Microarrays in the Diagnosis of Human Herpesvirus infections

Anne J. Jääskeläinen

Department of Virology
Haartman Institute
Faculty of Medicine
University of Helsinki

and

Department of Virology
Section of Clinical Microbiology
HUslab
Helsinki University Central Hospital

and

General Microbiology
Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki

ACADEMIC DISSERTATION

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Supervisors: Professor Antti Vaheri
Department of Virology
Haartman Institute
University of Helsinki
Helsinki, Finland

Docent Heli Piiparinen
Department of Virology
Haartman Institute
University of Helsinki
and Helsinki University Central Hospital
Helsinki, Finland

Reviewers: Docent Outi Monni
Institute of Biomedicine
University of Helsinki
Helsinki, Finland

Professor Veijo Hukkanen
Department of Microbiology
University of Oulu
Oulu, Finland

Opponent: Docent Raija Vainionpää
Department of Virology
University of Turku
Turku, Finland
To Ronja and Kalle 💕
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by Roman numerals (I-IV).


ABBREVIATIONS

BP  Bell's palsy
CMV, HCMV  cytomegalovirus, human cytomegalovirus
CNS  central nervous system
CPE  cytopathic effect
CSF  cerebrospinal fluid
DSN  diagnostic sensitivity
DSP  diagnostic specificity
EBV  Epstein-Barr virus
EIA  enzyme-linked immunosorbent assay
FP  facial palsy
gG  glycoprotein G
gE  glycoprotein E
GEv  genome equivalent
HHV-6A  human herpesvirus 6 variant A
HHV-6B  human herpesvirus 6 variant B
HHV-7  human herpesvirus 7
HSE  herpes simplex encephalitis
HSV-1  herpes simplex virus 1
HSV-2  herpes simplex virus 2
IE  immediate early
IFA  immunofluorescence assay
mRNA  messenger RNA
PAGE  poly-acrylamide gel electrophoresis
PCR  polymerase chain reaction
PHN  post herpetic neuralgia
pp65  phosphoprotein 65
pp150  phosphoprotein 150
PTLD  post-transplant lymphoproliferative disease
PUUV  Puumala hantavirus
QCMD  quality control of molecular diagnostics
qPCR  quantitative PCR
RHS  Ramsay Hunt syndrome
RT-PCR  reverse transcriptase polymerase chain reaction
SARS  severe acute respiratory syndrome
ssDNA  single stranded DNA
ssRNA  single stranded RNA
TBEV  tick-borne encephalitis virus
TRF  time-resolved fluorescence
VP  virus particle
VZV  varicella-zoster virus
SUMMARY

Currently, there are nine known human herpesviruses and these viruses appear to have been a very common companion of humans throughout the millennia. Of human herpesviruses, herpes simplex viruses 1 and 2 (HSV-1, HSV-2), causative agents of herpes labialis and genital herpes, and varicella-zoster virus (VZV), causative agent of chicken pox, are also common causes of central nervous system (CNS) infections. In addition, human cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpesviruses 6A, 6B, and 7 (HHV-6A, HHV-6B, HHV-7), all members of the herpesvirus family, can also be associated with encephalitis and meningitis. Accurate diagnostics and fast treatment are essential for patient recovery in CNS infections and therefore sensitive and effective diagnostic methods are needed.

The aim of this thesis was to develop new potential detection methods for diagnosing of human herpesvirus infections, especially in immunocompetent patients, using the microarray technique. Therefore, methods based on microarrays were developed for simultaneous detection of HSV-1, HSV-2, VZV, CMV, EBV, HHV-6A, HHV-6B, and HHV-7 nucleic acids, and for HSV-1, HSV-2, VZV, and CMV antibodies from various clinical samples.

The microarray methods developed showed potential for efficiently and accurately detecting human herpesvirus DNAs, especially in CNS infections, and for simultaneous detection of DNAs or antibodies for multiple different human herpesviruses from clinical samples. In fact, the microarray method revealed several previously unrecognized co-infections.

The microarray methods developed were sensitive and provided rapid detection of human herpesvirus DNA, and therefore the method could be applied to routine diagnostics. The microarrays might also be considered as an economical tool for diagnosing human herpesvirus infections.
Herpesviruksset ovat kulkeneet ihmisen kumppaneina vuosituhat toisensa jälkeen ja tällä hetkellä tunnetaan yhdeksän ihmiselle patogeenista herpesvirusta. Huuli- ja genitaaliherpestä aiheuttavat herpes simplex virus 1 ja 2 (HSV-1, HSV-2) sekä vesirokkoa aiheuttava varicalla-zoster virus (VZV) ovat ihmisen herpesviruksia, jotka voivat aiheuttaa myös vakavia keskushermostoperäisiä infektioita. Ihmisen herpesviruksista myös ihmisen cytomegalovirus (CMV), Epstein-Barr virus (EBV), ihmisen herpesvirukset 6 ja 7 (HHV-6, HHV-7), voidaan liittää aivokuumeeseen ja aivokalvontulehdukseen. Näiden virusten nopea ja tarkka diagnostiikka on oleellista oikean hoidon aloittamiseen ja potilaan toipumiselle varsinkin keskushermostoperäisissä infektioissa. Taudinaiheuttajien diagnoosoimiseksi tarvitaan herkkiä ja tehokkaita menetelmiä.

Tämän väitöskirjan tavoitteina oli kehittää uusia mikrosirupohjaisia menetelmiä ihmisen herpesvirusten aiheuttamien infektioiden diagnostiikkaan. Kehitetyillä mikrosiruilla tunnistettiin yhtä aikaa kahdeksan eri herpesviruksen nukleiinhappoa erilaisista kliinisistä näytteistä. Toisella serologisella mikrosirulla detektoitiin HSV-1, HSV-2, VZV ja CMV:lle spesifisiä vasta-aineita.

1. **HUMAN HERPESVIRUSES**

The tendency of herpesviruses to “creep” and spread is reflected in their name herpes, which comes from the Latin word *herpes* which, in turn, comes from the Greek word *herpein*, meaning to creep. The herpesvirus family is a large family of DNA viruses that cause diseases in humans and animals. Over 100 herpesviruses have already been isolated, and to date, there are nine known human herpesviruses [Strauss & Strauss 2002; Büchen-Osmond 2007].

The family Herpesviridae is divided into three subfamilies: Alphaherpesvirinae, Betaherpesvirinae, and Gammaherpesvirinae. Human herpesviruses 1 and 2 (HHV-1, HHV-2; also known as herpes simplex viruses or HSVs) belong to the genus *Simplexvirus*, and human herpesvirus 3 (HHV-3; also known as varicella-zoster virus, VZV) to the genus *Varicellovirus*, both members of the Alphaherpesvirinae subfamily. Human herpesvirus 5 (HHV-5; also known as human cytomegalovirus, HCMV or CMV) belongs to the genus *Cytomegalovirus*, and human herpesviruses 6A, 6B and 7 (HHV-6A, HHV-6B, HHV-7) belong to the genus *Roseolovirus*, both members of Betaherpesvirinae subfamily. Human herpesvirus 6 can be divided in two different variants: HHV-6A and -6B. Of these two different strains, HHV-6A shows more neurotropism than HHV-6B. Human herpesvirus 4 (HHV-4; also known as Epstein-Barr virus, EBV) and human herpesvirus 8 (HHV-8) belong to the Gammaherpesvirinae subfamily [Strauss & Strauss 2002; McGeoch, Rixon & Davison 2006; Büchen-Osmond 2007].

First breakthrough in herpesvirus characterization was in the early 20th century [Goodpasture 1929; Goodpasture 1942]. The first published
descriptions of elementary bodies of herpes simplex virus were made, and in 1949 electron microscopy of HSV was performed and the reactivation of HSV was suggested [Blank & Brody 1950; Coriell & Rake 1950]. VZV was isolated from the vesicular fluid of both chickenpox and zoster lesions in cell culture by T.H. Weller in early 1950 [Weller & Witton 1958]. CMV was observed and isolated by several scientists: T.H. Weller, M.G. Smith, and W.P. Rowe, in 1956-57 [Harnden et al. 1967; Ho 2007]. Epstein-Barr virus was named after Tony Epstein and Yvonne Barr, who first isolated EBV from Burkitt’s lymphoma samples in 1964 [Epstein, Achong & Barr 1964]. HHV-6 was discovered in 1986 and HHV-7 shortly thereafter in 1990 [Salahuddin et al. 1986; Frenkel et al. 1990]. HHV-8 was discovered by Chang and Moore in 1994-95 and was linked to malignancies, especially Kaposi’s sarcoma [Moore et al. 1996].

1.1. Structure and genome

The mature herpesvirus virion consists of an envelope, a tegument, a nucleocapsid, and a core [Roizman & Pellett 2001; Strauss & Strauss 2002; McGeoch, Rixon & Davison 2006; Büchen-Osmond 2007]. In general, herpesviruses are spherical to pleomorphic, and 120-200 nm in diameter. The surface of the virion is evenly covered with spikes that are distributed densely and can be seen as distinctive surface projections. The outermost part of the virion is an envelope which is a lipid bilayer containing viral glycoproteins and lipids that are of host cell origin. These lipids are derived during budding from the nuclear or other host cell membranes. The tegument layer is located between the envelope and capsid, and its proteins are present in variable amounts and arranged in a sometimes asymmetric manner. The capsid or nucleocapsid exhibits icosahedral symmetry (T=16) and appears round. The diameter of the capsid varies from 115 to 130 nm. The capsids consist of 162 capsomers and arrangement is visible in electron microscopy. The genome is a single molecule of linear double-stranded DNA
varying from 124-235 kbp, and it is wrapped around the core, which consists of a fibrillar reel. The core, with the DNA, is located inside the capsid shell, which is then called a nucleocapsid. The ends of the core fibers are anchored to the capsid shell. The viral genome encodes structural and non-structural proteins of the envelope, tegument and capsid. DNA molecules contain regions of unique sequence flanked by direct or inverted repeats [McGeoch, Rixon & Davison 2006].

1.2. Replication

In general, herpesvirus virions attach to the host cell by binding to glycosaminoglycans on the surface of the cell [Trybala et al. 1993; Roizman & Knipe 2001; Spear & Longnecker 2003]. For alpha- and gammaherpesviruses, binding can be mediated by a virion glycoprotein that is not required for entry. For example, glycoprotein gC for HSV and gp350 for EBV (in the case of B cells) are ligands for binding to cellular receptors of heparin sulfate and complement receptor 2, respectively.

The three glycoproteins gB, gH and gL are thought to be essential for the cell entry of all herpesviruses. In addition, gD and gp42 are proposed to be ligands for fusion for HSV and EBV, respectively [Spear & Longnecker 2003; Rey 2006]. For HSV, cellular receptors for fusion are, e.g., herpesvirus entry mediator (HVEM), which belongs to the tumor necrosis factor receptor family, and nectin-1 and -2, members of the immunoglobulin family. After binding, the viral envelope fuses to the cell plasma or endosomal membrane and the nucleocapsid of the virus enters the cytoplasm.

The nucleocapsid travels using the cytoskeleton of the cell to access the nucleus [Cohen & Straus 2001; Roizman & Knipe 2001; Garner 2003]. The capsid releases the linear viral DNA through nuclear pores into the nucleus.
The viral DNA then circularizes and the cellular RNA polymerase transcribes the viral DNA into mRNA [Cohen & Straus 2001; Roizman & Knipe 2001; Strauss & Strauss 2002]. Viral gene expression is regulated and can be divided in three steps. First, transcription of immediate-early (IE) genes, which generally encode regulatory proteins, is activated. The IE-proteins initiate the transcription of early genes encoding enzymes which are needed to increase the amount of nucleotides and for viral replication. The control of transcription is dependent both on the cell’s nuclear factors and proteins encoded by the virus. This system determines whether the infection will result in production of new virus particles and cell death (a lytic infection), persistent shedding of viruses (a persistent infection) or latency. Herpesviruses encode their own DNA polymerase, and some herpesviruses encode enzymes (such as thymidine kinase) that allow the virus to grow in non-dividing cells that do not therefore contain the precursors of DNA synthesis. Neurotropic herpesviruses cannot efficiently replicate without thymidine kinase (a phosphotransferase which can be found in most of the living cells) in nerve cells due to the low amounts of certain DNA precursors.

Finally, late genes, which encode the viral structural proteins, are activated and transcribed [Cohen & Straus 2001; Roizman & Knipe 2001; Mettenleiter, Klupp & Granzow 2006]. The transcription takes place in the nucleus, from which the mRNA is transferred to the cytoplasm to be translated into proteins. Depending on the protein, they can either go back into the nucleus, stay in the cytoplasm, or be transferred to the cell membrane, where they are incorporated. In the nucleus, the viral capsid proteins start to assemble and to form empty capsids. The full-length viral genome is copied and packaged into capsids to form nucleocapsids. Part of the nuclear membrane of the cell is covered with e.g. glycosylated proteins that trigger budding of nucleocapsid through the nuclear membrane. The nucleocapsid binds to this area and is budded to the cytoplasm. In the cytoplasm, the nucleocapsids are trapped by viral tegument proteins. At the final envelopment site of the endoplasmic reticulum, viral glycoproteins and outer tegument proteins
come together, and when the nucleocapsid with tegument proteins arrives there, accumulation begins and the final envelope is formed. Mature virions are then released by exocytosis.

