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Analytical characterization of an assay designed to detect and identify diverse agents of disseminated viral infection

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ABSTRACT

Background: Diverse viruses often reactivate in or infect cancer patients, patients with immunocompromising infections or genetic conditions, and transplant recipients undergoing immunosuppressive therapy. These infections can disseminate, leading to death, transplant rejection, and other severe outcomes.
Objectives: To develop and characterize an assay capable of inclusive and accurate identification of diverse potentially disseminating viruses directly from plasma specimens.
Study design: We developed a PCR/electrospray ionization mass spectrometry (PCR/ESI-MS) assay designed to simultaneously detect and identify adenovirus, enterovirus, polyomaviruses JC and BK, parvovirus B19, HSV-1, HSV-2, VZV, EBV, CMV, and herpesviruses 6–8 in plasma specimens. The assay performance was characterized analytically, and the results from clinical plasma samples were compared to the results obtained from single-analyte real time PCR tests currently used in clinical practice.
Results: The assay demonstrated sensitivity and specificity to diverse strains of the targeted viral families and robustness to interfering substances and potentially cross reacting organisms. The assay yielded 94% sensitivity when testing clinical plasma samples previously identified as positive using standard-of-care real-time PCR tests for a single target virus (available samples included positive samples for 11 viruses targeted by the assay).
Conclusions: The assay functioned as designed, providing simultaneous broad-spectrum detection and identification of diverse agents of disseminated viral infection. Among 156 clinical samples tested, 37 detections were made in addition to the detections matching the initial clinical positive results.

1. Background

Several families of DNA and RNA viruses are associated with disseminated viral infections among patients rendered immunocompromised by infectious disease, cancer, genetic defects, or immunosuppressive therapy [1–3]. Many of these viruses are endemic, with the majority of the human population experiencing infection during their lifetime. Such viruses include human herpesviruses (HHVs) [4–10], enteroviruses (HEVs) [11], polyomaviruses [12–14], adenoviruses [15,16], and parvovirus B19 [17,18]. During initial infection of immunocompetent individuals, some of these viruses cause mild acute symptoms followed by stable, asymptomatic, and essentially dormant carrier-state infections [3,19–22], whereas others normally cause only transient infections with relatively mild symptoms [20,22]. When the immune system is compromised these viruses can reactivate or reinfect from a secondary source, spread throughout the body, and threaten the survival of the patient [3,22]. Such infections may be limited by appropriate antiviral therapy, temporary withdrawal of immunosuppressive therapy, or other treatments [1–3,22]. The efficacy of such treatments is dependent on timely detection and identification of disseminating viruses.

Relevant diagnostic tools should be capable of broadly identifying responsible agents in sample types appropriate for detection of disseminated infection. Such sample types include fluids such as cerebrospinal fluid (CSF) [13,21] and plasma (or serum) [6–12,14,16,18,23,24]. Available assays for diagnosis of disseminated infection are usually limited to detection of single agents, and physicians generally test for single viruses suspected on the

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basis of presumptive empirical diagnosis. Recent data suggests that such strategies may be missing less easily recognized or relatively unexpected infections [3,22].

2. Objectives

Here we describe the principles and initial analytical characterization of a multiplex PCR/electrospray ionization mass spectrometry (PCR/ESI-MS) [25–28] test designed to detect and identify members of the human *Herpesviridae*, including *Simplexvirus* [herpes simplex viruses 1 and 2 (HSV-1 and -2 or HHV-1 and -2)], *Varicellovirus* [varicella zoster virus (VZV or HHV-3)], *Cytomegalovirus* (CMV or HHV-5), *Roseolovirus* (HHV-6 and HHV-7], *Lymphocryptovirus* [Epstein–Barr virus (EBV or HHV-4)], and *Rhadinovirus* [Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8)]. The test also detects human *Polyomavirus* (JC and BK), human *Enterovirus*, human *Mastadenovirus* (adenovirus), and human *Erythrovirus* (parvovirus B19). The test utilizes PCR reactions with primers targeted at sites conserved within viral families. Primer sites flank variable regions (as with ribosomal sequence analysis or multi-locus sequence typing), allowing identification of viruses through ESI-MS analysis of amplicons. Mass data from complimentary strands of each amplicon are computationally translated into base composition signatures [25–28]. Signatures are compared to a database of signatures generated from sequence database information and direct PCR/ESI-MS analysis of viral stocks. Result reporting is limited to the species (for herpes viruses 1–8 and polyomaviruses JC and BK) or genus (for adenovirus, enterovirus, and parvovirus) level to allow for tractable validation.

