JOURNAL OF CLINICAL MICROBIOLOGY, May 2002, p. 1728–1732 Vol. 40, No. 5
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.5.1728–1732.2002


# Utility of a Multiplex PCR Assay for Detecting Herpesvirus DNA in Clinical Samples

Julian Druce, Mike Catton, Doris Chibo, Kirsty Minerds, David Tyssen, Renata Kostecki, Bill Maskill, Wendy Leong-Shaw, Marie Gerrard, and Chris Birch*

*Victorian Infectious Diseases Reference Laboratory (VIDRL), North Melbourne, Victoria, Australia*

Received 31 October 2001/Returned for modification 24 December 2001/Accepted 23 February 2002

**A multiplex PCR was designed to amplify herpes simplex virus types 1 and 2, cytomegalovirus, and varicella-zoster virus DNA present in a diverse range of clinical material. The susceptibility of these viruses to in vivo inhibition by at least one antiviral drug was an important consideration in their inclusion in the multiplex detection system. An aliquot of equine herpesvirus was introduced into each specimen prior to extraction and served as an indicator of potential inhibitors of the PCR and a detector of suboptimal PCR conditions. Compared to virus isolation and immunofluorescence-based antigen detection, the multiplex assay yielded higher detection rates for all viruses represented in the assay. The turnaround time for performance of the assay was markedly reduced compared to those for the other techniques used to identify these viruses. More than 21,000 tests have been performed using the assay. Overall, the multiplex PCR enabled the detection of substantially increased numbers of herpesviruses, in some cases in specimens or anatomical sites where previously they were rarely if ever identified using traditional detection methods.**

Nucleic acid detection techniques such as PCR provide the potential for rapid and sensitive detection of serious, treatable virus infections, such as those caused by the herpes group of viruses. Detection of members of this group may comprise up to half the workload of many diagnostic virology laboratories. Virus isolation, shell vial-based assays, cytomegalovirus (CMV) pp65 antigenemia assays, and immunofluorescence (IF) assays all suffer from one or more limitations, including, respectively, slowness, insensitivity when applied to blood specimens, lack of suitability for high specimen throughput, and a requirement for infected cells in the specimen. PCR has the potential to overcome each of these limitations and also has applicability over a wide range of specimen types. Multiplex PCR assays have the additional advantage of combining primers that are specific for viruses associated with several potential differential diagnoses in the one test, thereby offering increased efficiency and cost-effectiveness. Multiplex PCR assays have been described for herpesviruses, although they vary in terms of the virus types represented and the specimens analyzed. For example, assays for the simultaneous detection of varicella-zoster virus (VZV), herpes simplex viruses (HSV), CMV, human herpesvirus 6, and Epstein-Barr virus in cerebrospinal fluid (CSF) (11) and assays for HSV and VZV in mucocutaneous specimens (6, 9) and CSF (12) have been reported, each with improved utility over existing methods in the diagnostic setting.

Our laboratory serves as a reference virus identification laboratory for a population of nearly 4 million people. A wide range of specimen types are received on a daily basis from hospital in- and outpatients as well as from those being served by general practitioners and doctors in specialized infectious-disease clinics. These patients have diverse clinical symptoms, including those associated with infections caused by one or more of the following: HSV type 1 (HSV-1) and HSV-2, CMV, and VZV. Because of the association of one or more of these viruses with central nervous system disease, ocular manifestations, and reactivation of virus in immunosuppressed and transplant patients, infections that are all potentially responsive to antiviral drug therapy, we developed a multiplex PCR assay capable of detecting them in clinical material. To control for possible inhibition of the PCR by substances present in the test specimens, an internal standard, equine herpesvirus type 4 (EHV-4) (2), was incorporated into all specimens prior to DNA extraction and PCR amplification. We present here the results of the assay validation, and our experience over the first 20 months using the test in the diagnostic laboratory.