1.3. Epidemiology and transmission

Generally, all human herpesviruses can spread via body fluids such as saliva. Herpesviruses which are latent in immune defense cells, e.g. CMV, EBV, HHV-6 and HHV-7, may also spread via transplantation. HSVs spread from person-to-person in close contact situations, for example, through blisters or lesions on skin or mucosa and also during sexual contact. VZV spreads via both contact and respiratory routes. EBV spreads via saliva, and CMV via several different routes, e.g. saliva, but also breast milk, body fluids, direct contact, blood transfusions and transplantation [Garner 2003; Hukkanen et al. 2003; Vetsika & Callan 2004; Ho 2007].

Generally, it can be said that human herpesviruses have been a very common companion of humans throughout the millennia. Nowadays, seroprevalences of human herpesviruses are surprisingly high, even though the state of hygiene, and residential density have changed within recent decades. Geographic location, changes in sexual behavior, child daycare, and breast-feeding may also have an influence on seroprevalence [Hukkanen et al. 2003; Svahn et al. 2006].

In Finland, HSV seroprevalence has been estimated to be currently about 50% among adults [Hukkanen et al. 2003; Alanen et al. 2005]. HSV-1 infection is more common than HSV-2 infection. A seroprevalence for VZV of over 80% has been calculated for Finnish children, and the percentage has been shown to increase to over 95% in adults [Aarnisalo et al. 2003; Nardone et al. 2007]. The CMV seroprevalence rises along with age and varies from 55% to 80% in adults in Finland. EBV infections are acquired during
REVIEW OF THE LITERATURE

childhood and adolescence, and seroprevalences of up to 90% have been estimated for adults in Finland. The seroprevalence for HHV-6 worldwide is over 90% and the infection is acquired during early childhood [Okuno et al. 1989].

1.4. Pathogenesis

1.4.1. Primary infection

All herpesviruses have primary target cells which they most efficiently infect. These target cells are often part of transmission routes. Mucoepithelial cells are primary targets of HSV-1, HSV-2, and VZV, which are all alphaherpesviruses [Garner 2003]. Several other cell types are preferred by betaherpesviruses, e.g. T lymphocytes for HHV-6 and -7 [De Bolle et al. 2005; Miyake et al. 2006], and epithelia, lymphocytes, or monocytes for CMV [Hummel & Abecassis 2002; Jarvis & Nelson 2002; Sinclair & Sissons 2006]. EBV, which is a gammaherpesvirus, can efficiently infect epithelial cells and B lymphocytes [Amon & Farrell 2005]. After replication of HSV-1 or -2 in the oral or genital mucosa, there is an uptake of virus into sensory nerve fibers followed by retrograde microtubule-associated transport to the cell body of neurons in the dorsal root ganglion near the spinal cord or into the trigeminal ganglion [Cunningham et al. 2006]. There the virus lapses into the latent state.

Symptoms of primary infection vary among herpesviruses. Tiny blisters called vesicles are commonly form around the mouth and nose (“cold sores”) due to HSV-1 infection, and in the genital area, most commonly as a result of HSV-2 infection. However, the epidemiology of the genital herpes is changing with evidence which indicate an increasing incidence of HSV-1 infections, particularly in young women 30 years of age or less [Ribes et al. 2001; Forward & Lee 2003]. VZV infection starts with symptoms of the
common cold, continuing with vesicle formation on various parts of the body (chicken pox) [reviewed in Garner 2003, and Hambleton & Gershon 2005]. Classic fever, pharyngitis and lymphadenopathy (throat pain and swollen lymph glands) are symptoms of mononucleosis, resulting from EBV infections [Kieff & Levine 1974; Vetsika & Callan 2004]. Occasionally, CMV, HHV-6, and HSV may cause mononucleosis-like illnesses [Hurt & Tammaro 2007]. EBV is also associated with post-transplant lymphoproliferative disorders (PTLD) and malignancies such as Burkitt’s lymphoma and nasopharyngeal carcinoma [Kieff & Levine 1974; Kieff 1995; Preiksaitis & Keay 2001; Junker 2005]. Roseola (also known as sixth disease, exanthema subitum, or roseola infantum) is a disease caused by HHV-6, and occasionally HHV-7 [Yamanishi et al. 1988].

In general, the severity of the primary infection depends on the immune status (immunocompetent vs. immunodeficient) and the age of the person. Symptoms of herpesvirus infections that occur during childhood are typically milder than in adolescence or adulthood. The primary infection, however, can also be asymptomatic, especially in children.

1.4.2. Latency and reactivation

Herpesviruses can evade or confuse the immune system so that it does not “clean” these viruses from the body and they can enter a latent state after primary infection and reactivate from this state. Different events, e.g. transplantation, cancer, infection, stress or other health conditions that lower the body’s defenses, can trigger the reactivation of latent herpesviruses.

Alphaherpesviruses, HSVs and VZV establish a latent state in neurons [Hill et al. 1990; Whitley 2001; Kennedy 2002; Efstathiou & Preston 2005; Steiner, Kennedy & Pachner 2007]. Betaherpesviruses, HHV-6 and HHV-7
switch from primary infection to latent state in T lymphocytes [De Bolle et al. 2005; Miyake et al. 2006] and CMV converts to latency in monocytes, lymphocytes and possibly other cell types [Hummel & Abecassis 2002; Jarvis & Nelson 2002; Sinclair & Sissons 2006]. In addition, HHV-6 is the only human herpesvirus known to integrate into host chromosome [Torelli et al. 1995; Daibata et al. 1999]. EBV, a gammaherpesvirus, establishes a latent state in B lymphocytes [Amon & Farrell 2005]. CMV reactivation results in serious morbidity and mortality in immunocompromised transplant recipients and reactivation has been suggested to be a multi-step process initiated by the response to the transplanted organ [see review Hummel and Abecassis 2002].

The immune system treats the neurons somewhat differently than other cell types. The ability to achieve latency in neuronal cells allows the maintenance of HSV for the lifetime of the host [Roizman & Knipe 2001; Whitley 2001; Garner 2003].

2. CLINICAL MANIFESTATIONS

Herpesviruses cause a wide variety of diseases. For HSVs the symptoms of reactivated infection are quite similar to those of primary infection and reactivations often occur. HSV-1 reactivations causing “cold sores” recur multiple times whereas, for example, VZV reactivation is not usually recurrent. In the case of VZV reactivation, the symptoms differ from those of a primary infection and the rate of occurrence is much lower than with HSVs. VZV causes herpes zoster, commonly known as shingles. It is most common in elderly, and causes lesions, nerve pain and occasionally small vesicles on the skin surface at the nerve ends. Post herpetic neuralgia (PHN), i.e., sustained nerve pain, is more frequent in the elderly, probably because of impaired cell-mediated immunity to VZV [Hambleton & Gershon 2005; Dworkin et al. 2008]. VZV reactivation tends to occur with increasing age
whereas HSV-1 reactivations tend to decrease with age [Steiner, Kennedy & Pachner 2007]. Zoster may cause abnormalities or complications of the central nervous system (CNS), i.e. mild mononuclear pleocytosis with a slight increase in protein levels. In general, all the human herpesviruses can also cause severe or even life-threatening diseases, e.g. CNS infections, which are fortunately not very common.

2.1. **Central nervous system infections**

Encephalitis, an acute inflammation of the brain, and meningitis, an acute inflammation of the protective membranes covering the CNS, are life-threatening diseases [Whitley 2006; Kovanen 2007a; Kovanen 2007b]. Encephalitis is often meningoencephalitis, where the inflammation has spread from the protective membranes to the brain. Myelitis, in which the CNS functions linking the brain and limbs are disrupted by swelling of the spinal cord, is also a severe disease. Of the herpesviruses, HSV, VZV and CMV are the most common causes of these life-threatening diseases, but EBV and HHV-6 can also be associated with encephalitis and meningitis [Aberle & Puchhammer-Stöckl 2002; Whitley 2006; Gilden et al. 2007; Mannonen et al. 2007]. HHV-7 has also been suspected to cause encephalitis but is not often looked for as a causative agent [Calvario et al. 2002; Ward et al. 2002]. Herpesviruses can also cause acute or chronic lymphocytic meningitis [Peltola & Valtonen 2003; Kupila et al. 2004].

The incidence of viral encephalitis is about 3/100 000 per year in Finland, and the main causes are HSV, VZV, and, particularly in the coastal regions of Finland, tick-borne encephalitis viruses (TBEV) [Koskiniemi et al. 2001; Hukkanen & Vuorinen 2002; Kupila et al. 2006; Kovanen 2007a; Kovanen 2007b]. Herpes simplex encephalitis (HSE) is a very severe infection of the CNS [reviewed by Whitley 2006]. Symptoms and signs of encephalitis can include fever, fatigue, headache, lethargy, irritability, confusion,
hallucinations, epileptic seizures, dysphasia, aphasia, and problems of cognition [Whitley 2006; Gilden et al. 2007; Kovanen 2007b]. These signs and symptoms reflect virus replication with accompanying inflammation in the medial temporal lobe and orbital surface of the frontal lobe. Mortality can be up to 70% without proper antiviral treatment. Mortality decreases to ~20% with treatment, and 50-60% of patients recover well or reasonably well. Survivors can be left with permanent seizure disorders, mental status changes, aphasia or motor deficits [Gilden et al. 2007]. In Finland, VZV has been suggested to be a major causative agent in CNS infections, followed by HSV and TBEV [Koskiniemi et al. 2001; Kupila et al. 2006]. VZV is also known to cause encephalitis in children after chickenpox and these symptoms occur typically 2-4 weeks after the onset of primary symptoms [Kovanen 2007b].

Viral meningitis is more common than bacterial meningitis. It is most often caused by enteroviruses, especially coxsackie and echoviruses, but other viruses, like HSV and VZV, can also be causative agents [Koskiniemi et al. 2001; Kupila et al. 2006; Kovanen 2007a]. The occurrence of viral meningitis is usually highest at the end of summer and beginning of autumn in Finland [Hukkanen & Vuorinen 2002]. Fever, headache, nausea and vomiting, stiffness of the neck, and problems with cognition can be symptoms of viral meningitis [Peltola & Valtonen 2003]. There is no antiviral treatment for enteroviral meningitis. For meningitis caused by herpesviruses, antiviral drugs can be administered. Patients usually recover well, and meningitis is considered to be less severe than encephalitis. However, meningitis can turn into encephalitis, and therefore patients have to be examined and monitored carefully.
2.2. Facial palsy

Herpesviruses, especially VZV, HSV and HHV-6, have been suggested to be associated with facial palsy (FP) [Furuta et al. 2005; Kanerva et al. 2007; Pitkäranta et al. 2000; Pitkäranta, Lahdenne & Piiparinen 2004]. HSV-1 and VZV are considered possible etiologic factors for idiopathic peripheral facial palsy (Bell’s palsy, BP), and VZV for Ramsay Hunt syndrome (RHS) [Furuta et al. 2005; Gilden et al. 2007; Murakami et al. 1998]. BP and RHS can present either in mononeuritic or polyneuritic forms, and occasionally inflammatory CSF findings are observed in BP patients, suggesting a CNS infection. FP is considered by some to be a result of CMV infection or in rare cases, EBV infection [Furuta et al. 2005].

Herpesvirus reactivation, rather than primary infection, is most often associated with FP [Mori, Nagai & Asanuma 2002; Furuta et al. 2005; Gilden et al. 2007]. In BP, the facial muscles are weakened or become paralyzed. This condition is not permanent and approximately half of BP patients will have essentially complete recovery in a short time. BP and RHS share most of the same symptoms. Pain, often in or behind the ear, can be acute and is one of the symptoms. It can start before muscle weakness is apparent and last a week or two, and with RHS even longer. Dizziness is another symptom that BP and RHS share. Hearing loss and blisters in the ear have been reported with RHS but not with BP. Vesicles is often the only clearly visible symptom that identifies RHS. BP is not contagious but RHS is when shingles vesicles are present.

There is another rare neurological disorder, Melkersson-Rosenthal syndrome appearing in childhood or early adolescence, which is characterized by, among other things, recurring facial paralysis and swelling of the face and lips [Ziem et al. 2000].
2.3. Treatment and antiviral drugs

Nucleoside analogs are the largest group of antiviral drugs which have been used to treat herpesvirus infections [Männistö & Tuominen 2000]. Of these acyclovir and its L-valyl ester valacyclovir are the most important. The other analogs are gancyclovir, pencyclovir, and famciclovir, the diacetyl ester of 6-deoxy pencyclovir. Nucleoside analogs enter cells that have been infected by viruses and compete with deoxynucleosides for the viral thymidine kinase, which phosphorylates the analogs to monophosphates. These monophosphates are converted by cellular kinases to the diphosphate and triphosphate forms. For example, acyclovir triphosphate competes with deoxyguanosine triphosphate. The triphosphates act as substrates for viral DNA polymerase which incorporates them into the growing DNA strand where they interrupt chain elongation by terminating it.

Acyclovir and valacyclovir have a limited effect against human herpesviruses [Erlich 1997; Männistö & Tuominen 2000]. However, HSV-1, HSV-2, VZV, and to some degree EBV are susceptible to it. Despite this narrow spectrum of activity, acyclovir has a very important role in prevention of disease and treatment of acute infections, e.g. common herpes in the facial area, genital herpes, varicella, herpes zoster, and encephalitis. Acyclovir can be administered locally but also orally or intravenously. One of its drawbacks is that herpesviruses develop resistance against acyclovir exceedingly fast and this phenomenon is a particularly serious problem to immunocompromised patients. The incidence of resistance has been estimated to be <10% in immunocompromised patients [Christophers et al. 1998]. Changes in thymidine kinase lead to incomplete phosphorylation of the drug. Mutations in viral DNA polymerase can also result in drug resistance.