The test described here is essentially qualitative (signal strength is used only to define reporting thresholds), and designed to detect viruses at levels associated with symptomatic disseminated infection. Plasma and serum are relevant sample types for detecting most of the viruses targeted by this assay [5–9,11,12,14,16,23,24], with the exception of JC virus, which is effectively diagnosed primarily from CSF [13,21]. The test is not designed to monitor low-titer viral levels in asymptomatic patients or to quantitatively track evolving infections.

In this study, the disseminated viremia assay was evaluated for analytical sensitivity, cross-reactivity toward potential viral and bacterial contaminants, inclusivity with regard to diverse strains, robustness with respect to interfering substances, and carryover between positive and negative samples. Banked clinical plasma specimens with existing positive results from diagnostic q-PCR tests were tested using the PCR/ESI-MS assay to measure sensitivity of the test with respect to confirmed infections, and healthy patient specimens were tested to estimate the rate of detectable asymptomatic carriage of the target viruses in healthy individuals.

3. Study design

The PCR/ESI-MS disseminated viremia assay (Ibis Biosciences, Carlsbad, CA) was performed in the same manner as other PCR/ESI-MS assays described in the literature [25–30]. In brief, samples were lysed and extracted using an automated total nucleic acid isolation system, followed by PCR amplification [29,30]. Unfragmented amplicons were analyzed by electrospray ionization mass spectrometry (ESI-MS). The resulting mass to charge spectra were converted to estimates of forward and reverse strand masses, which in turn were paired and translated to base compositions (base counts). Base count signatures were compared to known signatures in a curated on-board database to yield analyte identifications.

PCR amplification was performed in 8 wells of a 96-well PCR plate, per Table 1. Each well contained PCR reagents, primers, and a synthetic control template (amplification control) specific for one primer pair in that well. Failure to produce a minimum amount of amplicon from control or target templates was flagged as a well failure and potentially affected assays were specifically noted and repeated as necessary. An isolation control was added to each sample prior to extraction and used to monitor extraction efficiency, and samples for which the extraction control failed were flagged as invalid and repeated. The control consisted of an RNA transcript encapsulated by protein, and was designed to be unique relative to both human and targeted viral sequences. The control was amplified with a dedicated primer pair in the eighth well.

A report was produced for each sample that included the identity of any detected viruses along with a "level" derived from the ratios of target and control amplicon from all primers involved in the reported identification. Control amplicons were produced from synthetic target constructs consisting of nucleotide sequences designed to be amplified by one of the primer pairs in each well, which were included at a known concentration. The amplicons produced from these control sequences were readily discriminated from true viral amplicons by mass (base composition), as the constructs were designed to differ from natural target sequences through introduction of a small deletion. The level was semi-quantitative across a relatively short range of titers from the limit of detection upwards. This allowed approximation of relative concentration within the reportable level range and assignment of cutoffs to limit spurious low-level detections (apparent false positives). The report also provided a "Q score" (a relative measure of confidence) derived from parameters such as the number of primer pairs producing amplicons compared to the number expected, the closeness of match of those products to reference signatures, and consistency of signal amplitudes across multiple primer pairs.

Minimum cutoffs were applied to the level and Q-score for all reportable viruses to ensure that potentially spurious marginal detections from either contamination or spectral noise were not reported. Cutoffs were determined empirically during the course of assay development and testing, and were set at a level of 15 and a Q-score of 0.85 for enterovirus and polyomaviruses BK and JC, and a Q-score of 0.85 with no level cutoff for all other viruses. These cutoffs were chosen to minimize apparently spurious detections in healthy control specimens while maximizing sensitivity among clinical PCR-positive samples.