## MATERIALS AND METHODS

**Patients and specimens.** Specimens were received from individuals with a range of clinical presentations that included oral, skin, or genital lesions; keratitis; fever; encephalitis; and CMV-associated syndromes. Clinical material was sent to the laboratory as undiluted body fluids (CSF, feces, and anticoagulated blood and urine) or in virus transport medium (VTM) (swabs, saliva, nasopharyngeal aspirates [NPAs], bronchoalveolar lavages [BALs], nose/throat swabs [NTS], corneal scrapes, and biopsy tissue). On reaching the laboratory, biopsy tissue and feces were made to 10% (wt/vol) in VTM. Whole blood was separated into plasma and leukocyte fractions by low-speed centrifugation, and both fractions were tested by PCR. CSF and urine were tested without further dilution. The prospective study reported here was carried out on specimens received between October 1999 and May 2001.

**Validation of PCR.** Initial validation of the multiplex PCR included optimization of primers and cycling conditions, specificity checks against well-characterized virus-positive and virus-negative clinical material, and sensitivity determinations against virus isolates of known titer or commercially available quantitation assays. Clinical validation of the PCR was performed by prospective parallel testing on 656 specimens sent to the laboratory for detection of herpesviruses using the existing methods of virus isolation and/or IF. HSV-positive samples were confirmed using a PCR specific for the glycoprotein D gene of this virus; the specificity of the CMV component of the assay was confirmed in a second group of specimens using UL97-specific primers with sequencing of the product (results not shown). The majority of specimens tested were swabs from body sites (63%). Other specimen types included NTS/NPAs (7%), BALs (4%), leukocytes (3%), and saliva (3%). Biopsy, fecal, and pericardial samples were

* Corresponding author. Mailing address: VIDRL, 10 Wreckyn St., North Melbourne 3051, Victoria, Australia. Phone: (61 3) 93422626. Fax: (61 3) 93422666. Email: chris.birch@mh.org.au.

TABLE 1. Gene targets for HSV, CMV, VZV, and EHV-4 primer sequences used and their target product sizes

| Virus | Target gene | Product size[a] (bp) | Primer sequence[b] |
|---|---|---|---|
| HSV[c] | Glycoprotein B | | R1 5′GCACACCACCGACCTCAAGTACAA |
| | | 370 | R1 3′CCTGCCACTTGGTCATGGTG |
| | | | R2 5′ GCATGCTCGAGGAGGTTGGAC |
| | | 149 | R2 3′ TTGAAGCGGTCGGCGGCGTA |
| HSV-2 | US-4 | | R1 5′ACGTACTACCGCCTCAC |
| | | 258 | R1 3′CCACCTCTACCCACAAC |
| | | | R2 5′CCGCGCCTGCCGTCAGCCCATCCTC |
| | | 225 | R2 3′ AGACCCACGTGCAGCTCGCCG |
| CMV | UL123 | | R1 5′ CAAGCGGCCTCTGATAACCAAGC |
| | | 438 | R1 3′ CTCTTCCTCTGGGGCAACTTCCTC |
| | | | R2 5′ CCGATCCTCTGAGAGTCTGCTCTC |
| | | 190 | R2 3′ CAGCCACAATTACTGAGGACAGA |
| VZV | Matrix | | R1 5′ ATGTCCGTACAACATCAACT |
| | | 450 | R1 3′ GGAGACTGGATATGCACGCATC |
| | | | R2 5′ CGATTTTCCAAGAGAGACGC |
| | | 99 | R2 3′ GGTGGAGACGACTTCAATAGC |
| EHV-4 | Glycoprotein B | | R1 5′ CTGCTGTCATTATGCAGGGA |
| | | 509 | R1 3′ CGTCTTCTCGAAGACGGGTA |
| | | | R2 5′ CTGCTGTCATTATGCAGGGA |
| | | 323 | R3 3′ CGCTAGTGTCATCATCGTCG |

[a] Upper value represents first-round product size; lower value represents second-round product size.
[b] R1 = first-round primers; R2 = second-round primers.
[c] Primers for HSV are specific for both HSV-1 and HSV-2 DNA.

also tested but in low numbers. Only HSV, CMV, and VZV primers were included in the validation assay. Primers specific for HSV-2 were included at the commencement of prospective testing to enable differentiation of HSV-1 and HSV-2.