Gancyclovir and pencyclovir are also guanosine analogs [Männistö & Tuominen 2000]. Gancyclovir acts like acyclovir but it has activity against...
CMV and EBV. It is administrated intravenously and it also has severe side effects, such as neutropenia in over 35% of cases, and thrombocytopenia in ~10% of treated patients. Due to gancyclovir’s side effects, it is only administrated in severe CMV infections.

Pencyclovir’s phosphorylation to the monophosphate form depends on the thymidine kinase of HSV. HSV-1, HSV-2, and VZV DNA polymerases have a lower affinity for pencyclovir triphosphate than for acyclovir triphosphate, but pencyclovir invades the cell easier than acyclovir, thus, their relative activities are similar. Famciclovir is a diacetyl ester of 6-deoxy pencyclovir, and it is known to be the orally active form of pencyclovir [Fangman, Rao & Myers 2003; Männistö & Tuominen 2000]. Famciclovir is rapidly and extensively absorbed and converted to pencyclovir during transit through the intestinal wall and liver. Famciclovir has been discovered to be more effective against zoster than acyclovir [Erlich 1997].

A novel nucleotide analog called cidofovir contains a phosphonate group, and therefore does not require initial phosphorylation by a viral enzyme for activation like the other nucleoside analogs do. It has potent activity against, for example CMV [Fangman, Rao & Myers 2003].

Foscarnet is a pyrophosphate analog which attaches to the pyrophosphate-binding site of the virus-encoded DNA polymerase [Erlich 1997; Männistö & Tuominen 2000]. Binding to this site changes the conformation of the DNA polymerase. Foscarnet has activity against herpesviruses, but also against some retro-, and influenza viruses. Foscarnet is administered intravenously and it is used to treat herpes simplex and cytomegalovirus infections when acyclovir has lost its effectiveness.
3. **DIAGNOSIS OF HUMAN HERPESVIRUS INFECTIONS**

The method used for diagnosis of the herpesvirus infections depends on the clinical manifestations of the patient. In diagnostics, herpesvirus antibodies, the virus or some part of the virus are detected. Serological assays for detection of virus-specific antibodies are used to detect an increase or decrease in antibody levels and seroconversion, or to explore the history of patient immunity status [Hukkanen et al. 2003; McKendrick et al. 2007]. In acute cases, antigens may be detected from, for example, vesicle samples. Some of the herpesviruses can be cultured from a sample and afterwards identified with various methods [Leland & Ginocchio 2007]. The nucleic acids of herpesviruses can be detected from various sample types by polymerase chain reaction (PCR) [Piiparinen and Vaheri. 1991; Vesanen et al. 1996; Koskiniemi et al. 1997; Chiu et al. 1998; Read & Kurtz 1999; Espy et al. 2000; Pitkäranta et al. 2000; Read, Mitchell & Fink 2001; Aberle & Puchhammer-Stöckl 2002; Druce et al. 2002; Hudnall, Chen & Tyring 2004; Ihira et al. 2002; Piiparinen et al. 2002; Aalto et al. 2003].

In CNS infections, methods for diagnosis of herpesvirus infection include viral DNA detection with PCR and examination of intrathecally-produced antibodies [Koskiniemi et al. 2001; Aberle & Puchhammer-Stöckl 2002; Hukkanen et al. 2003; Schultze et al. 2004]. Generally, herpesvirus DNA and antibodies are not normally present in CSF, and therefore a positive DNA or IgM finding, or an increased ratio of IgG in CSF versus serum, can indicate CNS infection. However, the antibodies cannot be detected earlier than one week from onset of symptoms and therefore, during the first 7 to 10 days, methods detecting viral DNA are recommended in CNS infections [Hukkanen & Vuorinen 2002; Schultze et al. 2004; Kupila et al. 2006;]. In any case, serology is the method of choice in prolonged infections when nucleic acid detection may not provide sufficient information.
3.1. Serology

Detection of specific immunoglobulins is of importance for determining immunological status with regards to herpesviruses. Immunity can be determined from serum sample and is important, for example, to women of child-bearing age, and to young children for determining the need for VZV vaccination [McKendrick et al. 2007]. Serological tests can also be used at, for instance, clinics for sexually-transmitted diseases, as confirmatory tests for genital herpes with atypical symptoms [Ashley-Morrow, Krantz & Wald 2003]. The HSV-type specific serology has also significance in survey of genital herpes carriers and transmission.

A significant rise in the IgG antibody level (≥4-fold increase) and seroconversion, or IgM seropositivity, can indicate an acute infection [Yoshikawa et al. 2000; Hukkanen et al. 2003]. In herpesvirus reactivation, elevated IgG levels and IgM antibodies can be observed. IgG-avidity assays can be used to distinguish between recent and past infections [Kneitz et al. 2004].

During primary infection of the skin or mucosa, specific antibodies to HSV-1 and HSV-2 are often produced slowly and another serum sample should be tested 3-4 weeks after onset of symptoms with an enzyme-linked immunoassay (EIA). Therefore, the methods of choice for detection of acute infections of HSVs are virus culture and antigen detection. In acute chickenpox (varicella), shingles (herpes zoster), or obscure eczema with vesicles, EIAs for detection of antibodies to VZV are performed. If CMV infection is suspected during pregnancy, in transplantation or in immunosuppressed patients, or is thought to be suspected to be the causative agent of mononucleosis and fever, serological assays are performed. EIAs for EBV-antibody detection are used for instance when mononucleosis is suspected or sustained fever and lymphadenopathy are
observed. EBV antibodies can also be detected with an agglutination test of EBV IgM antibody, which is fast but not very accurate. It is, however, often sufficient for diagnosis [Hukkanen et al. 2003]. Primary HHV-6 or HHV-7 infection can be determined with serological assays if e.g. exanthema subitum, pityriasis rosea, fever, or lymphadenopathy are observed.

In serology, all the methods are based on interactions between antibody and antigen. Specific binding can be detected with various methods, some of which can also quantify the level of antigen-antibody binding. EIAs are often quantitative assays that can be used for examination of antibody levels [Schultze et al. 2004]. In EIA, the antigen can be coated onto wells and specific antibodies attached to antigens are detected with labeled anti-human IgG or IgM. In μ-capture EIAs, the human IgMs are coated onto the wells and antibodies attached to IgM are detected with labeled herpesvirus antigens [Oladepo et al. 2000]. To differentiate acute from past infections, avidity assays have been developed for herpesviruses [Hukkanen et al. 2003; Kneitz et al. 2004]. Avidity assays are based on the separation of low- and high-avidity antibodies, indicating acute and past infection, respectively. The separation is done using a denaturant such as urea.

EIAs based on recombinant antigen or virus lysate have been developed and are commercially available for several herpesviruses [Weinberg et al. 1996; Weber, Berger & Rabenau 2001; Ashley 2002; Wald & Ashley-Morrow 2002; Sauerbrei & Wützler 2006]. In EIAs that differentiate HSV-1 and HSV-2, glycoprotein G is often used as an antigen [Ashley 2002; Wald & Ashley-Morrow 2002]. However, HSV-2 strains comprising silent gG gene have been found which suggests that some mutated strain may not be detected with serological assays [Nordberg et al. 2007]. The envelope glycoproteins of VZV are highly immunogenic and glycoproteins such as VZV glycoprotein G have also been used in VZV-specific EIAs [Sauerbrei & Wützler 2006]. Recently, a new multiplex fluorescent microsphere immunoassay for the determination of EBV serologic status was evaluated [Martins, Litwin & Hill 2008].
Complement fixation (CF) tests can be used to reveal viral etiology in the case of unclear fever or rash of unknown origin. Of the herpesviruses, HSV, VZV, and CMV CF assays can be used in diagnostics [McHugh et al. 1985; Hukkanen et al. 2003]. In the CF test, herpesvirus antigen and complement are added to a diluted serum sample. An indirect immunofluorescence assay (IFA) can be used to determine antibody titers against herpesviruses [Landry et al. 1987; Yoshikawa et al. 2001]. The herpesvirus-infected cells are grown on glass microscope slides and diluted serum or CSF is added, so that the herpesvirus antibodies attach to the cells. The binding is detected with fluorescein-labeled anti-human IgG or IgM. However, this assay is unable to distinguish herpesviruses with cross-reacting antibodies, e.g. HHV-6 and HHV-7.

Other serological assays are immunoblotting, which specifically detects both IgG and IgM, and the neutralization test, which is generally considered to be a type-specific serological assay for most virus infections [LaCroix et al. 2000; Yoshikawa et al. 2001]. Radioimmunoassays and latex agglutination have also been used for screening of herpesvirus antibodies [Slomka et al. 1995; Svahn et al. 1997].

### 3.2. Viral culture

Virus isolation by culturing is used in HSV and VZV infections of the skin and mucosa. Cells from the eye or mucosa (vesicles) of the throat, cervix or urethra, can be sampled and inoculated into cell lines of choice to cultivate viruses. However, HSV-1, HSV-2, and VZV are not very commonly isolated from CSF [Hukkanen & Vuorinen 2002; Kupila et al. 2006]. CMV infection can be identified by culturing urine or throat swab samples. Virus isolation can be used for identification of perinatal or neonatal infection, and a positive finding indicates active virus infection [Kimberlin 2004].
Appropriate collection, transport and processing of clinical samples are important for virus isolation in cell cultures. After an appropriate time, the inoculated cell cultures are examined with microscopy for any cytopathic effect (CPE) appearing in cell monolayer, or virus is identified by detection of virus-specific antigens. The CPE is caused by viral replication and may be seen as swelling, rounding, shrinking, or clustering of the cells, syncytium formation or even as total destruction of the monolayer. HSVs may produce a visible CPE within 24 h of inoculation, but with CMV, it can take an average of 10–30 days, and therefore other, faster, culturing methods have been developed [Leland & Ginocchio 2007]. CPE formation by VZV is seen in 6-8 days.

The shell vial system was developed for rapid CMV isolation. At the time of inoculation, the vial is low-speed centrifuged, and after a brief incubation (~16-24 h) the cells are stained with a monoclonal antibody to early proteins of CMV [Gleaves et al. 1984]. Since it has proven successful, this shell vial system has been adapted to, for instance, HSV and VZV isolation. Rapid assays for CMV and HSV based on shell vial system have been used in diagnostics [Ziegler et al. 1988; Diamond et al. 2000]. The next advance in the diagnostic methods involved using different cell types, which were combined to grow together as a monolayer [Leland & Ginocchio 2007]. In this way, different viruses with different target cells could be cultivated simultaneously from the same sample. Currently, several commercially produced co-cultivated cell lines are available (Diagnostic Hybrids Inc., Athens, OH) and shell vial systems are also available commercially. In addition, EBV and HHV-6 can be cultivated in lymphocytic cell lines but they are cumbersome to culture, and therefore other methods are used for their detection [Hukkanen et al. 2003].
3.3. **Antigen detection**

Direct detection of herpesvirus antigens can be used in acute cases, e.g. primary infections with vesicle formation. For example, HSV and VZV antigen detection can be used for diagnosis of acute infection during childbirth or in newborns or immunosuppressed patients [Hukkanen et al. 2003]. HSV and VZV antigens can be detected from samples with an adequate number of cells containing virus antigens. The sample can be taken from the bottom of a broken blister, lesion or mucosa. The virus-specific antigens are detected by immunofluorescence microscopy and labeled virus-specific antibodies. Positive findings are an indication of primary infection, possible reactivation or reinfection. Occasionally, HSV carriers secrete the virus without any symptoms. In the acute phase, there can be only a few antigen-presenting cells. To increase sensitivity, it is recommended that virus is cultivated prior to viral antigen detection. The determination of phosphoprotein 65 (pp65) antigenemia has long been the reference test for monitoring CMV reactivation in transplant or immunosuppressed patients [Piiparinen et al. 2004; Cariani et al. 2007]. Monoclonal antibodies to pp65 are used for immunostaining of blood polymorphonuclear leukocytes. Real-time PCR methods for detection and quantification of CMV DNA have been suggested as a convenient alternative to the antigenemia test.

3.4. **Polymerase chain reaction methods – qualitative and quantitative**

Diagnostic tools have been developed for reliable and fast identification of herpesviruses. Polymerase chain reaction (PCR) and its numerous applications have been successfully used for laboratory diagnosis of e.g. CNS infections or monitoring of CMV and EBV DNA quantity in plasma. PCR can
be qualitative or quantitative and is used for detection of viral nucleic acid or quantification of the viral load in a sample.

Conventional PCR is based on amplification of a specific DNA sequence. Many PCR procedures have been developed, and a considerable number of them are used in diagnostics of human pathogens. The principles of PCR are thoroughly described elsewhere [Guatelli, Gingeras & Richman 1989]. In short, the genomic area of interest is amplified with PCR. First, the target genomic DNA is denatured. The specific primer pair (short ssDNA oligonucleotides) finds and anneals its complementary sequence in the genome. DNA polymerase uses the primer as a starting point and synthesizes the complementary strand by adding deoxyribonucleotides. The cycles of denaturating, annealing of primers to genomic DNA and extension are repeated and after final extension the product can be detected by visualization. The denaturation, annealing and extension are accomplished by changing the temperatures in amplification program.

