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Differentiation of *Variola major* and *Variola minor* variants by MGB-Eclipse probe melt curves and genotyping analysis

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ABSTRACT

Smallpox, caused by the *Variola major* virus, is considered to be one of the most lethal of all potential biological weapons and has far-reaching consequences. Real-time polymerase chain reaction (PCR) assays are available as a reliable diagnostic tool to detect members of the genus *Orthopoxvirus*. In addition real-time PCR assays specific for the variola virus have been developed that distinguish it from other orthopoxviruses. However, a positive identification of variola spp. does not classify the virus as the one that causes smallpox (*V. major*) or as the variant (*Variola minor*) that causes a much less severe form of the disease. This study reports the development of a real-time PCR minor groove binder (MGB)-Eclipse probe assay utilizing a sequence within the variola B9R/B10R gene complex that reliably differentiates *V. major* from *V. minor* by specific probe melting temperatures (T_ms) and genotyping analysis. The MGB-Eclipse probe assay is an important step beyond the standard TaqMan-MGB assay and we feel this is a significant addition to our current variola species identification algorithm with TaqMan-MGB assays that target the B9R and B10R genes. The probe T_ms for *V. major* and *V. minor* were 62.71 (±0.05) and 53.97 (±0.44) °C, respectively ($P = <0.001$). We also used the identical sequence to develop a TaqMan®-MGB assay that specifically detected *V. minor* but not *V. major* variants by qualitative analysis.

1. Introduction

Smallpox is a devastating illness caused by the *Orthopoxvirus Variola major*. There is no known cure or effective treatment for this highly contagious, disfiguring disease once symptoms develop. Prevention is only by isolation of infected persons and vaccination (http://www.mayoclinic.com/health/smallpox/DS00424). A vigorous campaign to eradicate smallpox by vaccination was successful with the last naturally occurring case recorded in 1977. In 1980 the World Health Organization (WHO) officially announced that smallpox had been eliminated worldwide. Stocks of viable virus are kept for research in vaccine and antiviral drug development, and diagnostic identification at two known locations: the Centers for Disease Control and Prevention (CDC) in Atlanta, GA and the State Research Center of Virology and Biotechnology, Novosibirsk, Russia. There are significant concerns that undeclared stocks of variola may be obtained by terrorists or rogue governments and that the relatively hardy double-stranded DNA virus, easily transmitted by aerosol, could be used as a biological agent. A deliberate release of smallpox virus would be devastating to today's highly susceptible, mobile population as well as generating widespread panic [1]. It is not surprising that today's physicians may not immediately recognize the signs and symptoms of clinical smallpox because it has been unseen for decades. If smallpox is suspected, PCR assays are available that provide rapid and definitive laboratory diagnosis to specifically differentiate variola virus from other orthopoxviruses that cause diseases with similar symptoms (rash and fever) [2–5]. However, the two principle variants of variola virus (*V. major* and *Variola minor*) differ greatly in severity with mortality rates of approximately 30% and 1% respectively (WHO fact sheet on smallpox, 2001). In this study, we developed a method to identify whether a variola virus is *V. major*, the causative agent for smallpox, or *V. minor* which causes a much milder disease known as alastrim or kaffir pox. During the development of real-time PCR variola-specific assays, a non-coding sequence located between the B9R and B10R genes of *V. major* and *V. minor* was identified. This intervening sequence (IS2) region is approximately 281 base pairs (bp) long in *V. major* and approximately 908 bp long in *V. minor*. The difference in size is due to a sequence of approximately 627 bp in *V. minor* that is not present in *V. major*. All *V. major* and *V. minor* genomes sequenced thus far include some portion of the IS2 sequence [6,7]. In order to differentiate the variants, we designed a real-time PCR assay

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14. ABSTRACT

Smallpox, caused by the Variola major virus, is considered to be one of the most lethal of all potential biological weapons and has far-reaching consequences. Real-time polymerase chain reactions (PCR) assays are available as a reliable diagnostic tool to detect members of the genus Orthopoxvirus. In addition real-time PCR assays specific for variola virus have been developed that distinguish it from other orthopoxviruses. However, a positive identification of variola spp. does not classify the virus as the one that causes smallpox (V. major) or as the variant (V. minor) that causes a much less severe form of the disease. This study reports the development of a real-time PCR minor groove binder (MGB) -Eclipse probe assay utilizing a unique sequence within the variola B9R/B10R gene complex that reliably differentiates V. major from V. minor by specific probe melting temperatures (Tms) and genotyping analysis. The probe Tms for V. major and V. minor were 62.71 (+/- 0.05) and 53.97 (+/- 0.44) °C, respectively (P = <0.001). We also used the identical sequence to develop a TaqMan®-MGB assay that specifically detected V. minor but not V. major variants by qualitative analysis.