**Nucleic acid extraction.** Nucleic acid was extracted using either of two methods. For undiluted body fluids, 100 μl of specimen was extracted using a commercially available column (Highpure Viral Nucleic Acid Extraction Column; Roche Diagnostics, Mannheim, Germany) followed by treatment of 40 μl of the column eluate with 2 μl of 1% Nonidet P-40 (NP-40) (BDH Chemicals Ltd., Poole, England) at 70°C for 45 s. Specimens sent in VTM were vortexed and then directly treated using the NP-40 procedure only.

**PCR amplification conditions.** The primers used in first- and second-round amplification and their target genes are shown in Table 1 and are based on those previously described for HSV (7), VZV (10), and CMV (14). Primers specific for HSV were designed to enable distinction between HSV-1 and HSV-2. One set (HSV) amplified both HSV-1 and HSV-2; the second set (HSV-2) amplified only HSV-2. As a result HSV-1 strains yielded a single second-round product (149 bp), while HSV-2 strains yielded two second-round products (149 and 225 bp) (Fig. 1). PCR primers specific for EHV-4 DNA were included in the multiplex assay to amplify an internal amplification control (see below). Mastermix preparation, sample manipulation, and addition of samples to mastermixes were performed in class 2 biological safety cabinets within a suite of four purpose-built PCR laboratories. PCRs were performed using eight-strip tubes and lids (Abgene, Surrey, United Kingdom). To avoid cross-contamination, only a single strip was opened at any time during these procedures, and all strip tubes were centrifuged at 6,300 rpm for 10 s in a bench top strip-tube centrifuge (model no. PMC-860; Tomy, Tokyo, Japan) prior to removal of lids.

For first-round PCR, 4 μl of extracted nucleic acid was added to 40 μl of a mastermix containing 500 nM first-round (R1) primer (Table 1), 1.8 mM $MgCl_2$, 2 μM deoxynucleoside triphosphate, and 0.3 U of *Taq* polymerase (Qiagen, Hilden, Germany). Cycling conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 30 s, with a final extension of 72°C for 3 min. For second-round PCR, 2 μl of first-round product was transferred to 40 μl of an identical mastermix but containing second-round (R2) primers as shown in Table 1. Cycling conditions were the same as for first-round PCR, but only 25 cycles were used. Electrophoretic separation of amplified products from clinical material was performed for 30 min at 80 mA on a 2% agarose gel in 1× TAE (40 mM Tris-acetate [pH 7.6], and 1 mM sodium EDTA). Molecular weight markers (Roche Diagnostics, Mannheim, Germany) were included on each gel. Gels were stained with ethidium bromide (Sigma Chemicals, Steinheim, Germany) and were photographed under UV illumination on a Gel Doc 2000 (Bio-Rad, Hercules, Calif.). All results were interpreted in a strictly qualitative fashion. Results were reported as either "(specific virus) DNA detected," "DNA not detected," or "specimen inhibitory to PCR." Clinical interpretation was not provided at the time of reporting.

**Assay controls.** A negative control consisting of nuclease-free water was included in every assay. Each positive control virus used in the assay (HSV-2, CMV, and VZV) was prepared from a known isolate serially titrated to its endpoint of PCR detection. An HSV-1 control was not routinely included because the design of the two primer sets used in the assay enabled distinction between both HSV-1 and HSV-2. Control viruses were used at 10 times the concentration of their last dilution positive by PCR. A typical gel of the multiplex PCR is shown in Fig 1. To control for the presence of inhibitors of PCR, EHV-4 was added to each test sample by incorporating it into the NP-40 used for extraction. This virus was chosen so that a standardized amount of a virus not expected to be present in any human clinical material could be added to all samples tested. The concentration of EHV-4 added was chosen so that it was slightly more sensitive to potential inhibition at its use dilution than any of the human herpesvirus targets. Therefore, specimens showing demonstrable inhibition of the EHV-4 control but with a human herpesvirus-specific product present