Sensitive PCR-based methods have been developed for detection of human herpesviruses, and they are widely used in diagnostics [Echevarria et al. 1994; Vesanen et al. 1996; Koskinemi et al. 1997; Pitkäranta et al. 2000; Koskinemi et al. 2002; Hukkanen et al. 2003a; Kupila et al. 2006; Hanson et al. 2007; Mannonen et al. 2007]. Multiplex-PCR methods for simultaneous detection of numerous herpesviruses have also been developed. In these applications, either one generic primer pair for the targets or several separate target-specific primer pairs are used. Multiplex-PCR is able to amplify several targets simultaneously in one reaction and therefore, it can be used in place of several sequential PCR reactions. One of the earliest multiplex-PCR methods for detection of herpesviruses was described in 1996 [Jackson et al. 1996]. Since then, multiplex-PCR methods or a multiplex nested-PCR method, in which two rounds of PCR reactions are performed, have been developed for simultaneous identification of several herpesviruses [Pozo & Tenorio 1999; Read & Kurtz 1999; Markoulatos et al. 2000;
Markoulatos et al. 2001; Calvario et al. 2002; Druce et al. 2002; Hudnall, Chen & Tyring 2004; Tafreshi et al. 2005]. They were specific, but the sensitivities of multiplex-PCR methods varied depending on detected herpesviruses. Today, the multiplex-PCR methods are developed aiming at faster, simpler and more cost-effective diagnostics [Bergallo et al. 2007].

PCR-based methods, in which amplification is monitored and visualized online, are called real-time PCRs. Qualitative real-time PCRs have been developed for identification of herpesvirus DNAs [Espy et al. 2000; Tang et al. 2007]. In addition, commercial real-time PCRs are already available, for instance for typing of HSVs [Podzorski 2006]. A few multiplex real-time PCR methods for HSV-1, HSV-2, and VZV have also been developed [O’Neill et al. 2003; Read, Mitchell & Fink 2001]. The sensitivities of multiplex real-time PCRs have been found to be similar to those of single or multiplex-PCRs. Possibly due to the difficulty of setting up a highly sensitive and specific multiplex real-time PCR for simultaneous identification of several herpesviruses in one reaction, separate real-time PCRs for single virus detection have been developed [Stöcher et al. 2003]. These PCRs for HSV-1, HSV-2, VZV, CMV and EBV DNA detection can be simultaneously amplified using the same parameters in separate reactions with virus-specific primer pairs.

Quantitative PCR-based methods are performed if CMV or EBV infections are suspected in an immunosuppressed patient [Aalto et al. 2003; Piiparinen et al. 2005]. For example, the amount of EBV DNA reflects the severity of EBV infection, and therefore viral DNA levels in clinical sample are followed during antiviral therapy. In addition to quantification of CMV and EBV DNA in clinical sample, quantitative real-time PCRs have also been published for diagnostics of HSVs, VZV, HHV-6, and HHV-7 [Kimura et al. 1999; Nitsche et al. 1999; Pevenstein et al. 1999; Ryncarz et al. 1999; Zerr et al. 2000]. Recently, a quantitative multiplex real-time PCR for EBV, CMV and HHV-6 was developed and tested with samples from transplant recipients [Wada et
al. 2007]. The results indicated that developed multiplex assay was as sensitive and specific as those of a single real-time PCR.

In 1991, a time-resolved fluorescence-based hybridization assay combined with PCR was described [Dahlen et al. 1991]. For herpesvirus detection, a time-resolved fluorometry (TRF) PCR assay was developed in 2000, which was further modified for simultaneous detection of HSV, VZV, CMV and enteroviruses in CNS infections [Hukkanen et al. 2000; Hukkanen & Vuorinen 2002]. A fully automated, homogeneous nucleic acid detection technique based on dry-reagent assay chemistry and time-resolved fluorometry was recently described, indicating the potential of this approach for nucleic acid detection and diagnosis [von Lode et al. 2007]. In the PCR-ELISA technique, labeled amplicons are hybridized to specific probes and colorimetric reaction is performed in microtiter wells [Musiani et al. 2007]. This technique has been applied for identification of human papillomaviruses and herpesviruses [Vesanen et al. 1996; Musiani et al. 2007].

4. MICROARRAY METHODS

Microarrays have been used in various fields of biosciences, such as studies of single nucleotide polymorphisms, gene expression and microbiology [Borsting, Sanchez & Morling 2004; Chambers et al. 1999; Huber et al. 2002; Pastinen et al. 2000; Sachse et al. 2005]. Microarrays consist of biomolecules attached to the solid support, which is typically glass, silicon, nylon membrane, or plastic [Cretich et al. 2006; Pastinen et al. 2000; Sachse et al. 2005]. The immobilized biomolecules can be oligonucleotides, PCR products, proteins, peptides, carbohydrates, or other small molecules. These biomolecules are arranged in predetermined order on a solid support, e.g. slides. In one array, there can be dozens or thousands of biomolecules. In microarray, there can be many separate arrays each targeted to different
sample, or just one large array on which only one sample is hybridized. The samples tested can be cellular extracts, PCR products, or antibodies, all of which can hybridize or attach to the biomolecules on the microarray.

Today, commercial nucleic acid microarrays are available for a number of applications such as gene expression studies and identification of nucleic acids from viral pathogens. Antibody microarrays are also commercially available for research purposes to study cellular processes such as apoptosis.

4.1. Nucleic acid microarrays for identification of viruses

The first viral microarrays were described a few years ago [Chizhikov et al. 2002; Wang et al. 2002; Wilson et al. 2002; Sengupta et al. 2003]. At the moment, there are numerous microarray methods which are used in viral studies. Some of these methods, especially the ones for herpesvirus detection, are presented in more detail in next chapter (Fig. 1). In short, all of these methods are based on the PCR assay and visualization of the PCR-product with different methods on the microarray [Wang et al. 2002; Wang et al. 2003; Lovmar et al. 2003; Boriskin et al. 2004; Klaassen et al. 2004; Korimbocus et al. 2004; Striebel et al. 2004; Albrecht et al. 2006; Lopez-Campos et al. 2007; Baek et al. 2008]. Microarray technology provides a promising approach for clinical microbiology, and a number of methods for detection and genotyping of adeno-, influenza-, corona-, zoonotic, rota-, noro-, astro-, entero-, papilloma- and respiratory tract viruses have already been introduced [Chizhikov et al. 2002; Wilson et al. 2002; Sengupta et al. 2003; Gemignani et al. 2004; Coiras et al. 2005; Nordström et al. 2005; Chou et al. 2006; Gheit et al. 2006; Jääskeläinen & Maunula 2006; Min et al. 2006; Ryabinin et al. 2006; Hsia et al. 2007; Liu et al. 2007].
All microarray methods for viral nucleic acid detection are based on PCR combined with microarrays with either oligonucleotides or longer DNA-fragments bound to the arrays. A common approach is to hybridize PCR product to oligonucleotides, and specific hybridization is visualized with a fluorescence or colorimetric reaction, i.e. enzyme catalyzed substrate precipitation. Microarrays provide a method to detect multiple PCR products in an efficient and sensitive manner. A small amount of amplicon is needed for detection, and scanners and readers for microarrays are capable of visualization even minimal fluorescence or color reactions. For example, ScanArray Express –laser scanner (PerkinElmer, Wellesley, MA), which is used for microarray reading, can reach the sensitivity of <0.1 fluorescent molecule per μm². With microarrays, numerous of viral targets may be identified simultaneously from various samples. These features make the
Microarray a very efficient and multifunctional tool for screening purposes and diagnosis.

Microarray assay for detecting 140 viruses, e.g. human rhino-, respiratory syncytial, parainfluenza, and adenoviruses, were described in 2002 [Wang et al. 2002]. The microarray consisted of 70-mer oligonucleotides. The RNA from samples was extracted and reverse-transcribed with a primer consisting of specific sequence followed by random sequence (Fig. 1). The PCR reaction was carried out using primers for the specific part of the first primer. The PCR-product was labeled afterwards in a separate reaction with random primers, enzyme and aminoallyl-labeled nucleotides, which were used to capture cyanine dye. After hybridization, fluorescence was detected. Wang et al. [2003] also published another microarray study, based on above presented publication. In this study, they increased the microarray from 140 viruses to a pan-viral microarray for ~1000 viruses with a total of 100 000 70-mer oligonucleotides on the microarray. This microarray was used as part of the global effort to identify the virus associated with severe acute respiratory syndrome (SARS). With this microarray, they captured the novel virus, which was further isolated from microarray spots and PCR amplified. This fragment was further cloned and sequenced to confirm that the captured virus was a new coronavirus.

A microarray method for identification of human rotaviruses was introduced by Lovmar et al. [2003], and this method was based on human rotavirus-specific oligonucleotides which were covalently immobilized on glass microscope slides (Fig. 1). Oligonucleotides were used to capture RT-PCR products, and the bound template was then was extended using fluorescent nucleotides and a thermostable DNA polymerase. Finally, the fluorescence intensity was measured using a microarray scanner.
A microarray method which was based on digoxigenin-labeled human papillomavirus-derived PCR amplicons was described by Klaassen et al. [2004]. The PCR-amplicons were hybridized onto type-specific biotinylated probes immobilized on streptavidin-coated glass slides. These hybridized amplicons containing digoxigenin were treated with a mouse monoclonal antibody specific for digoxigenin (Fig. 1). Alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins attached to the mouse monoclonal antibody, and alkaline phosphatase activity was detected using a substrate, i.e. colorimetric reaction. This microarray was designed for detection and subtyping of human papillomaviruses.

Boriskin et al. [2004] developed a microarray method in which they amplified herpesvirus, enterovirus and flavivirus nucleic acids with multiplex-PCR and RT-PCR. The PCR products were further labeled in a separate reaction with random primers, cyanine-labeled nucleotides and enzyme, followed by denaturation, overnight hybridization on the microarray and finally detection (Fig. 1). They used the same gel-purified PCR products as the probe elements on the microarray after generating them from the viral DNA or RNA template. Other studies using similar principles in PCR and microarray methods have been carried out. Striebel et al. [2004] described a microarray method for herpesvirus detection which was based on PCR with cyanine-labeled primers. These primers were branched at the 5´end, and each branch contained cyanine label (Fig. 1). In addition, Albrecht et al. [2006] used cyanine-labeled PCR-primers as a second primer set in the nested-PCR for human papillomaviruses, in which two sequential PCR reactions are performed using the PCR-product from first PCR as a template to second one (Fig. 1). They spotted sense and antisense oligonucleotides with spacer sequence on microarray. The denaturated PCR product was hybridized to the oligonucleotides on the microarray and signal was detected. Lopez-Campos et al. [2007] designed a microarray for human adenoviruses that was based on oligonucleotides on microarrays and nested-PCR. The labeling was performed using cyanine-labeled nucleotides in the second round of nested-
PCR followed by denaturation, hybridization on microarrays and detection (Fig. 1).

Korimbocus et al. [2005] introduced a microarray method for detection of herpes-, flavi- and enteroviruses in CNS infections. This method was based on three separate and generic PCR reaction products which were pooled labeled with meta-biotinphenylmethyl diazomethyl, followed by fragmentation with hydrochloric acid before hybridization to a microarray (Fig. 2). The hybridized fragments were stained using streptavidin labeled with R-phycoerythrin, which is a phycobiliprotein purified from red macroalgae and is used as a fluorochrome for fluorescent assays.

Recently, a microarray system using a bipolar integrated circuit photodiode array was described by Baek et al. [2008] for human papillomavirus
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detection. The amino-linked oligonucleotides were immobilized to the microarray and PCR with biotin-labeled PCR-primers was carried out. The denaturated PCR-products were hybridized to the oligonucleotide microarray, followed by reaction with an anti-biotin antibody-conjugated gold nanoparticle solution (Fig. 2). The silver enhancement reaction was performed before finally washing, drying and reading the microarray.

4.2. Serological microarrays

In serological microarrays, antigens are immobilized to solid supports for capturing the antibodies of choice. These analytical microarrays can be used to detect antibodies from serum samples for determination of immunity status, infection, allergy diagnostics or autoimmune diseases [Blixt et al. 2007; Bonhomme, Nappez & Raoult 2007; Cretich et al. 2006; Davies et al. 2008; Lin et al. 2007; Mezzasoma et al. 2002; Spisak et al. 2007]. Immunogenic proteins spotted on the microarrays can be used to detect disease with diagnostic potential. Antigens such as recombinant proteins or bacterial suspensions can also be immobilized on the microarray. The primary antibodies recognize specific antigens on the microarray and secondary antibodies with coupled fluorescent labels or other detection molecules are used to visualize the bound antibody.

Only a few serological microarrays for viral antibody detection have been developed. A microarray has been described for detection of antibodies directed against *Toxoplasma gondii*, rubella virus, CMV and HSV-1 and -2 (ToRCH antigens) [Mezzasoma et al. 2002]. Parallel detection of nucleic acids and antibodies to human immunodeficiency, hepatitis B and hepatitis C viruses with microarrays has also been described by Perrin et al. [2003]. Detection was based on nucleic acid probes, viral proteins and nonspecific proteins on a microarray (Fig. 3). The enzyme-labeled secondary antibody was used for detection of antibody that was captured by antigen on a
Microarrays have been designed for detection of SARS coronavirus antibodies using structural proteins as antigens [Lu et al. 2005]. Viral antibodies bound to the antigens on the microarray were detected by a cyanine-labeled secondary antibody. Sera from SARS patients were also tested using this microarray. Another coronavirus microarray was described in which entire or partial proteomes of the human SARS coronavirus and other human (HCoV-229E, HCoV-OC43), mouse, bovine, and feline coronaviruses were used as antigens [Zhu et al. 2006]. Human-anti-SARS antibodies were detected by cyanine-labeled goat anti-human IgGs acting as secondary antibodies.
An integrated protein microarray was developed for simultaneous detection of two viral antigens of hepatitis virus B and seven viral antibodies of human hepatitis B, C, D, E, and G viruses in human sera [Xu et al. 2007]. Another microarray was developed for hepatitis C antibody detection to be used for screening of, for example, blood banks [Kwon et al. 2008]. Four different antigens for hepatitis C virus were spotted into the wells of 96-well plates coated with sol-gel, and secondary labeled antibodies were used for detection. The captured hepatitis C antibody was detected by cyanine-labeled secondary antibody.

