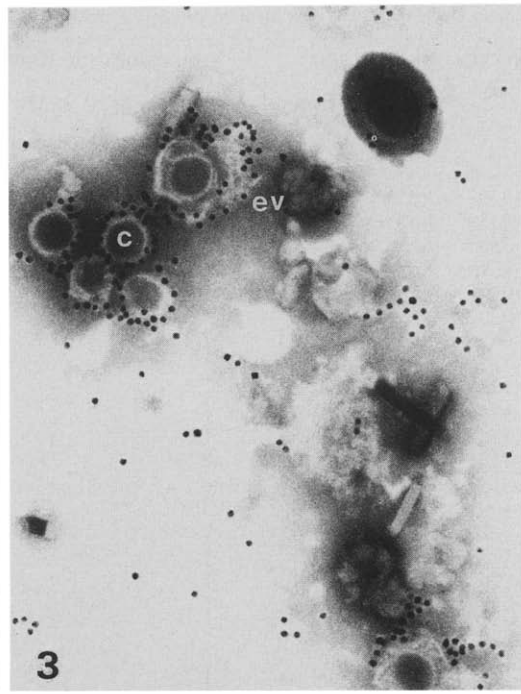


Fig. 2. Immunoelectron microscopy of specimen number T5-1745 (Table 3). Monoclonal antibodies, reactive with common HSV-1 and HSV-2 envelope antigens, were detected with gold labelled anti-mouse antibodies (60 000 ×). Fig. 3. Solid phase immuno-electron microscopy (SPIEM) of specimen number T5-1745 (Table 3). Virus particles, cores (c) and enveloped virus (ev) were adsorbed to EM grids coated with rabbit anti-HSV antibodies. Trapped virus particles were labelled with anti-HSV-1 monoclonal antibodies, followed by gold labelled anti-mouse antibodies. The efficient antigen trapping, also including viral proteins, is responsible for high background labelling (60 000 ×).

TABLE 2

Comparison of electron microscopy after virus concentration (EM), viral culture (VC) and Tzanck smear (TS) in 67 patients with clinical symptoms of HSV infection

Comparison			McNemar	Sensitivity 95% confidence interval
	EM+	EM−	$\chi^2 = 4.65$	6% to 23% difference in sensitivity
VC+	39	2	$P_2 = 0.03$	
VC−	14	12		
	EM+	EM−	$\chi^2 = 4.27$	1% to 20% difference in sensitivity
TS+	41	3	$P_2 = 0.04$	
TS−	12	11		
	VC+	VC−	$\chi^2 = 0.21$	−9% to 17% difference in sensitivity
TS+	33	11	$P_2 > 0.10$	
TS−	8	15		

TABLE 3

Comparison of viral culture, electron microscopy after virus concentration (EM) and immuno-EM at various virus typing levels after application of colloidal gold-tagged antibodies

