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Variola Virus-Specific Diagnostic Assays: Characterization, Sensitivity, and Specificity

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A public health response relies upon rapid and reliable confirmation of disease by diagnostic assays. Here, we detail the design and validation of two variola virus-specific real-time PCR assays, since previous assays cross-reacted with newly identified cowpox viruses. The assay specificity must continually be reassessed as other closely related viruses are identified.

Variola virus, a solely human pathogen, is the causative agent of smallpox, which was deemed eradicated in 1980 by the World Health Organization (WHO), leading to the cessation of routine smallpox vaccination. Although several vaccines exist, the threat of variola virus being used as a bioweapon still remains (1, 2). It would be contraindicated for certain individuals, such as the immunocompromised, to receive vaccination, due to serious vaccine adverse events that could be fatal (3, 4). Diagnosis based on clinical symptoms alone is challenging, since the symptoms of smallpox resemble those of numerous other rash-causing diseases and the majority of physicians today have never seen smallpox lesions. In the event of a release, quick and accurate clinical diagnosis would be critical to limit the spread of infection and contain a potential outbreak.

Real-time PCR has gained favor over historic diagnostic methods (5) due to its increased sensitivity, low cost, and more rapid results. This paper enumerates the design and validation of two variola virus-specific real-time PCR assays, which occurred after newly discovered orthopoxviruses identified a loss of specificity for previously authenticated variola virus-specific assays. These findings highlight the importance of continually verifying the assay's specificity to ensure a reliable diagnostic and to understand the assay's performance in identifying a high-consequence (select agent tier 1) pathogen.

Previously validated assays directed against the E9L and A13L (A14L in variola virus) genes (see the supplemental material) of variola virus were found to cross-react with newly identified isolates of cowpox virus (6) (Table 1). The alignments of these newly identified cowpox viruses predicted cross-reaction with the E9L assay; however, the A13L assay target region resembled the cowpox virus strain used in the original validation (see Fig. S1 and S2 in the supplemental material). The original validation was done with reagents and equipment that are no longer commercially available. In order to determine whether cross-reaction would occur, wet laboratory testing was performed with newer platforms and enzymes. The reaction mixtures and cycling conditions (see Table S2 in the supplemental material) were modified slightly to replace the master mix and for use on newer platforms, either the ABI7900 or ABI7500 fast Dx (Applied Biosystems, CA). As predicted, cross-reaction occurred with the E9L assay (Table 1; see Table S2C and E). Samples were considered positive if the fluorescent signal crossed the threshold within 40 cycles. The detection of the cowpox virus strains by the A13L assay was greatly reduced compared to their detection by the E9L assay (~10,000-fold) (Table 1). This low-level cross-reaction occurred with cowpox virus strains with sequences identical to that of the strain used in the original validation—the probe target region contains only 2 nucleotide differences, while most other non-variola orthopoxvirus sequences contain at least 3 nucleotide differences. Improvements in the efficiency of real-time PCR reagents and newer platforms may also account for this difference from the initial A13L validation (see Table S3). To fulfill the need for specific variola

TABLE 1 Cross-reactivity of E9L and A13L assays with newly identified cowpox virus strains

Strain (1 ng/μl)	GenBank accession no.	Avg positive C_T value in indicated assay[a]	
		E9L	A13L
FIN2000-MAN	HQ420893	Neg	Neg
GER1980-EP4	HQ420895	19.2	35.1
GER1991-3	DQ437593	18.0	34.2[b]
GER1998-2	HQ420897	16.7	Neg
Nor1994-MAN	HQ420899	Neg	37.3

[a] The average cycle when fluorescence crossed the threshold (C_T) is shown. Each sample was tested in triplicate. Neg, C_T value was not produced.
[b] GER1991-3 had a total DNA concentration of 4 ng instead of 5 ng.