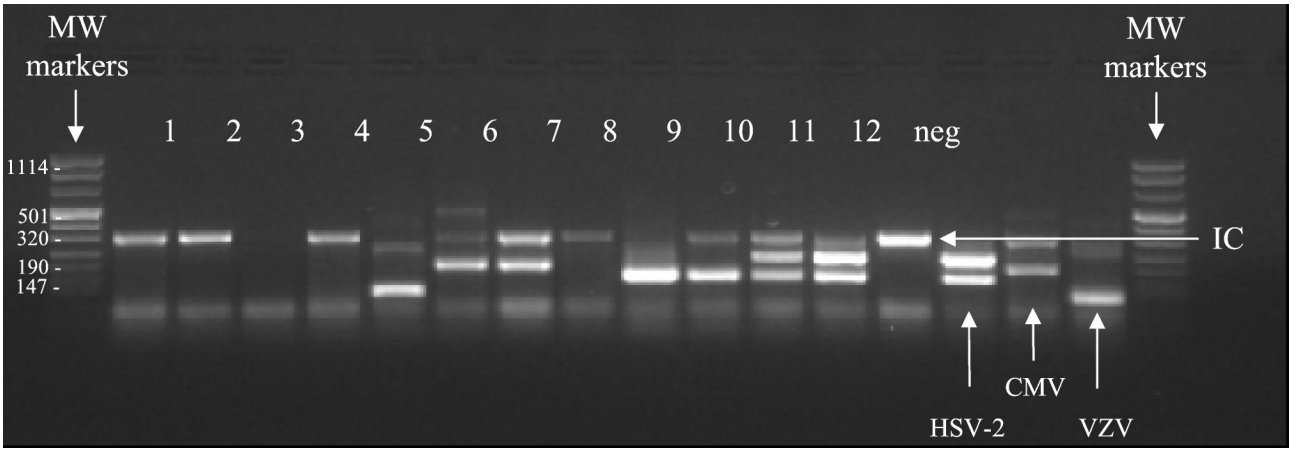


FIG. 1. Second-round PCR products from assay controls and 12 clinical specimens. Samples in lanes 1, 2, 4, and 8 are negative for the test viruses (internal control [IC] product visible only). Samples in lanes 9 and 10 are HSV-1 positive, in lanes 11 and 12 are HSV-2 positive, and in lanes 6 and 7 are CMV positive, and the sample in lane 5 is VZV positive. The sample in lane 3 is inhibitory to the PCR. Molecular mass (MW) markers (with selected sizes in base pairs) are shown in wells at both ends of the gel. neg = negative control (nuclease-free water).

TABLE 2. Comparison of multiplex PCR versus virus isolation and IF for the detection of HSV, VZV, and CMV in clinical samples during the validation phase

| Virus | PCR result | Isolation and IF positive[a] | Isolation and IF negative[a] |
|---|---|---|---|
| HSV[b] | Positive | 131 | 38 |
| | Negative | 0 | 487 |
| VZV | Positive | 22 | 1 |
| | Negative | 0 | 633 |
| CMV | Positive | 8[c] | 14[c] |
| | Negative | 0[c] | 634[c] |

[a] Results combined for virus isolation and IF.
[b] Data combined for HSV-1 and HSV-2.
[c] Includes extended CMV isolation (28 days) and shell vial detection.

(for example, lanes 5 and 9 in Fig. 1) were reported positive for that human herpesvirus. If specimens extracted using NP-40 were inhibitory, they were retested after column extraction. Specimens showing inhibition of the internal control after column extraction were reported as inhibitory.

**Direct IF.** Cells obtained from lesion swabs were spotted in triplicate onto glass slides and then dried and fixed in acetone. The cells in individual spots were stained for 30 min at 37°C with either HSV-1- or HSV-2-specific monoclonal antibodies (Trinity Biotech, Wicklow, Ireland) or VZV-specific monoclonal antibodies (Meridien Diagnostics, Cincinnati, Ohio) that were directly conjugated with fluorescein isothiocyanate. The slides were then washed three times with phosphate-buffered saline, mounted, and examined by fluorescence microscopy.