Specimen number	Site and type of lesions	Viral culture HSV type	Results of EM (positive/negative)					
			EM	Immuno-EM				
				aHu	aVZV	MCA1	MCA12	Virus type
T4-3537	lip, pustule (m)	nd	+	+	–	+	+	HSV-1
T4-3539	lip, vesicle (m)	1	+	#	#	+	+	HSV-1
T4-3603	face, vesicle (s)	1	+	–	–	+	+	HSV-1
T5-0299	lip, ulcer (m)	nd	+	+	#	+	+	HSV-1
T5-0301	natis, pustule (s)	1	+	#	#	+	+	HSV-1
T5-0302	lip, vesicle (m)	1	+	+	–	+	+	HSV-1
T5-0429	face, crusted (s)	1	+	+	–	+	+	HSV-1
T5-1686	lip, pustule (m)	1	+	+	–	+	+	HSV-1
T5-1745	penis, ulcer (s)	1	+	–	–	+	+	HSV-1
T5-1746	lip, pustule (m)	1	+	+	–	+	+	HSV-1
T5-2755	face, pustule (s)	1	+	+	–	+	+	HSV-1
T5-3186	face, pustule (s)	nd	+	+	–	+	+	HSV-1
T5-3187	lip, ulcer (m)	nd	+	+	–	+	+	HSV-1
T5-4091	face, pustule (s)	CPE	+	+	–	+	+	HSV-1
T4-3629	lip, pustule (m)	1	+	–	–	+	#	HSV-1
T4-3739	lip, vesicle (m)	1	+	–	–	+	#	HSV-1
T5-0163	lip, vesicle (m)	1	+	+	–	+	#	HSV-1
T5-0234	vulva, ulcer (m)	1	+	–	–	+	#	HSV-1
T5-0482	face, pustule (s)	1	+	–	–	+	#	HSV-1
T5-1100	trunk, pustule (s)	nd	+	+	–	+	#	HSV-1
T5-1101	face, pustule (s)	1	+	–	–	+	#	HSV-1
T5-0621	penis, ulcer (m)	2	+	–	–	–	+	HSV-2
T4-3580	lip, pustule (m)	1	+	–	–	#	#	HSV
T5-0398	vulva, pustule (s)	nd	+	+	–	#	+	HSV
T5-0708	lip, pustule (m)	1	+	#	–	#	#	HSV
T5-0709	lip, pustule (m)	1	+	+	–	#	#	HSV
T5-0883	vulva, pustule (s)	nd	+	–	#	#	+	HSV
T5-1010A	face, vesicle (s)	1	+	#	–	#	#	HSV
T5-1010B	natis, pustule (s)	2	+	e–\c+	–	#	#	HSV
T5-1267	lip, vesicle (m)	1	+	#	–	#	#	HSV
T5-1384	face, ulcer (s)	nd	+	#	+	#	–	VZV

(m) = mucous membrane; (s) = skin; CPE = cytopathological effect; nd = no virus detected; # = not tested; aHu = rabbit IgG anti-human (IgA,IgM,IgG); aVZV = human anti-VZV antibodies; MCA1 = monoclonal antibodies against HSV-1; MCA12 = monoclonal antibodies against HSV-1 and HSV-2; e = viral envelope; c = viral core.

virus typing was carried out by immuno-EM (Table 3). The specimen numbers are presented in a way as to create an overview of different typing levels. Since the electron microscope was available for a limited time during this study, the full range of typing tests could not be applied to all lesion samples. One VC (T5-4091) suggested VZV isolation based on the typical CPE (Table 3: column 3, CPE). Therefore, herpes virus typing was omitted but HSV-1 was clearly demonstrated by immuno-EM. In 7 cases, showing negative VC (Table 3: column 3, nd), immuno-EM was positive for HSV. In one sample (T5-1384), with negative VC, immuno-EM demonstrated VZV infection (Table 3: column

9, VZV). This case was included in the calculations for sensitivity of the tests for detecting herpes virus infection.

Incubation with colloidal gold tagged rabbit IgG anti-human IgA, IgM, and IgG antibodies (Table 3: column 5, aHu) to detect antivirus antibody complexes, was done in 25 lesion samples. Antiviral antibody-coated virus particles (labelled viral envelopes and viral cores) were seen in 15 samples, of which 6 correspond to negative VC. In one lesion sample (T5-1010B) only viral cores were labelled with antivirus antibodies but not viral envelopes.

The total number of lesion samples with negative VC and positive EM was 14 (Table 2). Eight samples out of these 14 were investigated by immuno-EM and are shown in Table 3 (column 3; nd). In the remaining 6 patients with negative VC, herpes virus infection was demonstrated by EM without further virus typing, which implicates that some of these samples may well contained VZV. A positive Tzanck smear was obtained by all three investigators in 4 of these 6 cases and a negative result in 1 patient; in 1 smear (T5-0004, Table 5) a positive Tzanck smear was obtained by two investigators. The sensitivities of EM and VC were compared for the 55 herpes virus proven lesion samples by either EM, VC or both tests (Table 4).