Recently, microarray techniques were also used for immunogenicity studies of a modified vaccinia virus Ankara. This virus was a highly attenuated vaccinia virus that is under consideration as an alternative to the conventional smallpox vaccine Dryvax. The antibody response for both modified vaccinia virus Ankara and the smallpox vaccine Dryvax was studied by comparing microarray profiles [Davies et al. 2008].

Protein microarray technology can be considered challenging due to the complex nature of proteins. The purity of the protein and maintenance of its native state when immobilized onto the surface of a microarray (including spotting and storage conditions) have an outcome on the effectiveness of the microarray. Recently, a new method which could overcome the problems of long-term storage or spotting of the replicate protein microarrays has been introduced by He et al. [2008]. In this method, PCR-amplified fragments encoding a set of tagged proteins are spotted on the solid support. When protein microarrays are needed, a permeable membrane carrying a cell-free lysate, capable of performing transcription and translation, is placed between cDNA microarray and the second slide, which is coated with tag-capturing reagent. The synthesized proteins diffuse through the membrane and become rapidly immobilized on the capture slide surface comprising a protein microarray corresponding to the cDNA microarray.
AIMS OF THE STUDY

The aims of the present study were:

- To develop a multiplex-PCR and microarray method for simultaneous detection of HSV-1, HSV-2, VZV, CMV, EBV, HHV-6A, HHV-6B, and HHV-7 from clinical specimens (I, II)

- To further evaluate and improve the microarray method for detection of HSV-1, HSV-2, and VZV from cerebrospinal fluid (III)

- To develop an antigen-based serological microarray for detection of HSV-1, HSV-2, VZV and CMV antibodies and to evaluate the effectiveness of the serological microarray as a fast screening tool (IV)
MATERIALS AND METHODS

1. MULTIPLY PCR AND MICROARRAY METHOD (I, II, III)

1.1. Clinical specimens

The clinical samples were from the Department of Virology, Helsinki University Hospital, Laboratory Services (HUSLAB). The clinical specimens consisted of whole-blood, cerebrospinal fluid (CSF), plasma and proficiency testing samples (described below). Ten whole-blood, 23 CSF, and 73 plasma samples were collected from solid organ and bone marrow transplant patients, and patients with neurological symptoms. Altogether, 10 samples from the VZV (4) and HSV (6) proficiency-testing program 2004 (QCMD, Glasgow, Scotland, UK) were included and all the specimens were screened in parallel with routine PCR methods and a multiplex-PCR and microarray method (I). The negative panel, consisting of 70 sera, 30 CSF and 11 proficiency testing samples from an enterovirus 2004 program, were also tested with the microarray method (I). Cell culture supernatants of HHV-6A strain GS and an in-house strain, and HHV-6B strain Z-29 (I) were tested with the microarray. Twenty CSFs positive and two negative for HSV-1, HSV-2 and VZV were collected and tested with the improved microarray (III).

Seventy CSFs, collected from the Department of Otorhinolaryngology, Helsinki University Hospital from 1998 to 2002, were tested with microarray method (II) in order to screen herpesviruses from FP patients and a control group. There were 33 peripheral FP patients (34 samples): 26 with Bell’s palsy (27 samples), five cases with simultaneous herpesvirus infections, one with puerperal FP (two weeks after delivery), and one with Melkersson-Rosenthal syndrome.
1.2. **Viral DNA controls**

Quantitated commercial viral DNA controls (I, III) were used to determine the sensitivity and specificity of the assay. The copy numbers refer to genome copy number, which has been determined by the manufacturer with quantitative PCR. Viral particles (VPs) refer to the viral particle count based on the amount of active virus prior to nucleic acid extraction, as determined by the manufacturer (Advanced Biotechnologies, Columbia, MD).

1.3. **Multiplex-PCRs**

1.3.1. **DNA extraction**

DNA was extracted from 200 μl of CSF (I) and 500 μl of whole blood (I) with phenol-chloroform. The precipitate from CSF was resuspended in 22 μl and that from whole blood in 100 μl of sterile water. DNA from 200 μl of CSF from a negative panel or sera/plasma (I) was extracted using the MagNA Pure LC instrument and a Total Nucleic Acid Kit (Roche Diagnostics, Basel, Switzerland), and DNA from 200 μl of CSF (II, III) was extracted using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics) according to the manufacturer’s instructions and with elution by 50 μl of elution buffer.

1.3.2. **Original and improved multiplex-PCRs**

The original multiplex-PCR-1 was used to identify HSV-1 and HSV-2 resulting in a 264-bp amplicon (I, primers in Table 1). Multiplex-PCR-2 contained primer pairs for amplification of CMV, EBV, VZV, HHV-6A, HHV-6B and HHV-7, resulting in 220 bp, 273 bp, 186 bp, 179 bp (both HHV-6A and HHV-6B) and 230 bp PCR-products (Table 1), respectively. The improved
multiplex-PCR-1 (III) contained a new primer pair for HSV amplification (a 249-bp amplicon, primers given in Table 1).

**TABLE 1.** Primers used in original and improved multiplex-PCRs (I, III).

<table>
<thead>
<tr>
<th>Primer (OP)</th>
<th>Sequences (5'→ 3'), MPLEX ID.</th>
<th>Gene</th>
<th>Genus (GenBank Acc. No), Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-FW (I)</td>
<td>AAGGAGCGCCCAAGGCGCCCG, M1</td>
<td>DNA pol.</td>
<td>* HSV-1 (X14112), 64750 – 64768; HSV-2 (Z86099), 65209 – 65229</td>
</tr>
<tr>
<td>HSV-RV (I)</td>
<td>PSeq-TGGGTTACAGGCTGCAAGT, M1</td>
<td></td>
<td>HSV-1 (X14112), 64976 – 64956; HSV-2 (Z86099), 65449 – 65429</td>
</tr>
<tr>
<td>HSV-FW (III)</td>
<td>AGCGAATTCGAGATGCTG(T/C)T, M2</td>
<td></td>
<td>HSV-1 (X14112), 64130-64150; HSV-2 (Z86099), 64591-64611</td>
</tr>
<tr>
<td>HSV-RV (III)</td>
<td>PSeq-CCTT(T/G)ATCTTGCTGCCTTC, M2</td>
<td></td>
<td>HSV-1 (X14112), 64335-64355; HSV-2 (Z86099), 64796-64816</td>
</tr>
<tr>
<td>VZV-FW (I, III)</td>
<td>CCATTCTCGCCGATTTTA, M3</td>
<td>DNA pol., ORF28</td>
<td>VZV (AY548171), 48508 – 48527</td>
</tr>
<tr>
<td>VZV-RV (I, III)</td>
<td>PSeq-GCCGCAATTGAAAGTTTAT, M3</td>
<td></td>
<td>VZV (AY548171), 48670 – 48651</td>
</tr>
<tr>
<td>CMV-FW (I, III)</td>
<td>GTACAACAGCGTGTCGCTGTC, M3</td>
<td>Processivity subunit of DNA pol, UL44</td>
<td>CMV (AY446894), 57044 – 57063</td>
</tr>
<tr>
<td>CMV-RV (I, III)</td>
<td>PSeq-CACCGGCAATCGTTTATC, M3</td>
<td></td>
<td>CMV (AY446894), 57240 – 57221</td>
</tr>
<tr>
<td>EBV-FW (I, III)</td>
<td>CGTAGATGACTCGAAGCTG, M3</td>
<td>DNA pol., BALF5</td>
<td>EBV (AJ507799), 154029 – 154047</td>
</tr>
<tr>
<td>EBV-RV (I, III)</td>
<td>PSeq-ACCATCTCGAAGCTG, M3</td>
<td></td>
<td>EBV (AJ507799), 154278 – 154261</td>
</tr>
<tr>
<td>HHV-6-FW (I, III)</td>
<td>CTCGATCGACTGAAAGCTG, M3</td>
<td>DNA pol., U38</td>
<td>HHV-6A (X83413), 59289 – 59308; HHV-6B (AF157706), 60409 – 60428</td>
</tr>
<tr>
<td>HHV-6-RV (I, III)</td>
<td>PSeq-CCGCTATGTTTATCGAGAC, M3</td>
<td></td>
<td>HHV-6A (X83413), 59444 – 59424; HHV-6B (AF157706), 60564 – 60544</td>
</tr>
<tr>
<td>HHV-7-FW (I, III)</td>
<td>AGGTCAACATGACAGTGTA, M3</td>
<td>DNA pol., U38</td>
<td>HHV-7 (AF037218), 56787 – 56806</td>
</tr>
<tr>
<td>HHV-7-RV (I, III)</td>
<td>PSeq-GGCAAGAAAGTGTGGGCTA, M3</td>
<td></td>
<td>HHV-7 (AF037218), 56993 – 56974</td>
</tr>
</tbody>
</table>

FW, forward primer (sense); RV, reverse primer [anti-sense, T3 RNA polymerase promoter sequence (PSeq)]<br>PSeq = AATTAACCTCATAAGGAGAA comes before the virus sequence; OP, Original publication where PCR primer was used; MPLEX ID indicates what primers are in the same PCR reaction [M1: HSV primers (I), M2: HSV primers (III), M3: other than HSV primers (both I and III)].<br>* Underlined AA nucleotides are match for HSV-2 and not included to HSV-1 position numbers.

The original (I) and improved (III) multiplex-PCR-1 methods were used to identify HSV-1 and HSV-2, and they were carried out in a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.01% (w/vol) gelatin, 0.2 mM dNTPmix (Finnzymes, Espoo, Finland), 0.6 µM of primers, 12.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) and 2 mM MgCl₂. The multiplex-PCR-2 was performed in a final volume of 53 µl containing 47.2 mM KCl, 9.4 mM Tris-HCl (pH 8.3), 0.009% (w/vol) gelatin, 0.19 mM dNTPmix (Finnzymes), 0.56 µM of each primer, 12.5 U AmpliTaq
Gold polymerase (Applied Biosystems) and 1.9 mM MgCl₂. The amplification consisted of denaturation at 95°C for 10 min, followed by 40 cycles of 96°C for 10 s, 55°C for 20 s and 72°C for 20 s and final extension at 72°C for 5 min. Five and 10 µl of template were used for multiplex-PCR-1s of HSVs, and multiplex-PCR-2 of VZV, CMV, EBV, HHV-6A, HHV-6B, and HHV-7, respectively.

1.4. **PCRs for daily diagnostics (I, III)**

Three qualitative PCRs for HSVs, HHV-6, and VZV, and two real-time quantitative PCRs (qPCRs) for CMV and EBV, were used in HUSLAB diagnostics in 2005. The HSV-PCRs were performed according to Piiparinen & Vahevi [1991] using the probes reported by Vesinanen et al. [1996]. The primers designed by Gopan et al. [1990] were used to carry out PCR for HHV-6, and the HHV-6 probe reported in Pitkäranta et al. [2000] was used to detect the HHV-6 amplicon. VZV-PCR was done according to Echevarria et al. [1994] and Koskinen et al. [1997]. Microplate hybridization with luminometric detection [Vesanen et al. 1996] was used for the detection of qualitative PCR products. qPCR for CMV was performed according to Piiparinen et al [2004]. EBV primers and a probe designed by Kimura et al. [1999] were used for the EBV qPCR based on a modification from Aalto et al. [2003].

1.5. **Microarray design**

Microscope glass slides activated as described by Guo et al. [1994], modified according to Pastinen et al. [2000] (I, II), or SAL-1-slides (Asper Biotech, Tartu, Estonia) (III) were spotted using a microarrayer (OmniGrid®, GeneMachines, Huntingdon, UK). Oligonucleotides were spotted in 20 µM concentration in 0.3 M sodium carbonate buffer (pH 9.0) (I) or 1×MSS (ArrayIt, TeleChem International, Inc., Sunnyvale, CA) (II, III) on microscope
slides. The oligonucleotides are described in Table 2. The spotting was performed at room temperature and at 50% humidity. The slides were stored overnight at room temperature before use.