All analytical studies except clinical specimen agreement studies and carryover used contrived samples consisting of plasma from apparently healthy adults (BioMed Supply Inc., Carlsbad, CA; ProMedDx, Norton, MA) spiked with cultured virus (ZeptoMetrix, Buffalo, NY; the HHV-6 Foundation, Santa Barbara, CA; and ATCC, Manassas, VA). Carryover analysis used Tris–EDTA buffer to avoid background virus occasionally present in plasma from healthy adults. Viral stocks were titered using quantitative PCR (Q-PCR) at ZeptoMetrix or Viracor-IBT Laboratories (Lee's Summit, MO) and maintained at −60 °C or lower. The clinical sample agreement study used clinically accredited quantitative real-time PCR reference lab results (Viracor-IBT) for comparator determinations. Clinical specimens were de-identified waste specimens obtained from infected patients (Viracor-IBT) or healthy adult plasma samples (ProMedDx, Norton, MA). Many samples from infected patients were diluted prior to testing to obtain sufficient volume.

Nucleic acid extraction for PCR/ESI-MS testing was performed using the Abbott *m*Sample Preparation System DNA Kit (Abbott Molecular, Des Plaines, IL) using a protocol designed to capture total nucleic acids. Extractions were performed on an automated system [30,31], and amplification was performed as previously described for other PCR/ESI-MS viral assays [29,30]. Post-PCR amplicon desalting and electrospray ionization mass spectrometry were performed using an automated platform (Ibis Biosciences,

Table 1
Target viruses and primer pairs. All primer pairs with the same well designation are multiplexed in a single well, for a total of 8 wells (A–H).

Primer designation	Well	Primer pair sequences (5′–3′)	Target virus
VIR3398	A	TCTGGAGTTTGACAGCGAATTCGAG TGTTGTAACCGGTGGCGAACTCGGG	HSV-1, HSV-2, VZV
VIR4849	A	TGCCTTTACTTCTAGGGCTGTACGG TAGTTTTGGCACTTGCACGGG	JC polyomavirus, BK polyomavirus
VIR4100	B	TCGCGCCCAGGTAGGC TGGCCCCGGCCTCGTAGTG	CMV
VIR943	B	TTGCAAGATGGCCACCCCATCGAT TGTGGCGCGGGCGAACTGCA	Human adenovirus
VIR3408	C	TGACTTTGCCAGCCTGTACCC TCAGGGTGGAGTAGCACAGGTT	CMV, HHV-8
VIR3118	C	TTACACAAGCCTGGGCAAGTTAGC TCCTGAATCCTTGCAGCACTGTC	Parvovirus
VIR3407	D	TCGTCCCCATCGACATGTAC TACTGTGTCCAGCTTGTAGTCTGA	EBV, HHV-8
VIR4843	D	TGATGGCCCCAACCAAAAGAAAAG TAGTTTTGGCACTTGCACGGG	JC polyomavirus, BK polyomavirus
VIR5155	D	TCGTTCCTGCCCTCACAGATCACG TAGGTCCGGCGACTGGCGTCAGT	Human adenovirus
VIR3405	E	TAAGCAGCAGCTGGCCATCAA TGCCACCCCCGTGAAGCCGTA	HSV-1, EBV, HHV-8
VIR3110	E	TGGGCCGCCAAGTACTGGAAAAAC TGTTTTCATTATTCCAGTTAACCATGCCATA	Parvovirus
VIR3758	F	TTCCTCCGGCCCCTGAATG TGAAACACGGGCACCGAAAGTAGT	Human enterovirus
VIR3760	G	TGGCTGCGTTGGCGGCC TAGCCGCATTCAGGGGCCGGA	Human enterovirus
VIR3400	H	TCCGCGCGGTATAATGCATGATGG TAGAACATACGCGGTTCCGAGTCACAAA	HHV-6, HHV-7
PLN4437	H	TGACGAGTTCATGAGGGCAGGC TCTGGCCTTTCAGCAAGTTTCCAAC	Isolation control

Carlsbad, CA), as previously described [30,31]. Hands-on time was approximately 1–1.5 h per batch for samples run in batches of 12–48, with time being spent primarily on reagent loading, sample set-up, and transfer between automated preparation and analysis platforms. Total time from sample to result was always less than 8 h for sets of 12 samples, and less than 10 h for sets of 48 samples processed together.

Strict separation of PCR set-up areas and amplification/analysis areas (including both personnel/equipment movement and airflow) was employed to prevent backflow of amplicon from the thermocycler, amplicon desalting, and mass spectrometry analysis components, per CLSI guidelines for open PCR processes.