**Virus isolation.** One hundred microliters of clinical material was inoculated into glass phials containing human embryonic cell monolayers in Eagle's minimal essential medium containing 2% fetal calf serum and was incubated at 35°C for 7 days. If clinical information suggested the possibility of infection with CMV, isolation attempts were performed over 28 days. For rapid CMV culture and detection, shell vials (Menzel Glaser, Freiburg, Germany) containing human embryonic cells were inoculated with 100 μl of specimen, centrifuged at 240 × *g* for 10 min, and then incubated at 35°C for 48 h. The cells were fixed in acetone and stained as described above using CMV-specific monoclonal antibodies directly conjugated with fluorescein isothiocyanate (Argene Biosoft, Varhiles, France) and were then examined by fluorescence microscopy.

## RESULTS

**Validation.** Comparison of the sensitivity of the multiplex assay versus virus isolation indicated that the PCR was at least 2 logs more sensitive than either method for HSV and VZV (results not shown). No attempt was made to analyze the sensitivity of the assay for these viruses in terms of genome equivalents. However, when directly compared to a commercial quantitative CMV PCR (CMV Monitor; Roche Diagnostic Systems, Branchburg, N.J.), the lower limit of detection of CMV in plasma using the multiplex assay was approximately 400 CMV DNA copies per ml (result not shown). Results comparing the multiplex PCR with virus isolation and IF are shown in Table 2. In clinical material from patients suspected of having an infection with one of the herpesviruses represented in the PCR assay, increased detection rates for HSV (combined HSV-1 and HSV-2) and CMV of 30 and 175%, respectively, were achieved. There was an increased detection rate of 5% for VZV. The sensitivity of the PCR versus isolation and IF combined was 100% each for HSV, VZV, and CMV.

**Prospective testing.** Since the introduction of the PCR to replace conventional isolation and IF, more than 21,000 specimens have been tested. The specimen types, numbers tested, and detection rates for all of the herpesviruses included in the assay are shown in Table 3. An analysis of the gender, age, and clinical symptoms of the patients from whom CSF samples were obtained is shown in Table 4. Of 1,598 such samples tested, 62 (4%) were positive for one of the test viruses (Table 3). HSV-1 was the most common virus detected in CSF (24 of 62 positive specimens [39%]), followed by VZV (34%), HSV-2 (18%), and CMV (10%). Encephalitis was the most common diagnosis in patients with HSV-1 detected in the CSF, whereas meningitis was a more common clinical diagnosis in patients with HSV-2 or VZV detected (Table 4).

Of 1,506 whole blood specimens tested, 305 (20%) were positive (Table 3). Most (85%) contained CMV, but HSV-1 (10%) HSV-2 (3%), and VZV (2%) were also detected. The majority of CMV-positive samples (57%) were from patients who had received bone marrow transplants. Of those blood samples positive for CMV, 20% were positive in plasma but negative in the accompanying leukocyte fraction.

Samples of biopsy tissue constituted 18% of the total number of specimens submitted for testing. CMV was the most common virus detected in these specimens (58% of all positives), most of which were obtained from transplant patients. CMV was also highly represented in BAL specimens (71% of all positives). The majority of these (68%) were also from patients who had undergone organ transplantation. In contrast, only 41% of HSV-1-positive BAL samples were from transplantation patients.

Swabs from genital sites, eyes, and unspecified anatomical sites represented the majority of specimens received and tested (69% of the total). HSV-1 predominated in eye swabs (87% of all positives). Of the genital swabs positive, HSV-2 was present

TABLE 3. Summary of specimen types and numbers positive for HSV-1, HSV-2, VZV, and CMV using the multiplex PCR

| Specimen type | No. tested | No. positive (% of positives) | | | |
|---|---|---|---|---|---|
| | | HSV-1 | HSV-2 | VZV | CMV |
| CSF | 1,598 | 24 (39) | 11 (18) | 21 (34) | 6 (10) |
| Blood (total)[a] | 1,506 | 31 (10) | 9 (3) | 7 (2) | 258 (85) |
| Leukocytes | | 7 | 3 | 1 | 77 |
| Plasma | | 18 | 5 | 4 | 52 |
| Plasma + leukocytes | | 6 | 1 | 2 | 129 |
| Biopsy tissue | 509 | 24 (26) | 6 (7) | 8 (9) | 53 (58) |
| BAL | 1,004 | 59 (28) | 3 (1) | 0 | 150 (71) |
| Urine | 929 | 6 (5) | 4 (3) | 3 (2) | 112 (90) |
| Saliva | 477 | 43 (43) | 3 (3) | 3 (3) | 52 (51) |
| Eye swabs | 946 | 188 (87) | 3 (1) | 22 (10) | 2 (1) |
| Genital swabs | 4,109 | 570 (37) | 925 (60) | 44 (2.8) | 5 (0.3) |
| Swabs (other)[b] | 9,402 | 1,811 (47) | 1,061 (27) | 919 (24) | 81 (2) |
| NPA/NTS | 409 | 68 | 2 | 6 | 12 |
| Total[c] | 21,037 | 2,839 (43) | 2,039 (31) | 1,039 (16) | 739 (11) |