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using MGB-Eclipse probe chemistry. The MGB-Eclipse probe is designed with a MGBDQ (minor groove binder-dark quencher) attached to the 5′ end and a FAM fluorescent reporter dye on the 3′ end [8]. This arrangement allows for the FAM fluorescence to be read during the amplification cycles but because the MGBDQ blocks exonuclease digestion of the probe, a probe T_m can be determined after the real-time PCR reaction is completed [9,10]. The difference in T_ms of *V. major* and *V. minor* is significant enough to distinguish each variant by its characteristic melting profile which allows the LC melting curve genotyping software to group samples with similar melting curves together and recognize each group as a genotype. We used this technology to make an objective identification of the specific variant. In addition to the differential assay, a TaqMan®-MGB assay specific for *V. minor* was developed. The target for this assay was the region of the IS2 in *V. minor* not present in *V. major*. Very importantly, by designing all the variola spp. assays within the B9R/B10R gene complex containing the IS2 sequence, a single cloned piece of variola DNA could be used as the positive control for all four assays.

2. Materials and methods

2.1. Target gene

The genome sequence targeted by this assay, *V. major*/*minor* IS2, is located within a non-coding region between the B9R and B10R genes of both *V. major* and *V. minor* strains. The complete genome sequence of *V. major* strain Congo 1970 (GenBank accession no. DQ437583) is 186,553 bp with the IS2 target sequence positioned at approximately nucleotide (nt) 161,893 (end of B9R gene) to nt 162,173 (the beginning of B10R gene) for a total length of 281 bp. The IS2 target sequence within the 188,062 bp of *V. minor* strain Brazil 1966 (GenBank accession no. DQ441419) is located at approximately nt 162,491 (end of B9R gene) to nt 163,398 (beginning of the B10R gene) for a total length of 908 bp. These sequences were used as targets for designing primers and probes for the specific MGB-Eclipse probe and TaqMan®-MGB assays.

2.2. Cloning and sequencing of variola strains

A fragment of *V. major* virus and *V. minor* virus containing the B9R/B10R genes, as well as the intervening non-coding sequences, were PCR amplified from the appropriate viral genomic DNA by using the forward primer 5′-ATG GAC ATT TCT TAT GTT ATT AAT G-3′ and reverse primer 5′-TCA AAA CGT GTA TCT CAT ATA TAC T-3′. The B9R/B10R genes of Bangladesh 1971 (V75-550), India 7124, Harper, Afghan Variolator 4, Congo 1970 (V70-46), Somalia, Minnesota 124, Garcia, and Brazil (V66-39) were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. All B9R/B10R plasmid cloning took place at the CDC. Clones were then transported to USAMRIID where we have permission to have possession of partial variola virus genes. The clones were grown up and the DNA extracted. Plasmid DNA was isolated from bacterial cultures by using a commercially available kit (Qiagen, Valencia, CA) and measured by optical density at 260 nm. The pCR2.1 clones containing B9R/B10R inserts were sequenced on an ABI Prism 3100 genetic analyzer (Foster City, CA).

2.3. Real-time PCR

2.3.1. Assay design

The specific primer, MGB-Eclipse and TaqMan®-MGB probe sequences were designed using Primer Express version 2.0 for Windows (Applied Biosystems, Foster City, CA). Software default parameters were used for primer and probe design. All available *V. major* and *V. minor* sequences were downloaded and the sequences were aligned and assembled using ClustalW2 (http://www.ebi.ac.uk/clustalw/index.html). The primer/probe sets selected represent the lowest scores and are located in regions that are highly homologous to the species. The program was then allowed to choose primer/probe sets. The primer/probe sets with the lowest penalty scores and optimal primer and amplicon lengths (<160 bp) were chosen. A lower penalty score indicated a primer/amplicon that satisfies a greater percentage of the parameters contained in the parameters page. Penalty scores were not absolute and were used only as a guide.