Tzanck smear

The three investigators EF, APO and JND obtained positive Tzanck smears

TABLE 4

Comparison of sensitivities of electron microscopy after virus concentration (EM), viral culture (VC) and Tzanck smear (TS) in detecting herpesvirus infection in 55 lesion samples that were positive in either VC or EM

Site and stage of lesions		Sensitivity (%)		
		EM	VC	TS*
Mucous membrane				
	oral cavity ($n=4$)	75	100	50
	lip ($n=23$)	96	78	87
	genitals ($n=6$)	100	67	67
	all lesions ($n=33$)	94	79	79
Skin				
	genitals ($n=3$)	100	33	67
	other ($n=19$)	100	74	74
	all lesions ($n=22$)	100	68	73
Lesion type				
	vesicule ($n=11$)	91	100	91
	pustule ($n=27$)	100	70	85
	ulcus ($n=16$)	94	63	56
	crusted ($n=1$)	nr	nr	nr
	all stages ($n=55$)	96	75	76

*Results obtained by investigator JND; nr, not relevant.

in 47, 46 and 44 smears, respectively ($n = 67$). We chose the results of JND with the least number of positive scores for statistical calculations and comparisons. This will not overestimate the sensitivity of the Tzanck smear. The sensitivity of the Tzanck smear in the 67 patients with clinically overt HSV lesions is 66% (95% confidence interval: 53–77%). In Table 2 the results of the Tzanck smears are compared with VC and EM. There was disagreement between the Tzanck smear results and VC in 19 cases while for EM disagreement arose in 15 cases. The sensitivities of the Tzanck smear and VC in this study for detecting herpes virus infection were about equal with no significant statistical difference (McNemar test: $\chi^2 = 0.21$; $P_2 > 0.10$; 95% confidence interval: -9% to 17%). The difference in sensitivity of Tzanck smear and EM was statistically significant (McNemar test: $\chi^2 = 4.27$, $P_2 = 0.04$; 95% confidence interval: 1–20% difference in sensitivity).

The Tzanck smear sensitivities obtained by investigators EF, APO (dermatovenereologists), and JND (cytotechnologist) for the HSV proven cases were 86% (47/55), 80% (44/55) and 76% (42/55), respectively (data of JND are listed in Table 4: all stages). In 4 patients with no proven herpetic infection by VC or EM (Table 5: T4/3385–T5/2871), the investigators APO and JND obtained 2 positive Tzanck smears in ulcerous mucous membrane lesions. The specificity was 83% (10/12). When the McNemar test was applied, no significant differences were demonstrated between the Tzanck smear results obtained by the three investigators ($P_2 > 0.10$).

TABLE 5

Interobserver discrepancies of the Tzanck smear in 67 patients with clinical signs of HSV infection combined with the results of viral culture (VC), and electron microscopy after virus concentration (EM)

Specimen number	Site and type of the lesion	Results laboratory tests (positive / negative)				
		VC	EM	Tzank smear		
				Observers		
				EF	APO	JND
	Mucous membrane					
T4-3580	lip, pustule	+	+	–	–	+
T4-3665	lip, ulcer	+	+	+	–	–
T5-0163	lip, vesicle	+	+	+	+	–
T5-0299	lip, ulcer	–	+	+	+	–
T5-2855	oral cavity, ulcer	+	+	+	–	+
T4-3385	oral cavity, ulcer	–	–	–	+	–
T4-3723	oral cavity, ulcer	–	–	–	+	–
T5-0970	penis, ulcer	–	–	–	–	+
T5-2871	lip, ulcer	–	–	–	–	+
	Skin					
T4-0301	natis, pustule	+	+	+	+	–
T4-3594	face, ulcer	+	+	+	–	–
T5-0004	natis, pustule	–	+	+	+	–

Discussion

Rapid diagnostic techniques for the detection of HSV and VZV are needed for optimal therapeutic management, especially for seriously ill patients. When VC is used for a confirmation test, tissue cultures should be inoculated shortly after collecting lesion samples, but this is generally only possible in university hospitals with laboratories close by. In the present study, because all samples of vesicular lesions were positive in VC (sensitivity 100%; Table 1), we concluded by approximation that the storage and transport conditions of all lesion samples were optimal.