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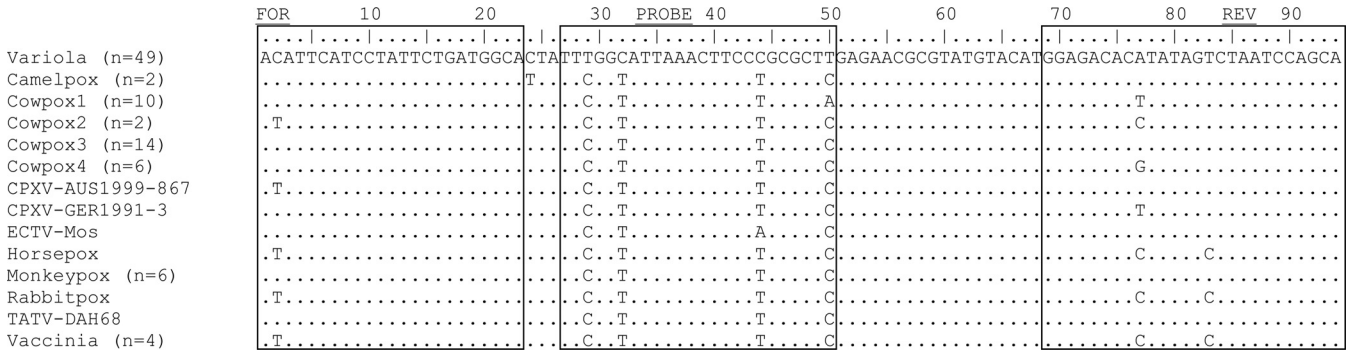

FIG 1 Sequence alignment of target regions for the A4L assay. The alignment of 99 strains was made using BioEdit Sequence Alignment Editor. Those strains within one species that were identical in the primer and probe target regions are condensed; the number in parentheses represents the number of strains included. GenBank sequence accession numbers are as follows (A4L sequences for RPXV-USA64-85A, SKPV-USA1978-WA, and VPXV-USA1985-CA are not available in GenBank): Camelpox virus (AY009089, NC_003391), Cowpox virus 1 (NC_003663, HQ420894, HQ420896, HQ420898, KC813511.1, KC813507.1, KC813494.1, KC813499.1, HQ420895.1, HQ420897.1), Cowpox virus 2 (X94355, HQ420893), Cowpox virus 3 (KC813509.1, KC813506.1, KC813504.1, KC813502.1, KC813500.1, KC813497.1, KC813498.1, KC813496.1, KC813495.1, KC813493.1, KC813492.1, KC813491.1, HQ420900.1, HQ420899.1), Cowpox virus 4 (KC813512.1, KC813510.1, KC813503.1, KC813501.1, KC813505.1, KC813508.1), Monkeypox virus (AY603973, DQ011156, DQ011157, DQ011155, NC_003310, DQ011154), Vaccinia virus (AY603355, M35027, NC_006998, AY678276). All other accession numbers can be found in Table S1 in the supplemental material. FOR, forward primer; REV, reverse primer; CPXV, cowpox virus; ECTV, ectromelia virus; TATV, taterapox virus. The probe contains 5′ 6-carboxyfluorescein (FAM).

virus assays, the conserved region of the genome, encoding essential genes, was evaluated for regions that demonstrated specificity to variola virus through bioinformatics analysis. Potential variola virus-specific targets were identified within the A4L and A36R genes (Fig. 1 and 2). The primers and probe sequences for the A4L and A36R assays were selected from the P4b precursor protein gene (A4L from BSH75 banu) and the enveloped virus glycoprotein gene (A36R from BSH75 banu), respectively, using Primer Express (version 2.0; Applied Biosystems, CA) (Table 2). The target regions for A4L and A36R were identical in all variola virus strains except for a single nucleotide polymorphism (SNP) in the A36R gene in CHN48 horn (Fig. 2). The reaction and cycling conditions are detailed in Tables 3 and 4. Samples were considered positive if the fluorescent signal crossed the threshold within 40 cycles.

Based on previous validations (see Table S4 in the supplemental material), bioinformatics analysis adeptly predicted the likelihood that a real-time PCR assay would detect a variola virus strain at a particular target region, due to low genetic variability found between variola virus strains (7). Additionally, previous validations demonstrated that real-time PCR assays remained sensitive regardless of sample type (see Table S4). Therefore, three variola virus strains of semipurified preparations were chosen for the inclusivity panel: VARV-IND64_vel5 from primary clade I, VARV-SLN68_258 from primary clade II, and VARV-CHN48_horn (primary clade I) due to the SNP (Fig. 2). To generate semipurified virus, crude virus preparations (virus-infected-cell lysates harvested at 48 or 72 h postinfection) were purified through 2 to 3 1,1,2-trichlorofluoroethane extractions followed by two sucrose cushion ultracentrifugations, as previously described (8–13).