### TABLE 2. Oligonucleotides used for herpesvirus identification on microarray (I, III).

<table>
<thead>
<tr>
<th>Oligos (OP)</th>
<th>Sequences (5’→ 3’), Microarray ID.</th>
<th>Genus (GeneBank Acc. no), Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1-T3 (I)</td>
<td>Am-TTTTTTTTTTTCCTTGACCACAATTTT, M1</td>
<td>HSV-1 (X141112), 64907 – 64922</td>
</tr>
<tr>
<td>HSV-2-T3 (I)</td>
<td>Am-TTTTTTTTTTTAGGAGGATATATTTTT, M1</td>
<td>HSV-2 (Z86099), 65279 - 65295</td>
</tr>
<tr>
<td>HSVG1-T3* (III)</td>
<td>Am-TTTTTTTTTTTAAGCTGACGGAGAATCCGTATCTCAT, M2</td>
<td>HSV-1 (X141112), 64311 - 64326; HSV-2 (Z86099), 64772 - 64787</td>
</tr>
<tr>
<td>HSVG2-T3* (III)</td>
<td>Am-TTTTTTTTTTGAACTCCATCTCCTATCAGTAAATACTGTCGGTCTC, M2</td>
<td>HSV-1 (X141112), 64907 – 64922</td>
</tr>
<tr>
<td>HSV-1-T3 (II)</td>
<td>Am-TTTTTTTTTTACCTCGTGTCCTTTTTG, M1</td>
<td>HSV-1 (X141112), 64311 - 64326</td>
</tr>
<tr>
<td>HSV-2-T3 (II)</td>
<td>Am-TTTTTTTTTTGAACTCCATCTCCTATCAGTAAATACTGTCGGTCTC, M2</td>
<td>HSV-2 (Z86099), 64772 - 64787</td>
</tr>
<tr>
<td>VZV-T3 (I, III)</td>
<td>Am-TTTTTTTTTTGAACATCAGGAGCCAGAAG, M2</td>
<td>VZV (AY548171), 46579 - 46597</td>
</tr>
<tr>
<td>VZV_1-T3 (III)</td>
<td>Am-TTTTTTTTTTGGGACATCAGGAGCCAGAAG, M2</td>
<td>VZV (AY548171), 46579 - 46597</td>
</tr>
<tr>
<td>CMV-T3 (I, III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>CMV (AY446894), 57122 - 57140</td>
</tr>
<tr>
<td>GCMV-T3 (III)</td>
<td>Am-TTTTTTTTTTGGGACATCAGGAGCCAGAAG, M2</td>
<td>CMV (AY446894), 57122 - 57140</td>
</tr>
<tr>
<td>EBV-T3 (I, III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>EBV (AJ507799), 154184 - 154201</td>
</tr>
<tr>
<td>GEBV-T3 (III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>EBV (AJ507799), 154184 - 154201</td>
</tr>
<tr>
<td>GHHV-6-T3* (III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>HHV-6A (X83413), 59409 - 59428; HHV-6B (AF157706), 56893 - 56909</td>
</tr>
<tr>
<td>HHV-6A-T3 (I, III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>HHV-6A (X83413), 59361 - 59377</td>
</tr>
<tr>
<td>HHV-6B-T3 (I, III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>HHV-6B (AF157706), 56893 - 56909</td>
</tr>
<tr>
<td>HHV-7-T3 (I, III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>HHV-7 (AF037218), 56893 - 56909</td>
</tr>
<tr>
<td>GHHV-7-T3 (III)</td>
<td>Am-TTTTTTTTTTTCAGTAAATACTGTCGGTCTC, M2</td>
<td>HHV-7 (AF037218), 56893 - 56909</td>
</tr>
</tbody>
</table>

T3, oligonucleotide (sense, 9xT spacer arm before sequence); Am, amino-link; Acc. no, accession number; OP, Original publication in which microarray oligonucleotide was used. Microarray ID indicates which oligos were spotted on the same microarray in different OPs (M1: oligos on the microarray in I OP; M2: oligos on the microarray in III OP). *Do not discriminate the types. **Underlined nucleotide A is match for HSV-1.
1.6. Microarray reactions

The reagents which differ in different microarray methods (I, III) are presented in Table 3. Two multiplex-PCR products were pooled before transcribing into ssRNA using AmpliScribe™ T3 High Yield Transcription Kit (described in I, II) and AmpliScribe™ T3 FLASH Transcription Kit (III) (Epicentre, Madison, WI) following the manufacturer’s instructions. Pooled multiplex-PCR solution which contained the improved multiplex-PCR1 was denatured for 2 min at 96°C before ssRNA transcription at 42°C for 1 h (III). The ssRNA was quickly denatured (95°C for 1.5 min) before adding NaCl to give final concentration of 1.5 mM, and allowing hybridization for 20 min at 42°C with oligonucleotides on arrays (Fig. 2).

<table>
<thead>
<tr>
<th>Reaction/Reagent</th>
<th>Reagents or Kit used in OP of I</th>
<th>Reagents or Kit used in OP of III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slides used for microarray</td>
<td>Microscope glass slides activated as described by Guo et al. [1994]</td>
<td>SAL-1-slides (Asper Biotech)</td>
</tr>
<tr>
<td>Oligonucleotide spotting buffer</td>
<td>Sodium carbonate buffer (pH 9.0) 1×MSS (ArrayIt, TeleChem International, Inc.)</td>
<td></td>
</tr>
<tr>
<td>SsRNA transcription</td>
<td>AmpliScribe™ T3 High Yield</td>
<td>AmpliScribe™ T3 FLASH</td>
</tr>
<tr>
<td></td>
<td>Transcription Kit (Epicentre)</td>
<td>Transcription Kit (Epicentre)</td>
</tr>
</tbody>
</table>

OP, Original publication in which the reagents or kits were used.
Before the extension reaction (20 min at 52°C), the microarrays were washed with array-washing buffer [0.5×TE (5 mM Tris, 0.5 mM EDTA), 0.3 M NaCl and 0.1% Triton X-100 (YA Kemia, Helsinki, Finland)] and sterile water (Fig. 2.) The extension solution, containing 55 mM Tris-HCl, 11 mM MgCl₂, 83 mM KCl, 11 mM DTT (Epicentre), 0.6 µM dATP, dGTP, ddATP, ddGTP, dUTP-CY5 and dCTP-CY5 (Amersham Bioscience, Little Chalfont, UK), 5 U MMLV reverse transcriptase (Epicentre), 0.5 M trehalose (Sigma-Aldrich) and 8.3% glycerol (MP Biomedicals, Irvine, CA), was added in a final volume of 4.5 µl (I, II, III). After the extension reaction, the microarrays were washed with array washing buffer and water, and then dried. Microarrays were dipped in 50 mM NaOH before the final washing step when SAL-1-slides (Asper Biotech) were used (III).
1.7. Analysis

The microarrays were analyzed using a ScanArray Express scanner with ScanArray™ and QuantArray™ software (PerkinElmer, Wellesley, MA). The cut-off value was determined for each subarray separately by calculating the mean value of local background-corrected intensities from non-specific oligonucleotides. The specific spot intensities were also corrected by subtracting local background. Intensities at least three times above the cut-off value were considered positive.

2. SEROLOGICAL MICROARRAY (IV)

2.1. Clinical specimens

For initial testing of the microarray, 61 patient sera were collected from patient serum panels previously tested for Puumala hantavirus antibodies at HUSLAB (Helsinki, Finland) during 2002. Altogether, 15 out of 61 were IgG-positive, 30 were IgG- and IgM-positive, one was IgM-positive, and 15 were IgG- and IgM-negative.

The herpesvirus study material included 122 serum samples (107 individuals) sent to HUSLAB (Helsinki, Finland) for analysis of HSV antibodies, 134 (118) for VZV antibodies, and 134 (117) for CMV antibodies during 2005 and 2006.

2.2. Viral antigens

The *Escherichia coli* derived recombinant proteins (ProsPec-Tany TechoGene Ltd, Rehovot, Israel) contained the CMV pp150 (UL32) immunodominant region (1011-1048 amino acids), HSV-1 gG (84-175 amino acids), HSV-2 gG
(525-578 amino acids) and VZV gE (amino acids 48-135). The recombinant proteins were purified by proprietary chromatographic techniques and a purity of >95% was determined by 10% polyacrylamide gel electrophoresis (PAGE) according to the manufacturer. Puumala hantavirus nucleocapsid protein (PUUV-N) was produced and purified according to Vapalahti et al. [1996]. The purity and concentration of PUUV-N were determined by PAGE using Precision Plus Protein™ Standards (Bio-Rad Laboratories, Espoo, Finland), and by spectrophotometry (280nm).

2.3. Microarray design

The viral antigens PUUV-N, HSV-1 gG-1, HSV-2 gG-2, VZV gE, and CMV pp150 (ProsPec-Tany TechoGene Ltd) in dilutions of 0.5-1.0, 0.25-0.50, and 0.05-0.1 μg/μl for PUUV-N; 10 and 30 μg/μl for HSV-1 gG1, HSV-2 gG2 and CMV pp150; and 0.1 and 0.3 μg/μl for VZV gE, were spotted in duplicate on nitrocellulose-coated FAST slides (Schleicher and Schuell BioScience Inc., Keene, NH) at Biomedicum Genomics (University of Helsinki) using a microarrayer (OmniGrid®, GeneMachines, Huntingdon, UK). Ten-fold dilutions of human IgM and IgG (Sigma-Aldrich Finland) were used as positive controls, and rabbit myosin (Sigma-Aldrich, Helsinki, Finland) and protein-printing buffer (TeleChem International Inc.) were used as negative controls on the microarrays.

2.4. Microarray reactions

After rinsing with PBS, the microarrays were blocked with blocking solution [2% bovine serum albumin (BSA, Sigma-Aldrich Finland) in PBS] for 90 min. IgG and IgM assays (Fig.3.) were performed in parallel. One microarray included 21 arrays, of which four were used for detection of positive- and one for negative-control samples.
Fig. 3 Herpesvirus-specific antigens were spotted on a microarray (A) and a dilution of a human serum sample was placed on the microarray. Human antibodies for specific herpesviruses attached to their specific antigens (B). Attachment was detected by labeled anti-human antibody (C) that attached to human antibodies, resulting in a signal at antigen specific spots (D).

2.4.1. IgG assays

Serum samples were diluted 1:200 in blocking solution, and 10 µl of each was transferred to a separate array and incubated for 30 min at room temperature. Before the washing steps, which were 3 times 3 min with washing buffer [0.2% Tween 20 (Fluka, Sigma-Aldrich Chemie GmbH, Buchs SG, Switzerland) in PBS], the serum dilution was removed by rinsing with...
the washing buffer. The microarrays were rinsed with PBS before incubating at room temperature for 15 min with 1 μg/mL Alexa 546-labelled goat anti-human IgG (Molecular Probes Europe BV, Leiden, The Netherlands) in PBS to detect antibodies attached to viral antigens (Fig. 3). Finally, the washing steps were repeated followed by a final rinse with sterile water and drying by centrifugation for 3 min at 1300 g in 50 ml Falcon tubes.

2.4.2. IgM assays

The protocol for the IgM test was identical to the IgG test with a few exceptions. Before dilution of a serum sample, the IgG from the sample was inactivated using GullSORB™ (Meridian Diagnostics Inc, Cincinnati, OH). One μg/ml Alexa 647-labeled goat anti-human IgM (Molecular Probes Europe BV) in PBS was used for antibody detection.

2.5. EIA and immunofluorescent assays

Puumala hantavirus IgG and IgM antibodies were previously tested with IFA and μ-capture EIA, respectively [Kallio-Kokko et al. 1998; Vapalahti et al. 1996]. Briefly, IF assays (IgG) were carried out using the PUUV Sotkamo strain grown in Vero E6 cells as an antigen and fluorescein isothiocyanate (FITC)-conjugated anti-human-IgG was used to detect the binding of PUUV antibodies to antigen. In μ-capture EIA, the plates were coated with goat anti-human IgM for capturing the IgM antibodies from serum samples. The specific PUUV-N recombinant protein was added and specific attachment of this antigen was detected with peroxidase-conjugated monoclonal antibody, which resulted in a visible reaction with substrate.

HerpeSelect® HSV-1 IgG and HSV-2 IgG ELISA kits (Focus Diagnostics Inc, Cypress, CA), and the EIagen HSV IgM test (Adaltis Italia S.p.A., Casalecchio
di Reno, Italia) were used for HSV antibody detection. HSV-1 and HSV-2 gG were used as antigens for the HerpeSelect® HSV-1 IgG EIA test and HSV-2 IgG EIA test (Focus Diagnostics Inc). The EIAs were coated with antigen and used to capture specific antibodies from serum samples. The captured antibodies were detected using peroxidase-conjugated anti-human IgG and tetramethylbenzidine (TMB) as substrate for the color reaction. In the EIAggen HSV IgM test (Adaltis Italia S.p.A.) the μ-capture principle was used. In this assay, IgM antibodies in the sample are first captured by the solid phase coated with anti-human IgM, and then after washing, the specific HSV IgM antibodies are detected by the addition of UV-light inactivated HSV-1 and HSV-2 particles that are labeled with a specific antibody conjugated to horseradish peroxidase (HRP). The substrate is added to reveal the bound conjugate, indicating a positive result. A sample dilution of 1:101 was used in these EIAs.

VIDAS CMV-IgG and -IgM Kits (bioMérieux sa, Marcy l’Etoile, France) and EIAggen Varicella Zoster IgM tests (Adaltis Italia S.p.A.) were used for CMV and VZV antibody detection, respectively. On VIDAS CMV-IgG and -IgM tests (bioMérieux sa), specific information on the antigen was not provided by bioMérieux sa. In both kits, the HCMV antigen was coated onto the solid phase and serum antibodies that attached were detected by conjugation and a substrate reaction. In the VIDAS system, 100 μl of sample was needed to perform the IgG test and another 100 μl for the IgM test.