2.3.2. MGB-Eclipse primers and probe for V. major and V. minor

The *V. major* (Congo 1970) 26 bp forward primer F279 (nt 161,941–161,966) is located in the non-coding region between the B9R and B10R genes and the 25 bp reverse primer R400 (nt 162,062–162,038) is located within the B10R gene. For *V. minor* (Brazil 1966) the F279 primer (nt 162,539–162,564) is located within the non-coding region between the B9R and the B10R genes (nt 163,228–163,398) and the R400 primer (nt 163,287–163,264) is located within the B10R gene. The 20 bp MGB-Eclipse probe was located from nt 161,984 to 161,993 in *V. major*. In *V. minor* 16 bp of the probe was complimentary to nt 162,582–162,597 with the remaining 4 bp complimentary to nt 163,236–163,239 (Table 1).

2.3.3. TaqMan®-MGB primer and probe for V. minor

The genomic DNA used for the design of the *V. minor* TaqMan®-MGB assay was Brazil 1966 (GenBank accession no. DQ41419). The primer and TaqMan®-MGB probe sequences were designed as described in Section 2.3.1 within the insertion sequence of *V. minor*. The forward primer (F419) was from nt 163,025 to 163,051, the reverse primer (R521) was from nt 163,127 to 163,105. The TaqMan®-MGB probe (p455S) was from nt 163,061 to 163,077 (Table 1).

2.3.4. PCR reaction conditions

The cloned B9R/B10R gene fragments from *V. major* (Bangladesh) and *V. minor* (Garcia) were used for primer and probe down-selection and optimization of the MGB-Eclipse assay. *V. minor* (Garcia) was used for the TaqMan®-MGB assay. The Roche LightCycler (LC) 1.5 (Roche Diagnostics, Indianapolis, IN) was used for testing at the CDC with the resulting data imported into the LC 4.0 software for analysis. All other real-time PCR amplifications were performed using the LC 2.0. All TaqMan®-MGB, and MGB-Eclipse assays were performed in 20 μL volumes with 15 μL of master mix (MM) and 5 μL sample added. The MMs were made up in PCR buffer (50 mM Tris, pH 8.3; 25 μg/mL of bovine serum albumin [BSA]) and 0.2 mM dNTP mix (Idaho Technology, Salt Lake City, UT). The probe concentrations were 0.1 μM. The final primer concentrations for the MGB-Eclipse probe assay were 0.1 μM for the forward primer and 0.5 μM for the reverse primer. The primer

Table 1
Primer and probe sequences for *Variola assays*.

Name	Length	Size (bp)	Sequence
V. major/*V. minor* primer and probe sequences			
F279	26		5′-CAAAAACTCCAACgAgATAAAAgAgA-3′
R400	24	122	5′-gTTTgTTgTACCCTgCATTTTgAT-3′
p331-ECL[a]	20		5′-BHQ[c] – CTgTCTACATAAgAATCAAg - 6FAM-3′
V. minor-specific primer and probe sequences			
F419	27		5′-AAAgAgTCgATgATAATCCATATAgCA-3′
R521	21	103	5′-CCAgCgACTggATTTTCTgAA-3′
p455sMGB[b]	17		5′-BHQ[c] – CTgTCTACATAAgAATCAAg - 6FAM-3′

[a] MGB-Eclipse probe.
[b] TaqMan-MGB probe.
[c] BHQ (black hole quencher).

concentrations were asymmetric in order to amplify more copies of the strand complementary to the probe. The TaqMan®-MGB assay primer concentrations were 0.5 μM for both forward and reverse primers. Thermal cycling in LCs 1.5 and 2.0 was performed as follows: one cycle at 95 °C for 2 min, followed by 45 cycles of 95 °C for 1 s, and 60 °C for 20 s. A fluorescence reading was taken at the end of each 60 °C step. For the MGB-Eclipse probe assays, a melt curve was generated at the end of the 45 amplification cycles: 95 °C for 1 s, 45 °C for 20 s, followed by an increase to 90 °C at a linear transition rate of 0.1 °C/s. A continuous read was made at wavelength 530 during the 0.1 °C/s ramping. The melt curve data were analyzed using the LC 4.0 software T_ms calling and genotyping analysis modules. TaqMan®-MGB results were analyzed with the qualitative detection module.

3. Results

3.1. Primer and probe selection

The primers and probes for the IS2 assays were initially developed using cloned-target DNA from *V. major* strain Bangladesh 1975 (V75-550) and *V. minor* strain Garcia. (NOTE: the IS2 sequence was part of the DNA that was cloned during the development of the B9R and B10R variola-specific assays.) Primer candidates were used to amplify 1 pg of target template in the presence of SYBR green dye. Melt curves and agarose gels were analyzed to eliminate inefficient and/or dimer producing primer pairs. Forward primer F279 and reverse primer R400 met the criteria for the IS2 major and minor MGB-Eclipse probe assay with F419 and R521 selected for the IS2 minor TaqMan®-MGB assay.