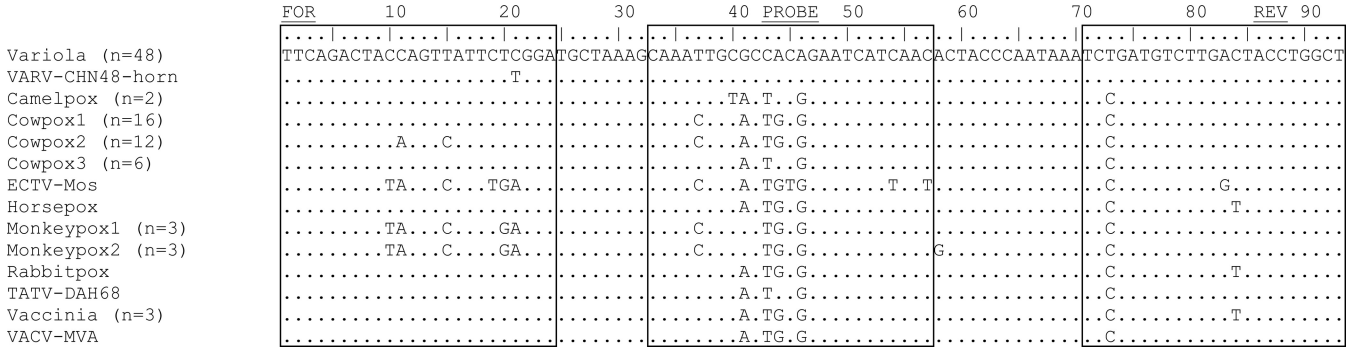

FIG 2 Sequence alignment of target regions for the A36R assay. The alignment of 99 strains was made using BioEdit Sequence Alignment Editor. Those strains within one species that were identical in the primer and probe target regions are condensed; the number in parentheses represents the number of strains included. GenBank sequence accession numbers are as follows (A36R sequences for RPXV-USA64-85A, SKPV-USA1978-WA and VPXV-USA1985-CA are not available in GenBank): Camelpox virus (AY009089, NC_003391), Cowpox virus 1 (HQ407377, HQ420893, HQ420895, HQ420898, X94355, KC813511.1, KC813507.1, KC813506.1, KC813504.1, KC813500.1, KC813498.1, KC813497.1, KC813495.1, KC813493.1, KC813492.1, KC813491.1), Cowpox virus 2 (NC_003663, HQ420894, HQ420896, DQ437593, HQ420897, HQ420899, HQ420900, KC813509.1, KC813502.1, KC813499.1, KC813496.1, KC813494.1), Cowpox virus 3 (KC813512.1, KC813510.1, KC813508.1, KC813505.1, KC813503.1, KC813501.1), Monkeypox virus 1 (AY603973, DQ011156, DQ011157), Monkeypox virus 2 (DQ011155, NC_003310, DQ011154), Vaccinia virus (NC_006998, M35027, AY678276). All other accession numbers can be found in Table S1 in the supplemental material. FOR, forward primer; REV, reverse primer; VARV, variola virus; ECTV, ectromelia virus; TATV, taterapox virus; VACV, vaccinia virus. The probe contains 5′ 6-carboxyfluorescein (FAM).

TABLE 2 Sequences of the primers and probes for the A4L and A36R assays[a]

Primer or probe	Sequence for:	
	A4L	A36R
Forward	5′ ACATTCATCCTATTCTGATGGCA 3′	5′ TTCAGACTACCAGTTATTCTCGGA 3′
Reverse	5′ TGCTGGATTAGACTATATGTGTCTCC 3′	5′ AGCCAGGTAGTCAAGACATCAGA 3′
Probe	5′ FAM-TTTGGCATTAAACTTCCCGCGCTT-BHQ 3′	5′ FAM-CAAATTGCGCCACAGAATCATCAAC-BHQ 3′

[a] Primer and probe sets were synthesized by the Biotechnology Core Facility (CDC, Atlanta, GA). The sequences are from BSH75 banu (GenBank accession number DQ437581). The amplicon lengths are 94 bp for A4L and 93 bp for A36R.

DNA was extracted using the AquaPure genomic DNA isolation kit (Bio-Rad, CA) and suspended in 50 μl of AquaPure DNA hydration buffer.