In the EIAggen VZV IgM test (Adaltis Italia S.p.A.), the solid phase is coated with anti-human IgM, which captures IgM antibodies from the sample. Incubation with partially purified VZV which was inactivated by treatment with β-propiolactone, in a complex with monoclonal antibodies conjugated with HRP detects the specifically bound VZV antigen when substrate is added. A sample dilution of 1:101 was used in the EIAggen VZV IgM test (Adaltis Italia S.p.A.). All herpesvirus EIAs were performed according to the manufacturers’ instructions.
An accredited in-house sandwich-EIA (HUSLAB, Helsinki University Hospital, Helsinki, Finland) with VZV glycoprotein EIA antigen (Institute Virion Ltd, Rüschlikon/Zürich, Switzerland) was used for VZV IgG testing. The VZV antigen was used for coating and capturing specific antibodies from the sample. Anti-human IgG labeled with HRP was used to detect antibodies bound to antigens and substrate was used for the color reaction. A sample dilution of 1:100 was used in VZV IgG EIA.

2.6. Analysis

Microarrays were analyzed using a ScanArray Express scanner with ScanArray™ and QuantArray™ software (PerkinElmer, Wellesley, MA). The cut-off value was determined separately for each array. Briefly, the spot intensity had the local background subtracted, and the cut-off value was determined by multiplying at least three times the mean value of intensities from negative control spots. Intensities at +/- 10% of the cut-off value were considered to give an equivocal result.
RESULTS AND DISCUSSION

1. DEVELOPMENT OF MULTIPLEX-PCR AND MICROARRAY METHODS

Our aim was to set up an efficient and sensitive multiplex-PCR and microarray method for detection of multiple herpesvirus DNAs, especially for use in viral DNA detection in CNS infections. At the moment, the diagnostic assay of choice in CNS infections is qualitative PCR. CSF samples are challenging due to the fact that diagnostic PCR must be able to detect viral DNA varying from $2 \times 10^2$ to $4 \times 10^7$ genome copies per ml in initial samples from encephalitis patients [Schloss et al. 2003]. Our results have indicated that microarray method is sensitive, and they can become the assay of choice for efficient detection of viral DNA from clinical samples.

One of our goals was to apply the developed microarray method to suit detection of viral infection in CSF as well as other clinical specimens such as serum, plasma and whole-blood. One aim was to optimize the microarray method so that it could be applied in diagnostics and would require only a single working day for completion. In addition, one of the goals was to use the microarrays to detect possible co-infections which may be missed using routine laboratory PCR methods. The advantage of microarrays is that they can be used to screen viruses from multiple samples simultaneously with accuracy and speed. Therefore, our aim was also to test the usefulness of the microarray method for detecting various herpesviruses from large series of samples.

In brief, in the method developed the viral DNA was amplified using multiplex-PCR. The PCR products were transcribed to ssRNAs before hybridizing to short oligonucleotides on a microarray followed by primer extension with fluorescent nucleotides. Similar methods have been reported
RESULTS AND DISCUSSION

previously but not for herpesvirus infections [Pastinen et al. 2000; Riise Stensland et al. 2005; Jääskeläinen & Maunula 2006].

1.1. Detection limits of multiplex-PCRs and microarray (I, III)

The aim was to amplify 8 herpesviruses in one reaction or under a single set of reaction conditions with the goal of detecting 10 or less viral copies or particles per reaction. First, the multiplex-PCR and microarray method was set up using the primer pair earlier described by Piiparinen & Vaheri [1991] as well as primer pairs and oligonucleotides designed specifically for this purpose (described in original publication I). However, this primer pair for HSVs did not work with all the other primers when commercial viral DNA controls were tested and therefore, the HSV amplification had to be carried out in a separate reaction to achieve optimal sensitivity. Finally, two multiplex-PCRs, one for HSV-1 and HSV-2, and another for VZV, CMV, EBV, HHV-6A, HHV-6B, and HHV-7, were optimized so that they could be performed in the same reaction conditions and detection limits were determined.

The microarray method had good detection limits varying from 1 to 7 viral genome copies, or from 2.5 to 9.1 VPs per reaction (Table III and Fig. 1 in original publication I). These detection limits indicate a quantity of between 2.5×10¹ and 1.75×10² genome copies of CMV, EBV, HHV-6A or HHV-7, and 6.25×10¹ to 4.55×10² VPs of HSV-1, HSV-2, VZV, or HHV-6B per ml of sample. Calculations were based on that DNA was extracted from 200 μl of sample, eluted in 50 μl, of which 5 and 10 μl were used as template for multiplex-PCR-1 of HSVs and -2 of other herpesviruses, respectively.

With high viral copy numbers, i.e. over ~4×10⁶ genome copies per ml, accurate herpesvirus identification was achieved with the microarray
RESULTS AND DISCUSSION

A method. However, some cross-reaction between HHV-6A and HHV-6B oligonucleotides was observed when over 10 000 HHV-6B VPs (i.e. 2.5x10^5 VPs per ml of sample) were added to the multiplex-PCR. At high concentrations of HHV-6A DNA, no cross-reactions were observed.

The multiplex-PCR and microarray method (I) was tested with patient samples. Three HSV-positive CSFs were tested, and one of these samples remained negative in the microarray while parallel diagnostic PCR gave a positive result. When the multiplex-PCR for HSVs was studied more closely, it was seen that the microarray was negative although the multiplex-PCR product could be visualized by agarose gel electrophoresis. In addition, the detection limits for microarray and multiplex-PCR of HSVs by agarose gel electrophoresis were the same. The results indicated that the method has to be improved for detection of HSVs. For other herpesviruses, the microarray was found to be the more sensitive detection method than agarose gel electrophoresis. The secondary structure of the HSV-PCR product was suspected to block efficient ssRNA production, and therefore effecting to results from microarray.

To improve the method, a new primer pair for HSVs, a multiplex-PCR (III) and detection oligonucleotides for the microarray were designed. In addition, new detection oligonucleotides (III) for VZV, CMV, EBV, HHV-6, and HHV-7 were also designed to increase the number of oligonucleotides for detecting possible diverse PCR amplicons. The improved microarray method (III) provided a detection limit of 5 copies per reaction for commercial viral DNA of HSV-1 and HSV-2, which in turn indicated 2.5x10^2 genome copies per ml of sample. This detection limit was comparable to the limits of 2x10^2 and 2.3x10^2 genome copies per ml reported by Schloss et al. [2003] and Aberle & Puchhammer-Stöckl [2002], respectively. The original method (I) was also retested with serial dilutions parallel with the improved one. However, an
accurate determination of detection limits was not successful due to the inconsistent results of the ssRNA transcription.

Boriskin et al. [2004] used microarrays to detect 13 different pathogens, including HSV-1, HSV-2, VZV, CMV, EBV, HHV-6A, HHV-6B, and HHV-7, using long probes consisting of purified multiplex-PCR products on microarrays. In addition, Korimbus et al. [2005] developed a microarray for detection of HSV-1, HSV-2 and CMV. The sensitivity of the multiplex-PCR and microarray method described by Boriskin et al. [2004] was determined by using CMV and echovirus as representatives for DNA and RNA viruses, respectively. The analytical sensitivities were 46 CMV DNA copies (1 ng) and 28 echovirus RNA copies (125 ag) per reaction. Korimbus et al. [2005] reported a sensitivity of 500 genome equivalents (Geq) per ml of CSF spiked with HSV-1, which is ~14 Geq per reaction (calculations based on information provided in their article: 100 µl of sample was extracted and eluted to 100 µl, and 27.5 µl of template was used in PCR). The sensitivities for other herpesviruses were not reported by Boriskin et al. [2004] or Korimbus et al. [2005].

These detection limits, including those for our microarray method, are directional and cannot be directly compared due to the different viral DNA origin and quantifications methods. However, it can be speculated that our method is as or more sensitive as these microarray methods. To provide a broad field of sensitivity, the detection limits were determined for all herpesviruses with our microarray method while Boriskin et al. [2004] and Korimbus et al. [2005] reported only for one or two herpesviruses.
1.2. Analysis of clinical specimens (I, III)

To define the applicability of the microarray method (I, III) in clinical diagnostics, patient samples were tested and results compared to parallel PCR results from daily diagnostics in HUSLAB (Helsinki, Finland). The microarray and diagnostic PCR results were compared in a qualitative manner.

With the first microarray method (Tables VI, VII and IX in original publication I), PCRs and microarrays gave concordant results for 214 out of 227 (94%) clinical samples. Altogether, there were 161 PCR-negative samples, and the microarray method gave a negative result for 154 of them, resulting in a specificity of 95.3%. Of 66 PCR-positive samples, the microarray method gave a concordant positive result in 62 cases, resulting in a sensitivity of 93.9%. Although, good sensitivity and specificity were achieved, more well-characterized samples were needed to demonstrate the sensitivity of the microarray method for CSF samples, and especially for detection of HSV-1 and HSV-2 DNA.

To improve the multiplex-PCR and microarray method, a new HSV-PCR procedure (III) and new oligonucleotides for herpesviruses (III) were designed. Twenty positive and two negative CSF samples for HSV-1, HSV-2, and VZV were collected from HUSLAB (Helsinki, Finland) and they were tested in parallel with the improved and original methods. The improved method (III) was superior, and concordant results (Table III in original publication III) with diagnostic PCR were achieved in all cases (100%). The original method (I) gave concordant result in 50% of cases.

The applicability of the microarray method was tested using plasma, whole-blood, and QCMD samples, and the method proved to be suitable for
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herpesvirus detection in these samples (Tables VI and VIII in original publication). When plasma samples were tested, microarrays also detected four possible new positives for CMV, EBV, or HHV-6B from diagnostic PCR-negative samples collected from transplant patients.

Several double and triple infections were detected with the microarray method (Table 4). The detected co-infections were studied more closely to confirm the suitability of microarray for detection of multiple infections. Two samples collected from renal and bone marrow transplant patients were observed to have simultaneously CMV and EBV DNA in the plasma based on microarray results (Table 3). Both of these patients were seropositive for CMV and EBV, and these findings were confirmed by detecting CMV and EBV DNA with diagnostic qPCR. Two possible double infections of EBV-HHV-7 and EBV-CMV were also detected from EBV-positive plasma samples (Table 4). These samples were from bone marrow and renal transplant patients. The possible EBV-CMV co-infection detected with microarrays was discovered from a bone marrow transplant patient who was EBV seropositive and had both CMV IgM and IgG antibodies. However, the CMV DNA could not be tested due to sample exhaustion. On the other hand, the serological background strongly suggested that this CMV DNA finding might be a true finding.

The other possible double infections were HHV-6B-EBV and HHV-6B-HHV-7 from whole blood samples, and VZV-EBV and VZV-HHV-6B from CSF samples (Table 4). Earlier studies had shown that the VZV-HHV-6B positive patient had intrathecal antibodies against both VZV and HHV-6 in the CSF. One double infection of VZV-HHV-6B in a QCMD sample and one possible triple infection of HSV-2, HHV-7 and HHV-6B in a CSF sample were found by the microarray method (Table 4). The putative triple infection with HSV-2, HHV-7 and HHV-6B in CSF had elevated leucocyte (970×10^6 cells/l) and erythrocyte (50×10^6 cells/l) counts, normal glucose values and elevated protein levels (597 mg/l). The blood cell levels were high and as a microarray
being a very sensitive method, latent herpesvirus DNAs may be detected due to the high blood cell counts. To establish the latent HHV-6 infection from active one, specific mRNA levels could be determined. In addition, Ward et al. [2007] have stated that HHV-6 positive CSF samples can be due to the HHV-6 integration into chromosome. To further determine this integration the CFS sample should tested for HHV-6 DNA level. The determination of HHV-6 DNA level in serum should also be confirmed for reason that the high-level serum HHV-6 DNA can indicate integration.

In daily diagnostics, HUSLAB (Helsinki, Finland) does not test for HHV-7 from clinical samples by PCR. Four specimens that were HHV-7 positive by the microarray method were seronegative for HHV-7 at the time of sampling and no follow-up samples were available. Therefore, acute infections cannot be conclusively ruled out.

Other studies have shown that co-infections could be relatively common in CNS infections. Koskiniemi et al. [2001] studied over 3000 patients with acute CNS symptoms and observed possible viral findings in 46% of the

---

**TABLE 4. Samples positive for multiple herpesviruses by diagnostic PCR or microarray method (f)**

<table>
<thead>
<tr>
<th>Diagnostic PCR result and number of specimens</th>
<th>Microarray result and number of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1HSV-2</td>
<td>HSV-2, HHV-7 and HHV-6B 1</td>
</tr>
<tr>
<td>1VZV/neg</td>
<td>VZV and HHV-6B 1</td>
</tr>
<tr>
<td></td>
<td>VZV and EBV 1</td>
</tr>
<tr>
<td>2CMV</td>
<td>CMV and EBV 2</td>
</tr>
<tr>
<td>2EBV</td>
<td>EBV and CMV 1</td>
</tr>
<tr>
<td></td>
<td>EBV and HHV-7 1</td>
</tr>
<tr>
<td>3HHV-6</td>
<td>HHV-6B and EBV 2</td>
</tr>
<tr>
<td></td>
<td>HHV-6B and HHV-7 2</td>
</tr>
<tr>
<td>4VZV</td>
<td>VZV and HHV-6B 1</td>
</tr>
</tbody>
</table>

neg, negative.

1CSF, 2Plasma, 3Whole blood, 4QCMD specimens.

*First former result of PCR, then parallel PCR result with microarray.

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cases. Overall, of confirmed and probable viral findings (>650), over 30 double infections of HSV and VZV were detected. Kupila et al. [2006] also observed HSV-1 and VZV co-infection from a patient with encephalitis. However, in these studies the PCR tests were carried out for HSV-1, HSV-2 and VZV, but not for other herpesviruses. Double CNS infections by other herpesviruses, e.g. HSV, VZV, EBV, CMV, HHV-6 and HHV-7, have also been identified [Sugaya et al. 2002; Weinberg et al. 2005].