3.2. IS2 MGB-Eclipse assay

3.2.1. Melt curve analysis

During assay development the *V. major* (Bangladesh) and *V. minor* (Garcia) specific MGB-Eclipse probes demonstrated an average T_m of 62.55 °C for *V. major* and 54.26 °C for *V. minor* with a ΔT_m of 8.29 °C (data not shown). Subsequently, all six *V. major* clones and three *V. minor* clones available to us were tested in triplicate (Fig. 1) with mean T_ms of 62.71 (±0.05) and 53.97 (±0.44) °C respectively (Table 2). Satterthwaite's T test for unequal variances demonstrated that the *V. major* T_ms differed significantly from *V. minor* T_ms; $P = <0.001$.

3.2.2. Genotype determination

The LC genotyping analysis software automatically called the genotypes by grouping T_ms of similar samples. The MGB-Eclipse probe assay discriminated between the melting temperatures of the amplicons of the *V. major* and *V. minor* isolates and these were automatically grouped into two distinct genotypes; one for each variant (Table 2).

3.3. IS2 TaqMan®-MGB

The TaqMan®-MGB probe designed within the insertion sequence for *V. minor* was run with the selected primer pairs on the LC 2.0 instrument. Data were analyzed by the LC 4 software using the qualitative detection module. No *V. major* clonal isolates (only *V. minor* strains) were detected by this assay (data not shown).

3.4. Testing of pox DNAs

In addition to testing the B9R/B10R clones in our laboratory, the *V. major*/*V. minor* MGB-Eclipse probe and *V. minor* TaqMan®-MGB assays were evaluated at the CDC by testing them against a blind panel consisting of both various concentrations of orthopox genomic DNAs and DNAs of organisms that are closely related to orthopoxes based on genetic analysis. Fig. 2 shows the LC real-time PCR results for that experiment. The assay was able to differentiate

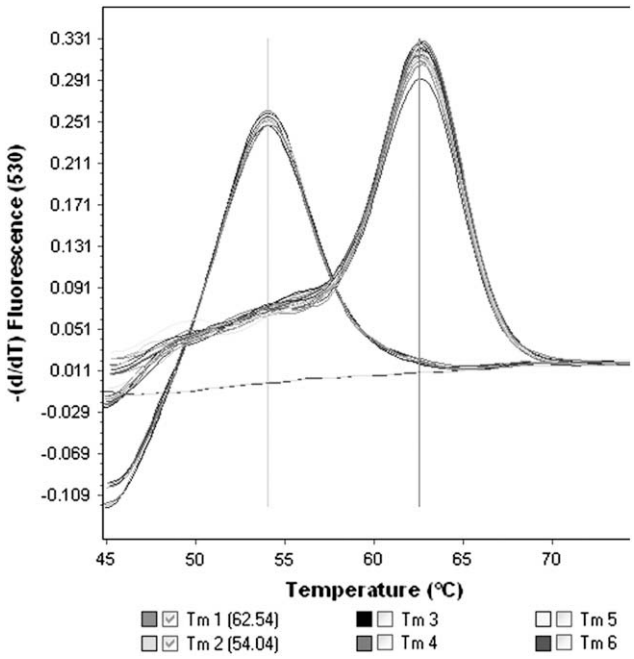

Fig. 1. Melting peaks of cloned *Variola* DNA by IS2 Eclipse probe assay. DNA from 10 isolates was run in triplicate. The T_m for *V. minor* is 53.97 °C ± 0.44 and the T_m for *V. major* is 62.71 °C ± 0.055 ($P = <0.001$).

Table 2
Cloned DNA of *Variola* strains with the resulting T_ms and genotypes.