A two-step approach was employed to determine the analytical limit of detection (LOD), defined as the lowest sample concentration producing a positive threshold cycle (C_T) value at least 95% of the time, for each inclusivity strain. Initially, an estimate of the analytical LOD for each variola virus strain was determined by preparing a 10-fold dilution series ranging from 500 pg to 0.05 fg of variola virus DNA per reaction. Based on the results, a narrower window of concentrations was selected to determine the analytical LOD. The lowest concentrations reproducibly detected were selected, along with the 2 log-fold-higher and -lower concentrations. A total of 20 reactions per concentration were run over 3 consecutive days by two operators for both assays to determine the analytical LOD for each strain, concentration, and assay. The analytical LOD for both assays was 50 fg, with standard deviations of 1.08 (A4L) and 1.44 (A36R) (Table 5). At the LOD concentration, the standard deviations of the observed C_T values between days ranged from 0.92 to 1.15 for A4L and from 0.85 to 1.89 for A36R. The standard deviations for each operator across all days were also calculated; operator 1 had a standard deviation of 1.06 for A4L and 0.91 for A36R. Operator 2 yielded a standard deviation of 1.12 for A4L and 1.83 for A36R. These data suggest the assay to be reproducible in the hands of multiple users.

To reduce the risk of potential false positives, an expansive exclusivity panel was defined, consisting of non-variola orthopoxviruses and non-orthopoxvirus agents that present with clinical symptoms of a rashlike illness. The origins of the viral, bacterial, clinical lesion material, and cellular isolates are documented in Table S1 in the supplemental material. Non-variola orthopoxvirus DNA was extracted using the QIAamp DNA blood minikit (Qiagen, CA) and resuspended in 50 μl of Qiagen AE elution buffer. Additionally, numerous human skin flora DNAs and human DNAs were evaluated to ensure that DNAs common in skin lesion clinical specimens (crust, swab of a lesion, touch preparation of lesion fluid, or fresh biopsy specimen) would not cross-react. Bacteria were propagated on anaerobic blood agar or 5% sheep blood agar and incubated at 35°C with or without CO_2. After a 24- or 48-h incubation period, harvested colonies were suspended in 0.85% sterile saline (0.5 McFarland turbidity) and extracted using the MagNA pure nucleic acid isolation kit I (Roche Applied Science, Indianapolis, IN) on the MagNA pure compact instrument (Roche Applied Science). Both assays demonstrated high specificity; none of the 38 non-variola virus agents screened produced a signal that crossed the threshold cycle value (Table 6).

Since smallpox is an eradicated disease, there are no recent clinical samples available to ascertain the diagnostic assay performance within clinical material from confirmed cases. Therefore, CDC Poxvirus program clinical sample remainders, collected from patients presenting with pustular or vesicular rash illness but ruled negative for poxvirus infection by real-time PCR, were selected for diagnostic assay validation. The clinical sample specimens consisted of the following sample types: swab, swab in viral transport medium, slide, tissue, and crust. Thirty-six of these clinical sample remainders were deidentified (IRB exempt protocol number 6423) and divided into three groups of 12 to be spiked with semipurified variola virus DNA (IRB protocol number 6447) from each of the three inclusivity strains (VARV-CHN48_horn, VARV-IND64_vel5, and VARV-SLN68_258). Each group was further subdivided into three subgroups of four to be spiked with 100 pg/μl (10,000× LOD), 1 pg/μl (100× LOD), or 100 fg/μl (10× LOD). DNA was extracted using the QIAamp DSP DNA blood minikit protocol (Qiagen, CA) and screened blindly by two operators in duplicate with A4L and A36R assays on the ABI7500 fast Dx (Applied Biosystems, Foster City, CA), using the cycling conditions listed in Table 4. The A4L and A36R assays generated positive results for 35/36 (97.2%) and 34/36 (94.4%) spiked specimens, respectively, for both operators (Table 7). Specimen 4 was not detected by either operator and was spiked with the intermediate amount of VARV-IND64_vel5 DNA (1 pg/μl, 100× LOD); however, the lowest spiked concentration of VARV-IND64_vel5 was detected 100% of the time by both assays and both operators. Specimen 35 was detected by 3/4 A36R reactions and was spiked

TABLE 3 Master mix reaction mixture for both the A4L and the A36R assays

Component	Amt and concn
LightCycler FastStart DNA Master HybProbe master mix (Roche Applied Science)	2.5 μl of 10× mix
Forward primer	1 μl of 25 μM stock
Reverse primer	1 μl of 25 μM stock
Probe	1 μl of 25 μM stock
Magnesium chloride	3 μl of 25 mM stock
Sample DNA	5 μl
Total vol	25 μl