The Tzanck smear is widely used in dermatovenereological practice, because it can give almost instantly information about the disease. Moreover, the Tzanck smear sensitivity and specificity in detecting herpes virus infection compares favorably with those of VC and immunofluorescence tests (Solomon, 1988). The Tzanck smear is not specific, which means that it cannot differentiate between HSV and VZV. A second drawback is that the test cannot be applied to detect herpes virus infections in patients with asymptomatic viral shedding.

Several immunological techniques have been developed for detection of HSV antigen. Although these tests are rapid, they are not as reliable as the standard cell culture technique. However, Dascal et al. (1989) introduced an enzyme immunoassay kit that is as sensitive as the viral culture technique. Cao et al. (1989) were able to rapidly detect cutaneous HSV infection with the polymerase chain reaction, but the sample collection in this study necessitates a punch biopsy. We have also tested lesion samples in commercially available ELISA kits, but the results were disappointing, the reason why EM was used as an alternative confirmation technique.

Electron microscopy is expensive and requires considerable experience. It is not available in most diagnostic laboratories, but it can rapidly and specifically diagnose herpetic infection in crude vesicle fluid of lesions (Almeida, 1962). This classical method, still in use by some EM laboratories, is based on detection of particles in suspensions made directly from smears. This technique might work well with lesion samples that largely consist of vesicle fluid and do not need a follow-up with gold labelling techniques. In addition, a large number of lesions are pustular and ulcerous and produce unreliable results with classical EM. The large amount of soluble proteins in such virus suspensions interferes with virus adsorption. The proteins adsorbed to the grid contain a lot of soluble viral antigens that make these grids unsuitable for successive virus labelling. Smith et al. (1962a,b) studied trypsin-chymotrypsin treatment of samples and agar filtration followed by virus detection in pseudoreplicas (Sharp 1960; Palmer 1975) to increase virus counts. These techniques are not suitable for the routine laboratory.

Ultracentrifugation methods based on the Beckman Airfuge, for more than 12 years in use in our laboratory, had not resulted in acceptable detection levels in other studies. Moreover, this method is hampered by excessive contamina-

tion of viruses on the grids with cellular material. Therefore, these grids cannot be used in colloidal gold labelling procedures.

Solid phase immuno-EM (SPIEM) can further enhance the sensitivity of virus detection by EM and is even more sensitive than ELISA (Kohn et al., 1985; Van Nieuwstadt et al., 1988; El-Ghorr et al., 1988). In SPIEM, the virus is picked up by a bilayer of protein A and capture antibody adsorbed to the EM grid (Kohn et al., 1985). The improvement of transmittable gastroenteritis (TGE) virus detection in faeces by SPIEM was at least 100 fold, compared with standard EM (Van Nieuwstadt et al., 1988). El-Ghorr et al. (1988) showed that this EM-adapted 'ELISA technique' can easily be combined with gold labelling techniques. Our studies indicated that this technique can also be used for herpes virus diagnosis (Fig. 3), and the 2 samples that were missed by EM in this study (Table 2) might have been detected in this way. SPIEM certainly will increase herpes virus detection levels. However, SPIEM gold labelling profiles are not consistent because background levels depend largely on the amount of soluble antigen present in a specific lesion sample. Therefore, it is not advisable to apply SPIEM-gold labelling methods directly to the crude tissue suspension, but labelling profiles will be better when carried out on ultracentrifugation-treated virus suspensions.