Sample #	Strain	T_m/°C	Genotype[a]
1	rNTC[b]		Negative
2	sNTC[c]		Negative
3	BSH	62.92	Major
4	Repl. of BSH	62.95	Major
5	Repl. of BSH	62.90	Major
6	Brazil	54.97	Minor
7	Repl. of Brazil	55.11	Minor
8	Repl. of Brazil	54.30	Minor
9	Congo	62.95	Major
10	Repl. of Congo	62.94	Major
11	Repl. of Congo	62.86	Major
12	Harper	62.97	Major
13	Repl. of Harper	62.89	Major
14	Repl. of Harper	62.89	Major
15	Garcia	54.37	Minor
16	Repl. of Garcia	54.28	Minor
17	Repl. of Garcia	53.94	Minor
18	Minnesota	54.87	Minor
19	Repl. of Minnesota	53.97	Minor
20	Repl. of Minnesota	54.05	Minor
21	Somalia	62.83	Major
22	Repl. of Somalia	62.84	Major
23	Repl. of Somalia	62.87	Major
24	Variolator	62.92	Major
25	Repl. of Variolator	62.99	Major
26	Repl. of Variolator	62.90	Major
27	India	62.87	Major
28	Repl. of India	62.84	Major
29	Repl. of India	62.91	Major
30	7124	62.99	Major
31	Repl. of 7124	62.96	Major
32	Repl. of 7124	63.04	Major

[a] As determined by LC 4.0 software genotyping analysis module.
[b] Reagent no template control.
[c] Sample no template control.

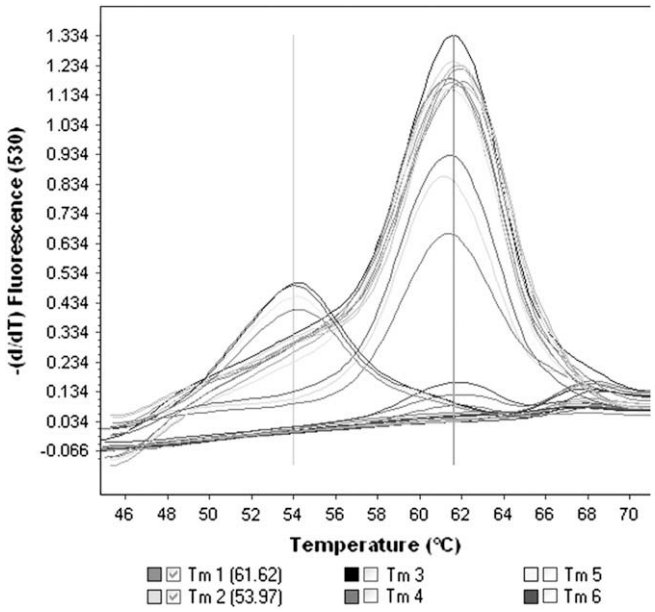

Fig. 2. Melting peaks of *Variola* genomic DNA from CDC blinded panel. The T_m for *V. minor* is 53.97 °C ± 0.4 and the T_m for *V. major* is 61.62 °C ± 0.33.

several *V. major* DNAs from *V. minor* DNAs at various DNA concentrations (Table 3). The T_ms of the MGB-Eclipse probe used (p331-ECL) differentiated the variants from each other 17 out of 18 times. (NOTE: the concentration of the only missed sample was 1 fg/μL).

Table 3
Genotyping results of CDC blinded panel.

Sample #	Blinded #	Organism	Concentration	Genotype[a]
1		NTC	NA	Negative
2	7	Variola V73-175	10 fg/μL	Major
3	8	HFEM	1 ng/μL	Negative
4	9	Variola V73-175	100 pg/μL	Major
5	13	Ectomelia	1 ng/μL	Negative
6	15	Variola 7125	1 fg/μL	Major
7	18	RCN	1 ng/μL	Negative
8	20	Saureus II	1 ng/μL	Negative
9	21	Sup T	1 ng/μL	Negative
10	22	Volepox	1 ng/μL	Negative
11	26	Caml. 2379 E1	1 ng/μL	Negative
12	27	Variola 7125	1 pg/μL	Major
13	28	VCA EP1	1 ng/μL	Negative
14	29	Webster	1 ng/μL	Negative
15	31	Vac. IHDW	1 ng/μL	Negative
16	34	Vac. Lister	1 ng/μL	Negative
17	42	BSC 40	1 ng/μL	Negative
18	56	Variola V73-175[b]	1 fg/μL	Negative
19	57	Variola V73-175	100 fg/μL	Major
20	58	Variola V73-175	10 pg/μL	Major
21	64	Variola 7125	100 fg/μL	Major
22	68	Variola 7125	100 pg/μL	Major
23	81	Caml. V78-I-903	1 ng/μL	Negative
24	83	Variola V73-175	1 pg/μL	Major
25	85	Variola 7125	10 fg/μL	Major
26	93	Variola 7125	1 ng/μL	Major
27	95	Variola V73-175	1 ng/μL	Major
28	97	Variola 7125	10 pg/μL	Major
29	JH	Variola Butler[c]	1/5 dil	Minor
30	JH	Variola Butler[c]	UD	Minor
31	JH	Variola Garcia[c]	1/5 dil	Minor
32	JH	Variola Garcia[c]	UD	Minor

[a] Genotypes determined by the LC 4.0 software genotyping analysis module.
[b] Sample below the limit of detection of the assay.
[c] Genomic *V. minor* samples (Butler and Garcia) were provided by Dr. John Huggins and were not part of the CDC blinded panel.