Viral stock quantitation was done per standard q-PCR protocols at ZeptoMetrix (enterovirus inclusivity panel) or Viracor-IBT (other viral stocks). PCR quantitation assumed homogenous primer site target sequences in all strains of a given viral species, however variation in apparent limit of detection (LOD) was expected due to the effects of primer site variability on both the PCR/ESI-MS assay and the reference PCR test used to quantify the strain. A subset of results was excluded, and substitute samples tested, due to secondary detections of unspiked viruses in spiked samples and negative controls, per the study protocol's predefined criteria. All samples yielding unexpected secondary detections were replaced and retested, and matrix (plasma or diluent) lots yielding repeated unexpected detections were replaced and the affected samples were then rebuilt in the new matrix lot and retested. Matrix-associated secondary detections were primarily of parvovirus B19, which occurs sporadically in healthy donor plasma [32].

Table 2
Limits of detection.

Virus	Limit of detection (copies/ml)	Valid detections at LOD
HSV-1	4.0×10^2	19/20
HSV-2	6.0×10^2	20/20
VZV	1.9×10^3	19/20
EBV	1.4×10^3	20/20
CMV	1.5×10^3	19/20
CMV (WHO standard)	6.3×10^3	20/20
HHV-6	9.0×10^2	19/19[a]
HHV-7	3.0×10^2	20/20
HHV-8	1.3×10^3	19/20
BK polyomavirus	6.6×10^3	20/20
JC polyomavirus	5.7×10^3	20/20
Human adenovirus	3.8×10^4	19/19[a]
Human enterovirus	3.0×10^3	19/20
Parvovirus B19	1.2×10^3	20/20

[a] One replicate was determined to be invalid. Given that 19/20 detections satisfy the 95% detection rate for LOD, retesting was not performed.

4. Results

4.1. Limit of detection

LOD was determined by testing pooled plasma spiked with a range of concentrations of virus. The LOD for each virus was defined as the lowest titer tested that produced at least 95% detection across 20 replicates. The LOD for CMV was also tested using the WHO International Standard for Human Cytomegalovirus. Data are shown in Table 2.

Table 3
Interfering Substances and Test Concentrations.

Substance pool	Substances tested[a]
1	Zidovudine, valsartan
2	Abacavir sulfate, peginterferon alfa-2b, ribavirin
3	Tenofir disoproxil fumarate, lamivudine, ganciclovir, valganciclovir HCl, acyclovir
4	Stavudine, efavirenz, lopinavir, ritonavir, enfuvirtide, ciprofloxacin
5	Nevirapine, azithromycin, valacyclovir HCl
6	Adefovir, didanosine, entecavir, cidofovir, mycophenolate mofetil
7	Famotidine, cyclosporine
8	Prednisone, tacrolimus, azathioprine
9	Atenolol, amlodipine besylate, lisinopril, rabeprazole
10	Acetaminophen, abacavir, lamivudine, amikacin, metronidazole
11	Dexamethasone, atanavir sulfate, fluconazole, ceftazidime
12	Dobutamine, emtricitabine, tenofovir, itraconazole
13	Rapamune, fosamprenavir, doxycycline, piperacillin
14	Vinblastine, lamivudine, zidovudine, gentamicin, vancomycin
15	Warfarin, amphotericin B, imipenem, sulfamethoxazole, trimethoprim
16	Bilirubin
17	Gamma globulin
18	Hemoglobin
19	Triglycerides

[a] All substances were tested at concentrations representing either CLSI-recommended test levels or three times their researched peak plasma concentration (C_{max}) [33].

4.2. Reproducibility

Reproducibility was assessed using three lots of sample preparation and PCR reagents run over 5 days by three operators using three machines. Lots and machines were paired with users. Each user/lot/machine performed three analyses of each specimen type on each of the five testing days for a total of 45 replicates of each specimen type. Specimen types (panel members) included one strain of each of the 13 viruses listed in Tables 1 and 2, each of which was tested at two concentrations (3× and 10× LOD). Some viruses were tested in pairs, yielding a total of 16 specimen types. All valid tests were positive, providing a point estimate of 100% reproducibility for each virus.

4.3. Carryover

Positive samples of each target virus were prepared at 100× LOD and processed adjacent to negative samples by alternation of positive and negative samples in a checkerboard pattern in the sample plate. Each of the 190 valid negative samples yielded negative results, providing a point estimate of 0% carryover. The test concentration was chosen on the basis of availability of evenly titered stocks across the range of target viruses, and was not representative of the highest titers seen in clinical specimens. Further testing will be required to address the potential for carryover at higher titers.