Interference of proteins present in the virus suspension with virus adsorption is diminished by dilution of the sample with water or 10 mM Tris buffer. This probably contributed to the high virus particle count observed after diluting the virus suspension 15–20 times to approximately 400 μl. In addition, we found evidence that the use of sand and the effect of short sonication greatly facilitates herpes virus release from cell fragments and improves the detection of equine herpes viruses in the organ tissues of aborted foetuses (unpublished observations). Therefore, we assume that the use of sand facilitates the release of viruses from all types of HSV lesions. Taking into account the above considerations, the improvement of detection by a factor 10–12 after centrifugation through tartrate was no surprise. Moreover, protein-free virus suspensions after ultracentrifugation make all successive gold labelling steps more easy to interpret and will give more reliable and reproducible results.

The immuno-gold labelling data obtained from this study and the data presented in our previous studies confined to VZV (Folkers et al., 1989; Vreeswijk et al., 1988) indicate that EM can diagnose rapidly HSV and VZV infection in skin and mucous membrane lesions, even in cases where VC fails. Therefore, EM must still be considered as an important tool for research and diagnostic laboratories.

Vesicles contain their highest titres of virus within the first 24–48 h (Spruance et al., 1977). In this study the sensitivity of VC for vesicular, pustular and ulcerous lesions (in 55 proven herpetic specimens) was 100%, 70% and 63%, respectively (Table 4). Thus, viral shedding seemed to depend on the stage and duration of the herpetic lesions. However, a relation cannot be found between

the lesion type and the presence of human (IgA, IgM, IgG) antiviral immunecomplexes in EM preparations (Table 3), which implies that we cannot explain a decrease of VC sensitivity simply by circulating anti-HSV antibodies, which may have penetrated into the lesions and neutralized the viruses in the final stages of the disease. Since all samples with negative VC, except one, showed viral immune complexes in EM, antibodies may well have played a role in virus inactivation in VC.

In all patients with anti-virus immune complexes, viral cores as well as labelled envelopes were observed. In one patient (Table 3: T5-1010B), however, the viral envelopes were not labelled. In recent EM studies (unpublished observations) we found that anti-viral core antibodies of the IgG class can persist for a long time (several months or even much longer) after anti-viral envelope antibodies have disappeared. Since the patient with specimen number T5-1010 suffered from recurrent infections, a high level of anti-viral core antibodies may have persisted from earlier outbreaks.

In the present study, the overall sensitivity (in 67 clinical specimens) of the Tzanck smear was 66%, and that of VC 61%, with statistically no significant difference when the McNemar test was applied ($\chi^2 = 0.21$; $P_2 > 0.10$). To further study the difference in sensitivity between Tzanck smear and VC the 95% confidence interval was calculated (Gardner et al., 1989). The difference in sensitivity ranging from -9% to 17% (Table 2) implies that both tests have about equal sensitivities, but due to the limited sample size in this study a difference in sensitivity may be as high as 26%.

Compared to VC, the sensitivity of the Tzanck smear also depends on the stage of the lesion from which the test sample was collected and was 91% in detecting virus infected epithelial cells in vesicular lesions (Table 4). These findings confirmed our earlier results (Oranje et al., 1986; Folkers et al., 1988). However, EM sensitivity did not seem to have a relation with the stage of the lesion (Table 4). Therefore, we conclude that EM was the proper confirmation test for VC and TS in this group of patients. The data presented in Table 5 summarize the TS discrepancies between 3 observers. In 55 smears there was full agreement about the final diagnosis ($+/+/+$ or $-/-/-$). The results indicate that reliable sensitivity values can be reached if a second opinion is part of the medical diagnostic strategy.

The present study updates the diagnostic value of electron microscopy, viral culture and Tzanck smear for HSV diagnosis with special attention to the importance of EM as a rapid confirmation technique in special cases where we cannot rely on other laboratory tests. The use of ultracentrifugation-treated virus suspensions is crucial for obtaining reliable results in immuno-electron microscopy. Although new and more sensitive laboratory tests are under way, such as for instance the polymerase chain reaction, and each of them having their own specific drawback, improved EM techniques can still be of practical use for sensitivity and specificity studies.

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