[a] Plasma and cell fractions combined.
[b] Anatomical site not specified.
[c] More than one virus-specific DNA type was detected in 22 (1.04%) of specimens.

in 60%, HSV-1 in 37%, and VZV in nearly 3%. Approximately half of the genital specimens containing VZV were from patients whose clinical diagnosis was recorded as a likely HSV infection. Although many of the specimens from unspecified anatomical sites were likely to have been genital swabs, a more even distribution of virus types was observed in this group (47% HSV-1, 27% HSV-2, and 24% VZV).

## DISCUSSION

The multiplex herpesvirus PCR that we describe resulted in increased detection rates for HSV-1, HSV-2, CMV, and VZV compared to those for previously used methods for detection of these viruses. Overall, 32% of specimens tested were positive for at least one of these viruses. The turnaround time from receipt of specimen to issuing of results was also improved, although specimens that could be examined for the presence of VZV or HSV antigens by IF were also tested rapidly if their numbers were low. In general, turnaround time for HSV detection was reduced from 4 days to 1 day, from 7 to 14 days to 1 day for VZV, and from between 4 days (shell vial) and up to 28 days (isolation) to 1 day for CMV. During the validation phase the greatest increases in detection rates were seen with HSV and CMV. Increases of 30 and 175%, respectively, were seen in detection rates by PCR from those by traditional techniques (including shell vial assay and long-term culture for CMV). The improvement in PCR detection rates for VZV was modest, suggesting that the existing IF method for detection of this virus was of similar sensitivity to PCR on the specimens evaluated. However, since introduction of the PCR as a routine test into the diagnostic laboratory, a wider range of samples (including CSF and blood) has been tested for the presence of VZV, resulting in an increased detection rate for this virus compared to the rate in our previous experience.

Use of multiplex PCR instead of virus isolation has also facilitated the detection of multiple viruses in individual specimens. Albeit rare, this was most commonly seen in specimens containing HSV-1 or HSV-2 (0.1% of positive specimens). Prior to the availability of PCR, only the more rapidly growing virus type (presumably HSV) would likely have been detected in such samples. Although the lack of HSV-1-specific primers in our assay did not enable dual HSV-1/HSV-2 infections to be detected simultaneously, such infections are rare and would be unlikely to be detected using virus isolation techniques.

The prospective use of the multiplex PCR has enabled the testing of a wide range of specimen types, including blood and tissues for the detection of HSV. Such specimens were rarely if ever sent to the laboratory before the availability of the molecular assay. The assay's ability to detect these viruses in such specimens enhances its utility in the clinical setting. Similarly, detection of HSV DNA in CSF has been made possible using the PCR, where previously isolation of HSV from this site was not successful in cases of herpes encephalitis. In this context our data are very similar to those previously reported on the differences in clinical presentation between alpha-, beta-, and gammaherpes viruses (15). All viruses represented in the PCR assay were found on at least one occasion in CSF specimens, indicating that HSV screening alone by PCR may be insufficient when central nervous system involvement occurs. In agreement with studies by others (11), we found a need for nested PCR in such cases, since single-round PCR was not of sufficient sensitivity to detect HSV in all CSF samples tested from patients with this infection (results not shown). The better performance offered by nested PCR in such cases suggests a need for caution when using real-time capillary PCR, which has otherwise been applied with good results in the diagnosis of VZV (3) and HSV (4) in vesicular lesions. Our results using the multiplex PCR for detection of HSV in genital lesions was in agreement with other studies showing improved sensitivity, turnaround time, and cost-effectiveness (1, 8).

