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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,

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