4.4. Interference and cross-reactivity

Sixty-four potentially interfering substances combined in 19 pools (shown in Table 3) were tested in plasma, with and without each target virus spiked at 3× LOD, in triplicate. Similarly, 16 yeast, bacteria, and viruses (shown in Table 4) were tested in triplicate for cross-reactivity. None of the substances or cross-reactors tested interfered with the ability of the assay to detect the target viruses, and none generated spurious detections in the absence of a target virus.

Table 4
Cross-reactor test concentrations.

Organism	Minimum concentration tested
Candida albicans	1.0×10^6 copies/ml
Group B streptococcus	1.0×10^6 TCID50/ml
Hepatitis A virus	9.0×10^3 copies/ml
Hepatitis B virus	5.9×10^4 IU/ml
Hepatitis C virus	8.1×10^3 IU/ml
HIV-1	8.9×10^3 copies/ml
HTLV-1	1.0×10^5 copies/ml
Human papillomavirus	9.1×10^4 copies/ml
Human rhinovirus	1.3×10^4 TCID50/ml
Morbillivirus (measles)	1.0×10^5 TCID50/ml
Mumps virus	1.0×10^5 TCID50/ml
Mycobacterium species	1.0×10^6 copies/ml
Staphylococcus aureus	1.0×10^6 copies/ml
Staphylococcus epidermidis	1.0×10^6 copies/ml
Vaccinia virus	1.0×10^5 copies/ml
West Nile virus	1.0×10^5 copies/ml

4.5. Inclusivity

Multiple recognized strains or multiple independent clinical isolates of each target virus were analyzed using the disseminated viremia assay across a range of concentrations. Strains were then retested in triplicate as spikes in human plasma at moderate concentrations (those resulting in assay-reported "levels" of approximately 500 in the initial dilution series). All tested strains were detected and correctly identified in triplicate at the test concentrations shown in Table 5, and no tested levels resulted in incorrect identifications.

4.6. Clinical specimen specificity

Plasma EDTA specimens from 109 apparently healthy adults were analyzed with the PCR/ESI-MS disseminated viremia assay in order to explore the background carriage rate and to estimate the specificity of the assay for clinically relevant infections. The PCR/ESI-MS assay was negative for 107 specimens, one specimen was positive for HHV-6, and one was positive for both EBV and HHV-6. The unresolved specificity of the assay was thereby determined to be approximately 98%. Both positive samples were subjected to resolution PCR testing at an independent reference laboratory (Viracor-IBT). Both HHV-6 detections were confirmed by PCR, whereas the EBV result was not confirmed, offering a resolved specificity estimate of 99% (107/108, with one original sample – the one for which both PCR/ESI-MS and reference laboratory results were positive for HHV-6 only – excluded as a true positive).

4.7. Clinical specimen sensitivity

Sensitivity was evaluated by testing 158 plasma EDTA waste specimens from symptomatic patients which had previously tested positive by clinically accredited real-time PCR (Viracor-IBT) for one of the viruses targeted by the disseminated viremia assay. Technicians performing PCR/ESI-MS were blind to the associated PCR test results. Each of these samples had been tested for only one of the 13 targeted viruses, as ordered by the requesting health care providers. In most cases, insufficient sample volume was available to perform PCR/ESI-MS testing and comparator testing, hence these samples were diluted to approximately 2.5 ml to provide sufficient volume. Due to extended storage time and the dilution, all samples (158) were retested by Viracor-IBT for the initially detected viruses, and only those confirmed positive upon retesting (156/158, 98.5%) were used in this study. No HHV-7 or HHV-8 positive specimens were obtained. Of the 156 included samples, 147 yielded positive PCR/ESI-MS results matching those of the Viracor-IBT PCR test

Table 5
Viral strains tested as spikes in donor plasma.