4. Discussion

While the genomes of the variola spp. contain several gene targets that may distinguish *V. major* from *V. minor*, IS2 was selected because of the availability of the entire cloned B9R/B10R gene complex of several variola strains that were obtained at the CDC in the process of developing the variola-specific B9R and B10R TaqMan®-MGB assays [2]. The availability of these clones allowed us to evaluate assays for *V. major* and *V. minor* before advanced validation testing using genomic variola DNA on site at the CDC.

The *V. minor* primers and TaqMan®-MGB probes designed within the IS2 sequence assay reliably detected only *V. minor* DNA. However, another set of primers designed on either side of the IS2 sequence deletion/insertion with the probe spanning the site easily detected *V. major* DNA, but an unacceptable low-level signal with *V. minor* DNA (data not shown) was also produced. This prompted the decision to evaluate other real-time probe chemistries to positively identify a variola sample as *V. major* and not the result of an indirect determination from a negative reaction for *V. minor*. The MGB-Eclipse probe system designed to discriminate between single nucleotide polymorphisms (SNPs) by the change in T_m caused by the destabilizing effect of base pair mismatch was selected. The TaqMan®-MGB probe designed for the *V. major* assay was modified to function as an MGB-Eclipse probe by switching the FAM fluorescent dye to the 3′ end and a MGBDQ at the 5′ end of the probe. In the MGB-Eclipse IS2-specific assay, the probe was an exact match to the gene sequence for *V. major* strains spanning the site where the deletion occurred. However in the *V. minor* strains, 16 bp of the 5′ hybridization sequence was separated by 749–800 bases from the remaining 4 bp of the 3′ hybridization sequence resulting in a mismatched probe/amplicon duplex. In some cases when the temperature of the reaction is significantly above the T_m of the probe, amplification curves may not be generated. For this reason, amplification curves with this assay cannot be relied on to differentiate *V. major* from *V. minor* or to generate a limit of detection (LOD). However, the primers still bind and an amplicon is generated (even though the probe may not initially bind during the 60 °C of the amplification cycles). Lowering the reaction temperature to 45 °C prior to the melt cycle allows the probe to hybridize even to mismatched amplicon sequences.

The MGB-Eclipse assay for *V. major*/*minor* and the TaqMan®-MGB assay for *V. minor* are not intended to be a screening assay for variola spp. but to be used after variola-specific assays have identified an orthopoxvirus as variola [2,3,11]. All other pox virus DNA would have been eliminated by one or more variola-specific assays. The risk algorithm flowchart on the CDC website (www.bt.cdc.gov/agent/smallpox/diagnosis/riskalgorithm) portrays the testing protocol of orthopox suspect samples. A *pan*-orthopox and variola PCR positive sample would be a candidate for the *V. major*/*minor* assay and the *V. minor* TaqMan®-MGB assay would serve as an additional confirmation. Also, the Eclipse probe assay would likely be run on a cultured isolate of the variola spp.; therefore sensitivity is not an issue.

The design of the assay spanning the deletion/insertion site in the variola B9R/B10R gene complex produced a chromosome-based real-time PCR assay that provides differential identification of these two closely related organisms. MGB-Eclipse assays have been used successfully to distinguish organisms that could not be differentiated by conventional TaqMan® chemistry such as *Mycobacterium chelonae* from *Mycobacterium abscessus* [9] and *Yersinia pestis* from *Yersinia pseudotuberculosis* [12]. Both studies describe simple and rapid differentiation after species identification had been determined by other methods.

The most important aspect of this assay is for the T_ms of the Eclipse probe to be significantly different enough as to be able to clearly differentiate *V. major* from *V. minor*. Previously developed TaqMan®-MGB real-time PCR assays that detect all orthopox viruses and assays that selectively detect variola virus provide rapid identification in the event of an aerosol release of smallpox virus [1]. Once a preliminary diagnosis of variola infection is made, immediate and correct recognition as to which of the two variants is responsible (*V. major* or *V. minor*) would provide healthcare officials with critical insight into the management of an outbreak and implement extensive emergency control measures, if necessary.

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