In studies not reported here, we have found that undiluted body fluids often contain inhibitors that are not removed by the simple NP-40 DNA extraction procedure used for this PCR. Although most of these specimens are likely to have inhibitors removed during extraction through commercially available columns, it remains important to include an internal control to detect potential PCR inhibitors in all specimens tested. As well as indicating the presence of such inhibitors, the low concentration of EHV-4 used as the internal control also allows variation in mastermix preparations or suboptimal cycling conditions to be detected. For these reasons, it is important to use the internal control at such a concentration that its product is eliminated when specimen preparation and assay conditions are less than optimal.

The ability of multiplex PCR to yield unexpected results has been of particular interest and often of considerable clinical benefit. Specific details of these results are to be published separately, but they include the occasional detection of HSV and VZV in plasma, urine, saliva, and biopsy specimens from transplant patients when CMV was expected to be present and the detection of VZV in nearly 3% of genital lesions in patients suspected of having herpes simplex infection. The increased

TABLE 4. Gender, age range, and clinical symptoms at presentation of patients in whom HSV-1, HSV-2, CMV, or VZV was detected in CSF specimens using multiplex PCR

| Virus detected | No. of positive samples | Age range in yr (no. of patients) | No. of males/no. of females | Presenting symptom (no. of patients) |
|---|---|---|---|---|
| HSV-1 | 24 | 0–10 (1)<br>11–20 (4)<br>21–50 (6)<br>>50 (13) | 14/10 | Encephalitis (10)<br>Meningitis (3)<br>Febris (4)<br>Not specified (7) |
| HSV-2 | 11 | 21–50 (10)<br>>50 (1) | 4/7 | Meningitis (5)<br>Headache (2)<br>Confusion (1)<br>Not specified (3) |
| CMV | 6 | 30–40 (6) | 6/0 | HIV positivity (6)<br>Encephalitis (1)<br>Meningitis (1)<br>Headache (1)<br>Lymphoma (1)<br>Bell's palsy (1)<br>Not specified (1) |
| VZV | 21 | 0–10 (1)<br>11–20 (3)<br>21–50 (5)<br>>50 (12) | 13/8 | Meningitis (9)<br>Encephalitis (1)<br>Confusion (1)<br>Seizures (1)<br>Drowsiness (1)<br>Other/not specified (8) |

detection rates of all targeted viruses (but particularly of CMV) in the blood of transplant patients also provided early indications of infections or reactivations, often prior to the onset of symptoms. Of interest was our observation that 20% of all CMV PCR-positive blood specimens occurred in the plasma fraction alone. It is likely that this is attributable to a reservoir of CMV distinct from leukocytes in these patients, possibly in endothelial tissue, as has been suggested by others (5, 13).

The increased sensitivity and turnaround times of PCR-based assays have the potential to significantly affect patient management, particularly if such assays are designed to include viruses for which antiviral drug therapy is available. When throughput is high, as is the case in our laboratory, multiplexed PCR assays are also cost-effective and provide a wider differential diagnosis than do individual assays. Finally, the use of a PCR system such as we describe challenges virologists and clinicians to think more about the relationship between viruses and the infections that they may cause. A specific, sensitive multiplex PCR assay has the capability to detect viruses in specimens and body sites where previously they were rarely if ever seen.

## ACKNOWLEDGMENTS

We thank Linda Hobday and Penny Tresise for their assistance performing PCR assays and thank Mike Studdert and Carol Hartley (School of Veterinary Science, University of Melbourne) for supplying EHV-4.