Virus (as reported by PCR/ESI-MS)	Strain or unique identifier	Tested titer (copies/ml)
Herpes simplex virus 1 (HHV-1)	HF	5.0×10^2
	KOS	1.0×10^3
	MacIntyre	5.0×10^2
	ATCC # VR-1383	5.0×10^2
Herpes simplex virus 2 (HHV-2)	G	5.0×10^2
	MS	1.0×10^3
Varicella-zoster virus (HHV-3)	AV92-3:L	5.0×10^3
	Zeptometrix #306640	1.0×10^4
	Oka	1.0×10^5
Epstein–Barr virus (HHV-4)	B95-8	1.0×10^5
	HR 1	1.0×10^5
Cytomegalovirus (HHV-5)	AD-169	5.0×10^5
	Davis	5.0×10^5
	Merlin	1.0×10^5
Roseolovirus (HHV-6)	6A HSB I	4.2×10^3
	6A U1102	8.0×10^3
	6A WA	8.0×10^3
	6A HSB2 CO1	2.6×10^3
	6A HSB2 GS	1.1×10^4
	6A CBMC SIE	2.0×10^3
	6B 400	1.7×10^2
	6B CBMC MAR	1.7×10^3
	6B MT4 HST	1.8×10^2
Roseolovirus (HHV-7)	Supt-1 J1	9.3×10^2
	SATO	1.3×10^1
	H7-4 (Isolated DNA)	1.2×10^4
Kaposi's sarcoma-associated herpesvirus (HHV-8)	Zeptometrix # 305247	1.0×10^5
	B0704	5.0×10^4
BK polyomavirus	ATCC # VR-837	1.0×10^4
	Zeptometrix # 306685	1.0×10^4
JC polyomavirus	MAD-1	5.0×10^4
	MAD-4	1.0×10^5
Human adenovirus	Type 1	5.0×10^5
	Type 3	1.0×10^5
	Type 4	5.0×10^5
	Type 5	5.0×10^5
	Type 7A	5.0×10^5
	Type 8	5.0×10^5
	Type 10	5.0×10^5
	Type 12	1.0×10^5
	Type 14	1.0×10^5
	Type 18	1.0×10^4
	Type 31	1.0×10^6
	Type 40	1.0×10^5
	Type 41	1.0×10^4
Human enterovirus	CV-A1	5.0×10^5
	CV-A2	5.0×10^5
	CV-A4	5.0×10^5
	CV-A8	5.0×10^5
	CV-A9	1.0×10^4
	CV-A10	2.3×10^4
	CV-A16	1.0×10^6
	CV-A17	1.0×10^6
	CV-A21	2.5×10^4
	CV-B3	1.0×10^4
	CV-B4	5.0×10^2
	CV-B5	5.0×10^5
	E-6	1.0×10^3
	E-11	4.0×10^4
	EV-68	5.0×10^4
	EV-69	5.0×10^5
	EV-70	5.0×10^5
	EV-71	1.0×10^3
	PV-3	5.7×10^3
Parvovirus	B19 Zeptometrix # 306652[a]	1.0×10^4
	B19 Zeptometrix # 306879[a]	5.0×10^4
	B19 Zeptometrix # 306880[a]	5.0×10^4
	B19 Zeptometrix # 306881[a]	5.0×10^4

[a] A positive clinical sample was used in lieu of cultured virus, if cultured virus was unavailable.

results, generating a point estimate for sensitivity of 94%. The samples yielding false negative results by PCR/ESI-MS tended toward the low end of the titer range of the tested clinical specimens, with the exception of one HSV-1 false negative (the remaining 8 false negatives averaged approximately 2×10^3 copies/ml, as compared to an average of 2.5×10^6 for all clinical positive qPCR results). The noted HSV-1 false negative was an extremely high positive sample (>10^8 copies per ml by qPCR) which was reported as a

Table 6
Sensitivity of PCR/ESI-MS assay evaluated using samples from symptomatic patients.

Virus ID by PCR[a]	Number of specimens positive by initial PCR (Q-PCR titer range of tested dilution, in copies/ml)	PCR/ESI-MS positive matched to initial PCR result	Additional PCR/ESI-MS positives among samples previously untested for the indicated virus (# confirmed by PCR)[b]
HSV-1	11 (1×10^2 to $>10^8$)	10	1 (0)
HSV-2	3 (1×10^5 to $>10^8$)	3	2 (0)
VZV	9 (8×10^2 to $>10^8$)	9	2 (0)
EBV	12 (5×10^2 to 4×10^5)	9	13 (6)
CMV	17 (1×10^3 to 1×10^5	17	5 (2)
HHV-6	25 (3×10^2 to 5×10^4)	24	0
HHV-7	0	0	0
HHV-8	0	0	3 (0)
BK polyomavirus	26 (9×10^2 to 4×10^6)	26	8 (7)
JC polyomavirus	7 (1×10^3 to 9×10^4)	6	1 (1)
Human adenovirus	23 (2×10^2 to 3×10^6)	23	2 (1)
Human enterovirus	2 (8×10^2 to 9×10^2)	2	0
Parvovirus B19	21 (3×10^2 to 3×10^5)	18	0
Total	156	147	37 (17)

