Characterization of a Novel Melt Curve by Use of the Roche LightCycler HSV 1/2 Analyte-Specific Reagent Real-Time PCR Assay: Frequencies of This Novel (Low) Melt Curve and Commonly Encountered (Intermediate) Melt Curves

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We characterize a novel probe binding-site polymorphism detectable solely by melt curve analysis using the Roche LightCycler HSV 1/2 analyte-specific reagent real-time PCR assay. The frequencies of this novel (47°C) and previously described intermediate (60 to 62°C) melt curves were 0.016% and 4.9%, respectively.

The clinical spectrum of herpes simplex virus (HSV-1, HSV-2) infection ranges from subclinical mucosal shedding to vesicular or ulcerative lesions of skin and mucous membranes, hepatitis, keratitis, pneumonitis, sepsis, and meningoencephalitis (1). For laboratory diagnosis, laborious cell culture techniques have been largely supplanted with real-time PCR (qPCR) due to marked improvements in test sensitivity and turnaround time. Various laboratory-developed and commercially available qPCR products (analyte-specific reagents [ASR]; FDA approved) exist. In the United States, the LightCycler HSV 1/2 ASR real-time PCR assay (HSV qPCR; Roche Diagnostics, Indianapolis, IN) is commonplace, being used by about 30% of clinical laboratories according to a recent College of American Pathologists (CAP) participant summary (2). HSV qPCR uses fluorescence resonance energy transfer (FRET) hybridization probes for detection and melt curve-based genotyping, whereby predictable polymorphisms within the amplicon yield expected melt curves for HSV-1 (~55°C) and HSV-2 (~68°C). Occasionally, probe binding-site polymorphisms yield “intermediate” melt curves (60 to 62°C); these have been described previously (3–6). Herein, we describe a novel, previously uncharacterized probe binding-site polymorphism detectable solely by melt curve analysis (~47°C). The frequencies of this novel and previously described intermediate melt curves are described.

A 24-year-old female was evaluated for vaginal discomfort of 5 days' duration; vesicular lesions were noted on her labia majora. Clinical material was submitted in M4-RT viral transport medium (Remel, Lenexa, KS) to Beaumont Laboratory. HSV qPCR was performed using the LightCycler 1.2 (Roche Diagnostics, Indianapolis, IN). In brief, 5 µl of LightCycler HSV 1/2 recovery template (Roche Diagnostics, Indianapolis, IN) was added to 200 µl of patient specimen to serve as an extraction/inhibition control and then extracted on the NucliSENS easyMag automated system (bioMérieux, Durham, NC) according to the manufacturer's instructions. Amplification and detection of a 215-bp fragment of the DNA polymerase (*pol*) gene of HSV-1 (GenBank accession no. [X03181](#)) and HSV-2 (GenBank accession no. [M16321](#)) utilized Roche analyte-specific reagents. The reaction master mix included 2 µl of HSV primer pair containing 7 µM primer 1 (5'-GCT CGA GTG CGA AAA AAC GTT C-3') and 7 µM primer 2 (5'-CGG GGC GCT CGG CTA AC-3'), 2 µl of 2 µM HSV probe 1 (5'-

GCG CAC CAG ATC CAC GCC CTT GAT GAG C-fluorescein-3'), 2 µl of 4 µM HSV probe 2 (5'-LC-Red-640-CTT GCC TCC GCA GAT GAC GCC-3'), 2 µl of 4 µM HSV probe C-3 (5'-LC-Red-705-GAA CGT GAF CGT CTA CTG CGG-3'), 2.4 µl of 25 mM MgCl₂, 0.25 µl of a 2-U/µl concentration of uracil DNA glycosylase (UNG) and 2 µl of 10× LightCycler DNA Master Hybprobe mix combined with 2.35 µl DNase-free H₂O for a final volume of 15 µl. The extracted patient specimen (5 µl) was added to the master mix for a total reaction volume of 20 µl. After an initial UNG treatment (40°C for 10 min, 95°C for 10 min), amplification was performed for 45 cycles (95°C for 10 s, 55°C for 15 s, 72°C for 15 s), followed by melt curve analysis (59°C for 20 s, decrease to 40°C at a rate of 20°C/s, hold at 40°C for 20 s, increase to 80°C at a rate of 0.2°C/s). Before patient results were reported, PCR amplification results and melt curves for each specimen were reviewed independently by two medical technologists. The ELVIS HSV rapid shell vial culture/D³ DFA HSV identification kit test (Diagnostic Hybrids/Quidel Corporation, Athens, OH) was performed according to the manufacturer's instructions. Conventional cell culture (A549/MRC-5 cell lines; 2% fetal bovine Eagle's minimal essential refeed medium containing gentamicin, penicillin, streptomycin, and amphotericin B) was incubated in a roller drum (35 to 37°C, non-CO₂) until a cytopathic effect was observed. Cell monolayers were then scraped and affixed to glass slides for identification using the MicroTrak HSV 1/HSV 2 culture identification/typing test (Trinity Biotech, Bray, Co., Wicklow, Ireland). DNA sequencing was performed at the Molecular Biology Core facility at Mayo Clinic (Rochester, MN). In brief, the isolate was amplified using the previously described HSV qPCR primer/probe sequences. After amplification, the following were added to two 0.2-ml tubes per sample: 4 µl of amplified product,

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FIG 1 HSV qPCR amplification (top panel) and melt curve (bottom panel) analysis. Curve A in the top panel, positive control containing both HSV-1 and HSV-2. HSV genotyping, based upon differences in FRET probe annealing kinetics with amplified target DNA, yields distinct and predictable melt curves for HSV-1 (~55°C; curve A1 in bottom panel) and HSV-2 (~68°C; curve A2 in bottom panel); curves B, previously described variant of HSV-2 yielding an intermediate melt curve (~62°C); curves C, previously described variant of HSV-1 yielding an intermediate melt curve (~60°C); curves D, newly described variant of HSV-1 yielding the novel melt curve (~47°C). Note the absence of a PCR amplification curve (top panel); E, negative control.