Rapid and simultaneous detection of herpesviruses is important for proper drug treatment and efficient patient recovery. The antiviral treatment is immediately started if CNS infection is suspected. To achieve efficient patient recovery specific antiviral treatment should be administrated as soon as possible. Among herpesviruses some drug resistance has occurred and there is no mutual antiviral treatment for all herpesviruses, although acyclovir is effective for several herpesviruses. To optimize testing time and sensitivity, some changes were made to the first microarray method (I, III). More efficient reagents were used, e.g. for ssRNA transcription, microarray spotting and slides. The total time needed for the assay was approximately 5 h, thereby providing results within a single working day. The assay consisted of steps from sample processing to array scanning, including the DNA extraction with the High Viral Nucleic Acid Kit (Roche Diagnostics) (~20-30 min), multiplex-PCR amplification (~1.5 h), ssRNA transcription (1.5-2 h), hybridization and primer extension reaction on the microarray, scanning and analysis (1-1.5 h).

In conclusion, we optimized the microarray method (III) for herpesvirus DNA detection from CSF, plasma, serum and whole-blood and for completion within a single day, and therefore it could be considered a candidate for use in diagnostics. Confirmed co-infections indicated that the microarray method had the potential to be used as a method for detection of multiple infections. Of the human herpesviruses, HSV and VZV in particular are often involved
RESULTS AND DISCUSSION

in CNS infections and the most severe CNS infection is caused by HSV [Whitley 2006; Hanson et al. 2007]. The improved microarray method was shown in particular to detect HSV-1, HSV-2, and VZV in CSF samples. The advantage of microarray methods is also that numerous different viral DNAs can be detected from a small amount of extracted sample. Stöcher et al. [2003] developed four different real-time PCRs for detection of five human herpesviruses in a single run with the same parameters. These PCR reactions were sensitive but needed to be performed separately, increasing the amount of extraction needed for parallel testing of several viruses. In addition, the relative time and cost for analysis could be higher than using the microarray method.

1.3. Facial palsy study group (II)

The microarray method (I) was used for screening purposes before it was modified and improved. The main goal was to screen 70 CSF samples from FP patients and a control group. Kanerva et al. [2007] previously screened this same material for HSV, VZV, and HHV-6 DNA by conventional PCR methods and found one patient with HHV-6 DNA. Results are described in more detail in original publication II. Briefly, from the study group two positive results were obtained. A 13-year-old girl with FP and no additional symptoms was found to be positive for HHV-7 DNA, and a 33-year-old woman who had given birth two weeks prior to FP was found to be simultaneously positive for HHV-6A and -6B DNA in the CSF (Table I in original publication II). Four other patients had intensities barely below the cut-off level for positivity. From these samples, the microarray method the simultaneous presence of HHV-6A and -6B DNA in one sample, two samples were positive for HHV-6B DNA, and one sample was positive for EBV DNA (Table I in original publication II).
The CSF sample reported by Kanerva et al. [2007] to be positive for HHV-6 was no longer positive by the microarray method (II). The reason for this discrepant result could be due to degraded DNA as a result from several freezing and thawing cycles of sample. The patients with positive or possibly positive for HHV-6 by microarray some presented elevated blood cell levels. This can have an influence to results as HHV-6 and HHV-7 can alter to latent state in leucocytes. In addition to latency, HHV-6 can be found integrated into chromosome and therefore HHV-6 positive CSF samples can also be due to the integration [Ward et al. 2007]. To establish the latent HHV-6 infection from active one, specific mRNA levels could be determined [Yoshikawa et al. 2003]. Ward et al. [2007] noted that integrated HHV-6 DNA could also be found in other cells than blood cells, even in hair follicles. The integrated HHV-6 DNA level can be quantified in CSF and serum. The high-level serum HHV-6 DNA may indicate integration. Due to the retrospective nature of this study, there were no serum samples available and most of the CSF samples were exhausted during the study.

Our result showed that microarrays are sensitive methods and efficient in screening of herpesvirus DNAs in a large panel of samples.

2. **SEROLOGICAL MICROARRAY FOR HSV, VZV, AND CMV (IV)**

The aim of this study was to set up an easy screening tool for herpesvirus antibodies. A successful combination of preferred immunological assays on one platform and simultaneous detection of several herpesvirus antibodies is the goal. A microarray method was considered appropriate for achieving this goal and so a serological microarray was designed (IV). Briefly, a microarray was designed, evaluated and compared to the commercial and in-house accredited EIAs used in daily diagnostics by HUSLAB (Helsinki, Finland).
The PUUV-N recombinant protein was first immobilized on a microarray for single virus antibody detection from previously tested serum samples. The results from PUUV IgG and IgM antibody testing gave excellent specificities and sensitivities. The specificities were 100% for both PUUV IgG and IgM, and the sensitivities were 84.1% and 90.6% for PUUV IgG and IgM, respectively.

The high level of purity of recombinant proteins was found to be essential for accurate screening. Therefore, a serological microarray for HSV-1, HSV-2, VZV, and CMV antibodies was set up using commercial high-purity recombinant proteins and tested with patient samples. The HSV G glycoproteins gG1 for HSV-1 and gG2 for HSV-2, which have been used in serological assays to distinguish between HSV-1 and HSV-2, were used as antigens for HSVs in microarrays [Ashley 2002, Ashley et al. 1998, Wald, Ashley-Morrow 2002]. VZV gE, which has been found to be efficiently recognized by the immune system, and CMV pp150, a protein of viral tegument that has been found to be highly immunogenic, were immobilized on the microarray [Fields BN, Knipe DM & Howley PM 1996; La Rosa et al. 2005, Landini et al. 1991].

The specificities for HSV-1, HSV-2, VZV and CMV IgG were 75.9%, 94.7%, 82.6%, 81.4%, respectively (Table 5). The specificity of HSV-1 IgG was below 80% because the microarray method detected 14 new positives for HSV-1 IgG out of 58 negative with EIA. Of these 14, five had HSV IgM antibodies as determined by EIA, which may indicate that microarray detected possible IgG antibodies earlier than commercial EIA. The sensitivities for HSV-1, HSV-2, VZV and CMV IgG were 93.3%, 65.2%, 76.1%, and 83.0%, respectively (Table 5). These sensitivities, except for HSV-2, could be considered to be good.
TABLE 5. Results from herpesvirus-specific EIAAs compared to microarrays, and calculated diagnostic specificities and sensitivities.

<table>
<thead>
<tr>
<th>Herpesvirus EIA, no of samples</th>
<th>MICROARRAY Results</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G+</td>
<td>G-</td>
<td>E</td>
<td>DSP</td>
<td>DSN</td>
</tr>
<tr>
<td>HSV-1 G+</td>
<td>49</td>
<td>44</td>
<td>4</td>
<td>1</td>
<td>75.9%</td>
</tr>
<tr>
<td>HSV-2 G+</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>94.7%</td>
</tr>
<tr>
<td>HSV-1, and -2 G+</td>
<td>13</td>
<td>13 of which 9* double and 3 HSV-1 pos, 1 HSV-2 pos</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HSV-1 G-</td>
<td>58</td>
<td>14</td>
<td>44</td>
<td>-</td>
<td>75.9%</td>
</tr>
<tr>
<td>HSV-2 G-</td>
<td>95</td>
<td>5</td>
<td>90</td>
<td>-</td>
<td>94.7%</td>
</tr>
<tr>
<td></td>
<td>G+</td>
<td>G-</td>
<td>E</td>
<td>DSP</td>
<td>DSN</td>
</tr>
<tr>
<td>HSV M+</td>
<td>15</td>
<td>4</td>
<td>11</td>
<td>-</td>
<td>95.9%</td>
</tr>
<tr>
<td>HSV M-</td>
<td>98</td>
<td>4</td>
<td>94</td>
<td>-</td>
<td>94.7%</td>
</tr>
<tr>
<td>HSV M E</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>VZV G+</td>
<td>110</td>
<td>89</td>
<td>18</td>
<td>3</td>
<td>82.6%</td>
</tr>
<tr>
<td>VZV G-</td>
<td>23</td>
<td>4</td>
<td>19</td>
<td>-</td>
<td>82.6%</td>
</tr>
<tr>
<td></td>
<td>G+</td>
<td>G-</td>
<td>E</td>
<td>DSP</td>
<td>DSN</td>
</tr>
<tr>
<td>VZV M+</td>
<td>58</td>
<td>46</td>
<td>6</td>
<td>6</td>
<td>43.5%</td>
</tr>
<tr>
<td>VZV M-</td>
<td>69</td>
<td>39</td>
<td>30</td>
<td>-</td>
<td>82.6%</td>
</tr>
<tr>
<td>VZV M E</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>82.6%</td>
</tr>
<tr>
<td>CMV G+</td>
<td>91</td>
<td>73</td>
<td>15</td>
<td>3</td>
<td>81.4%</td>
</tr>
<tr>
<td>CMV G-</td>
<td>43</td>
<td>8</td>
<td>35</td>
<td>-</td>
<td>81.4%</td>
</tr>
<tr>
<td></td>
<td>M+</td>
<td>M-</td>
<td>E</td>
<td>DSP</td>
<td>DSN</td>
</tr>
<tr>
<td>CMV M+</td>
<td>14</td>
<td>12</td>
<td>2</td>
<td>-</td>
<td>99.1%</td>
</tr>
<tr>
<td>CMV M-</td>
<td>118</td>
<td>1</td>
<td>113</td>
<td>4</td>
<td>85.7%</td>
</tr>
<tr>
<td>CMV M E</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G, IgG; M, IgM, E, equivocal; +, positive; -, negative; DSP, diagnostic specificity; DSN, diagnostic sensitivity.

*Nine samples had dual antibodies against HSV-1 and -2.

Good specificities for HSV and CMV IgM (95.9% and 99.1%, respectively) were achieved but the specificity for VZV IgM was only 43.5% (Table 5). Sixty-nine samples were VZV-IgM negative by immunoassay but 39 of these samples were positive according to the microarray. However, 31 out of the 39 samples had high levels of VZV-IgG antibodies, and it could be speculated that the false positives might be due to IgG adsorbing before IgM in the microarray. Sensitivities of the microarray method for HSV, VZV and CMV IgM varied being 26.7%, 88.5%, and 85.7%, respectively (Table 5).

The EIA for HSV IgM detection used in HUSLAB (Helsinki, Finland) does not differentiate the HSV-1 and HSV-2 IgM antibodies. With the microarray, HSV-1 and HSV-2 antibodies were both detected, and differentiation was achieved when IgG antibodies for both were present in the sample. The
sensitivity of the HSV-2 IgG was only 65.2% with the microarray. The lower sensitivity of HSV IgM could be due to a failure to properly detect that the sample was positive for HSV-2 IgM. According to the manufacturer (Prospec-Tany Technogene Ltd.), the recombinant protein was immunoreactive with sera of HSV-infected individuals. However, the recombinant protein can be denatured during microarray spotting and storage, thereby losing some of its activity and ability to bind specific antibody. One possibility could also be that glycoprotein G is not the best possible antigen for efficient antibody detection due to the gG deficient HSV-2 strains [Nordberg et al. 2007]. At the moment, the commercial kits contain other antigens such as UV-light inactivated HSV-1 and HSV-2 particles, which are used in HSV IgM tests (Adaltis Italia S.p.A).

The sensitivities and specificities of our assay were promising, but some optimization is still needed to achieve a reliable serological tool for diagnosis. However, this microarray method might be used as a fast screening test for detection of IgG antibodies to HSV-1, VZV and CMV, in order to provide an immunological history. The herpesvirus-specific EIAs are often quantitative assays and used to monitor antibody levels of sample. Accurate EIAs are commercially available, but when used as a screening tool they could be laborious, expensive and sample-consuming. Some specific EIAs even need 100 µl of sample, e.g. the VIDAS-system (bioMérieux sa) for CMV antibody detection. The microarray method uses sample efficiently, and only 1 µl is needed for simultaneous detection of different antibodies. Microarrays cannot not yet be considered a highly reliable quantitative method, although some published studies of viral antibody detection have been carried out [Lu et al. 2005; Mezzasoma et al. 2002]. However, as a screening tool, the microarray could be a superior method. After screening, some of the results can be confirmed by specific EIA to reveal quantitative antibody levels for diagnosis of acute infections. This could potentially reduce the amount of expensive EIAs used in daily diagnostics.
CONCLUDING REMARKS AND FUTURE PROSPECTS

Microarray methods have the potential to replace some of the assays used in diagnostics. For example, the multiplex-PCR and microarray method (III) developed for detection of eight human herpesviruses can be further modified to be used in daily diagnostics of CNS infections. Sequential PCRs can be replaced with one microarray test. The microarray method is a qualitative assay, at least for now. However, microarrays can be used as screening tools in other than CNS infections, e.g. for CMV and EBV DNA detection from transplant patient samples, after which quantitative PCRs could be carried out on the microarray-positive samples. Therefore, fewer quantitative PCRs can be carried out, and in potential multi-infections, quantification can be performed for all the microarray-screened viruses with quantitative PCR. Serological microarrays could be used as a fast screening tool. In the future, when microarray technology has evolved and been tested more, perhaps serological assays such as EIAs could be replaced by microarrays. Developing of spotting equipments and reagents is proceeding, and reproducibility can be achieved faster.

In conclusion, microarray-based methods have showed the potential to be an efficient diagnostic assay. In the future, it is likely that the technique will be further developed to meet the requirements of routine diagnosis of viral infections.
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