[a] Virus identified from clinical sample in Viracor-IBT PCR test.
[b] Some confirmations could not be performed due to insufficient sample volume.

high-level HSV-2 detection by PCR/ESI-MS. The PCR/ESI-MS signature clearly matched HSV-2 rather than HSV-1. Resolution of this apparent misidentification (on the part of either PCR/ESI-MS or qPCR) was not pursued as such resolution was not defined in the clinical sample study protocol. As shown in Table 6, the PCR/ESI-MS also yielded 37 additional detections for viruses for which Viracor-IBT had not performed clinical testing. In cases where sample volumes permitted further testing, the samples yielding these extra detections were sent back to Viracor-IBT for confirmatory testing for the extra viruses. Seventeen (46%) of these 37 extra detections were confirmed by PCR.

4.8. Other characterizations

Using plasma spiked with approximately 3× LOD of each targeted virus, it was demonstrated that samples could be stored at 15–30 °C for up to 16 h, at 2–8 °C for up to 3 days, or at −20 to −80 °C for up to one month without compromising detection of virus by the PCR/ESI-MS assay. Throughout the course of the studies described here, approximately 1% of samples were invalidated and repeated as a result of isolation control failures, and approximately 1% were invalidated and repeated on the basis of well failure criteria.

5. Discussion

This paper presents the initial analytical characterization of a PCR/ESI-MS assay designed to detect and identify agents of disseminated viral infection seen in immunocompromised patients. The study was limited to plasma sample analysis, and clinical specimen analysis was limited to retrospective testing of waste specimens associated with clinical PCR results but without clinical diagnostic and treatment data.

The assay appears to be sufficiently sensitive to identify the targeted viruses in plasma at titers associated with symptomatic infection. Analysis of samples from healthy adults suggests that the assay is not so sensitive that viruses will be commonly detected in asymptomatic individuals. Highly sensitive and quantitative RT-PCR assays are available for monitoring very low (carrier-state) levels of some of the viruses targeted by the assay described here (cytomegalovirus is an example) [34] for purposes of predicting reactivation-associated dissemination, and for monitoring changes in viral load in response to treatment. The PCR/ESI-MS assay described here is not intended for such purposes.

The unique value of the PCR/ESI-MS disseminated viremia assay lies in its ability to simultaneously detect and identify clinically relevant titers of many viral species commonly associated with disseminated infection in immunocompromised patients. Currently, providers generally order tests for only one or a small subset of the 13 viruses targeted by the PCR/ESI-MS assay, requiring subjective determination of the virus identity prior to testing. Although many of these viruses are associated with specific symptoms, atypical presentations are possible and the presence of one disseminated virus may mask the presence of others. In such cases, only a broad spectrum test can offer the information needed to define appropriate treatment.

The broadly targeted primers and permissive PCR conditions employed in the disseminated viremia assay and detection of most viruses by multiple primer pairs limits the chances that mutations or polymorphisms will cause assay failure and result in false negative results [26,35]. Permissive PCR may be more prone to spurious amplification of host sequences or untargeted biological entities, but the risk of such reactions leading to false positive detection and identification by ESI/MS is mitigated by the requirement that amplicons match sequence-derived base composition signatures.

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Conflict of interest

This work described was performed and the manuscript was written by the employees of Ibis Biosciences, a Division of Abbott, the maker of the device described herein.

Ethical approval

Human plasma used as matrix or as healthy patient control samples was obtained from qualified contract research organization (CRO) vendors (BioMed Supply Inc., Carlsbad, CA; ProMedDx, Norton, MA), as were positive clinical plasma samples (obtained as deidentified waste specimens from Viracor-IBT Laboratories, Lee's Summit, MO). These samples were procured under the approval of the CRO's Institutional Review Boards (IRBs) and used under the approval of Ibis Bioscience's protocol Pro00006845 by Chesapeake Research Review's IRB.

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