1 μ l of shrimp alkaline phosphatase (SAP; USB product no. 70092Z), and 1 μ l of exonuclease 1 (Exo 1; USB product no. 70073X) or 2 μ l ExoSAP-IT (USB product no. 78201). After a quick spin, the tubes were placed in a thermocycler using the following conditions: 37°C for 15 min and 80°C for 15 min. After a quick spin, 1 μ l of 1.6 μ M primer (1:30 dilution of the 50 μ M stock) was added to each tube—one tube for forward primer and one tube for reverse primer. The treated samples underwent fluorescently labeled dideoxynucleotide chain termination-based sequencing using an ABI 3730 XL DNA analyzer with ABI Sequence Analysis software v5.2 (Life Technologies, Foster City, CA).

HSV qPCR results for the patient specimen were uniquely positive, being detected solely by melt curve analysis (Fig. 1). HSV-1 was cultivated and identified by both cell culture methods, and HSV qPCR results on the cultivated isolate confirmed the novel 47°C melt curve. Sequence analysis revealed a novel probe binding-site polymorphism (Fig. 2). This polymorphism precluded

FIG 2 DNA sequence alignment within FRET probe annealing region. The FRET probes are designed to have identical sequence alignment with HSV-2. The presence of polymorphisms within the HSV polymerase gene allows for genotypic distinction of HSV-1 from HSV-2. Less commonly encountered polymorphisms will lead to intermediate melt curves for HSV-1 or HSV-2. HSV-1, HSV-1 control (~55°C), with two polymorphisms (positions 34 and 41); HSV-2, HSV-2 control (~68°C), with no polymorphisms; Patient, novel variant (~47°C), with two polymorphisms (positions 41 and 43); HSV-1 Int, HSV-1 intermediate melt curve (~60°C), with one polymorphism (position 41); HSV-2 Int, HSV-2 intermediate melt curve (~62°C), with three polymorphisms (positions 10, 13, and 22).

FRET probe annealing during the elevated temperatures ($\geq 55^{\circ}\text{C}$) of iterative thermocycling but facilitated annealing at the lower temperatures encountered during the initial phases of melt curve analysis. A retrospective review of 22,609 HSV qPCR results from the Beaumont Laboratory (October 2003 to May 2013) revealed a total of 6,176 HSV-positive results. The frequencies of expected HSV-1 ($\sim 55^{\circ}\text{C}$), expected HSV-2 ($\sim 68^{\circ}\text{C}$), HSV-intermediate (60 to 62°C), and the novel (47°C) melt curves were 49.7% (3,070/6,176 positive results), 45.4% (2,800/6,176), 4.9% (305/6,176), and 0.016% (1/6,176), respectively. Our observed frequency of HSV-intermediate (60°C to 62°C) melt curves was identical to the 5% frequency reported by Issa et al. (6) The vast majority of these retrospective specimens were from mucocutaneous sites (i.e., lip, mouth, eye, genitalia) from equal proportions of male and female patients (2 months to 85 years). Patient age, gender, and site of specimen collection for the retrospective specimens were not associated with the incidence of encountering expected or intermediate melt curves. Age, gender, and site of specimen collection from the patient harboring the unique HSV-1 strain were similar to those of the retrospective patient specimens. The fitness of this unique HSV-1 strain did not appear to be compromised, as it was cultivated as readily as other HSV-1 and HSV-2 strains producing expected and intermediate melt curves (data not shown). Whether or not this unique HSV-1 strain alters the lower limit of detection (LLOD) of the Roche HSV qPCR assay is undetermined. Studies to determine this would require special cell culture techniques (i.e., 50% tissue culture infective dose [TCID₅₀] method), which is beyond the scope of this report. However, the unique polymorphism described herein is isolated to the FRET probe binding region. In theory, qPCR assay kinetics should proceed uninhibited with minimal impact on the LLOD as long as the medical technologist remembers to review the melt curve.

The origin of this unique HSV-1 strain is unknown, but it is likely to have arisen from random mutations within the HSV-1 genome that were undetected by internal proofreading/repair mechanisms. It is also possible that it evolved from HSV therapy-derived selective pressure, since the relevant therapeutic agents (i.e., acyclovir, valacyclovir) target the HSV thymidine kinase and DNA polymerase genes. The 47°C melt curve described herein is

extremely rare but is noteworthy as it highlights several important points. First, as listed in a recent CAP participant summary, about 30% of participants utilized the Roche HSV qPCR assay, increasing the likelihood that this novel polymorphism has been or will be encountered by others (2). Second, the importance of appropriate qPCR design and test interpretation is paramount. FRET probe-based qPCR assays should always be accompanied by melt curve analysis, as clinical laboratory personnel may fail to review melting curves in the absence of a visual amplification curve. Of note, if the Roche HSV qPCR assay utilized a TaqMan probe, the patient specimen would have been falsely reported as negative since TaqMan probes, which by design cannot undergo melt curve analysis, would be unable to anneal during iterative thermocycling.

In conclusion, the Roche HSV qPCR assay yields robust detection and genotype results in the vast majority of cases. Intermediate melt curves are uncommon, and polymorphisms conferring novel melt curves are rare. When variant melt curves are encountered, genotyping can be performed by DNA sequencing, cell culture techniques, or alternative qPCR assays.

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