## REFERENCES

1. **Coyle, P. V., A. Desai, D. Wyatt, C. McCaughey, and H. J. O'Neill.** 1999. A comparison of virus isolation, indirect immunofluorescence and multiplex polymerase chain reaction for the diagnosis of primary and recurrent herpes simplex type 1 and type 2 infections. J. Virol. Methods **83:**75–82.
2. **Crabb, B. S., and M. J. Studdert.** 1995. Equine herpesviruses 4 (equine pneumonitis virus) and 1 (equine abortion virus). Adv. Virus Res. **45:**153–190.
3. **Espy, M. J., R. Teo, T. K. Ross, K. A. Svien, A. D. Wold, J. R. Uhl, and T. F. Smith.** 2000. Diagnosis of varicella-zoster virus infections in the clinical laboratory by LightCycler PCR. J. Clin. Microbiol. **38:**3187–3189.
4. **Espy, M. J., P. N. Rys, A. D. Wold, J. R. Uhl, L. M. Sloan, G. D. Jenkins, D. M. Ilstrup, F. R. Cockerill, R. Patel, J. E. Rosenblatt, and T. F. Smith.** 2001. Detection of herpes simplex virus DNA in genital and dermal specimens by LightCycler PCR after extraction using the IsoQuick, MagNA Pure, and BioRobot 9604 methods. J. Clin. Microbiol. **39:**2233–2236.
5. **Fish, K. N., C. Soderberg-Naucler, L. K. Mills, S. Stenglein, and J. A. Nelson.** 1998. Human cytomegalovirus persistently infects aortic endothelial cells. J. Virol. **72:**5661–5668.
6. **Jain, S., D. Wyatt, C. McCaughey, H. J. O'Neill, and P. V. Coyle.** 2001. Nested multiplex polymerase chain reaction for the diagnosis of cutaneous herpes simplex and herpes zoster infections and a comparison with electron microscopy. J. Med. Virol. **63:**52–56.
7. **Lakeman, F. D., and R. J. Whitley.** 1995. Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. J. Infect. Dis. **171:**857–863.
8. **Marshall, D. S., D. R. Linfert, A. Draghi, Y. S. McCarter, and G. J. Tsongalis.** 2001. Identification of herpes simplex virus genital infection: comparison of a multiplex PCR assay and traditional viral isolation techniques. Mod. Pathol. **14:**152–156.
9. **Nogueira, M. L., J. B. Amorim, J. G. Oliveira, C. A. Bonjardim, P. C. Ferreira, and E. G. Kroon.** 2000. Comparison of virus isolation and various polymerase chain reaction methods in the diagnosis of mucocutaneous herpesvirus infection. Acta Virol. **44:**61–65.
10. **Puchhammer-Stockl, E., T. Popow-Kraupp, F. X. Heinz, C. W. Mandl, and C. Kunz.** 1991. Detection of varicella-zoster virus DNA by polymerase chain reaction in the cerebrospinal fluid of patients suffering from neurological complications associated with chicken pox or herpes zoster. J. Clin. Microbiol. **29:**1513–1516.
11. **Quereda, A., I. Corrall, F. Laguna, M. E., Valencia, A. Tenorio, J. E. Echeverria, E. Navas, P. Martin-Davila, A. Moreno, V. Moreno, J. M. Gonzalez-Lahoz, J. R. Arribas, and A. Guerrero.** 2000. Diagnostic utility of a multiplex herpesvirus PCR assay performed with cerebrospinal fluid from human immunodeficiency virus-infected patients with neurological disorders. J. Clin. Microbiol. **38:**3061–3067.
12. **Read, S. J., and J. B. Kurtz.** 1999. Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. J. Clin. Microbiol. **37:**1352–1355.
13. **Sinzger, C., A. Grefte, B. Placher, A. S. Gouw, T. H. The, and G. Jahn.** 1995. Fibroblasts, epithelial cells, endothelial cells and smooth muscle cells are major targets of human cytomegalovirus infection in lung and gastrointestinal tissue. J. Gen. Virol. **76:**741–750.
14. **Studahl, M., T. Bergstrom, K. Ekeland-Sjoberg, and A. Ricksten.** 1995. Detection of cytomegalovirus DNA in cerebrospinal fluid in immunocompetent patients as a sign of active infection. J. Med. Virol. **46:**274–280.
15. **Studahl, M., L. Hagberg, E. Rekabdar, and T. Bergstrom.** 2000. Herpesvirus DNA detection in cerebral spinal fluid: differences in clinical presentation between alpha-, beta-, and gamma-herpesviruses. Scand. J. Infect. Dis. **32:**237–248.