TABLE 4 Cycling conditions on the ABI7500 Fast Dx for the A4L and A36R assays

Step	Cycling conditions (temp [°C], time, no. of cycles) for:	
	A4L	A36R
Activate polymerase	95, 8 min, 1	95, 8 min, 1
Denature	95, 5 s, 40	95, 5 s, 40
Elongate	60, 25 s, 40	60, 25 s, 40

TABLE 5 Analytical LOD determined for A4L and A36R real-time PCR assays with three variola virus strains[a]

Strain	Concn of sample	A4L		A36R	
		No. positive/ total no. of replicates	Avg positive C_T value	No. positive/ total no. of replicates	Avg positive C_T value
CHN48 horn	500 pg	3/3	20.8	3/3	22.3
CHN48 horn	50 pg	3/3	24.0	3/3	26.0
CHN48 horn	5 pg	3/3	27.7	3/3	29.8
CHN48 horn	500 fg	3/3	31.4	20/20	32.7
CHN48 horn	50 fg	20/20	35.1	19/20	36.2
CHN48 horn	5 fg	20/20	38.4	10/20	39.1
CHN48 horn	0.5 fg	1/20	39.4	0/20	Neg
CHN48 horn	0.05 fg	0/20	Neg	0/20	Neg
CHN48 horn	0.005 fg	0/20	Neg	—	—
IND64 vel5	500 pg	3/3	19.0	3/3	20.1
IND64 vel5	50 pg	3/3	22.6	3/3	23.8
IND64 vel5	5 pg	3/3	26.3	3/3	27.9
IND64 vel5	500 fg	3/3	29.8	20/20	30.9
IND64 vel5	50 fg	20/20	33.4	20/20	34.4
IND64 vel5	5 fg	18/20	37.4	20/20	37.4
IND64 vel5	0.5 fg	8/20	39.2	5/20	38.8
IND64 vel5	0.05 fg	0/20	Neg	0/20	Neg
IND64 vel5	0.005 fg	0/20	Neg	—	—
SLN68 258	500 pg	3/3	21.4	3/3	23.2
SLN68 258	50 pg	3/3	25.2	3/3	26.9
SLN68 258	5 pg	3/3	28.7	3/3	30.9
SLN68 258	500 fg	3/3	32.3	20/20	33.4
SLN68 258	50 fg	20/20	35.4	20/20	36.9
SLN68 258	5 fg	14/20	39.2	6/20	39.6
SLN68 258	0.5 fg	0/20	Neg	0/20	Neg
SLN68 258	0.05 fg	0/20	Neg	0/20	Neg
SLN68 258	0.005 fg	0/20	Neg	—	—

[a] Each sample was tested in triplicate. Neg, C_T value was not produced; —, not tested.

TABLE 6 Specificity of A4L and A36R real-time PCR assays screened against non-variola orthopoxviruses, rash-causing agents, and normal skin flora[a]

Species[b]	Strain	Concn of sample	Avg positive C_T value for:	
			A4L	A36R
Camelpox virus	Somalia-1978-2379	500 pg	Neg	Neg
Cowpox virus	FIN2000-MAN	500 pg	Neg	Neg
Cowpox virus	GER1980-EP4	500 pg	Neg	Neg
Cowpox virus	GER1991-3	500 pg	Neg	Neg
Cowpox virus	GER1998-2	500 pg	Neg	Neg
Cowpox virus	Nor1994-MAN	500 pg	Neg	Neg
Ectromelia virus	Mos	500 pg	Neg	Neg
Monkeypox virus	RCG-2003-358	500 pg	Neg	Neg
Monkeypox virus	USA-2003-044	500 pg	Neg	Neg
Raccoonpox virus	USA64-85A	500 pg	Neg	Neg
Skunkpox virus	USA-1978-WA	500 pg	Neg	Neg
Taterapox virus	DAH68	500 pg	Neg	Neg
Vaccinia virus	ACAM 2000	500 pg	Neg	Neg
Vaccinia virus	BRZ SERRO	500 pg	Neg	Neg
Vaccinia virus	Copenhagen	500 pg	Neg	Neg
Vaccinia virus	WR	500 pg	Neg	Neg
Volepox virus	USA-1985-CA	500 pg	Neg	Neg
Escherichia coli		5×10^3 CFU	Neg	Neg
Enterococcus faecalis		5×10^3 CFU	Neg	Neg
Herpes simplex virus type 1	F	5×10^6 genomes	Neg	Neg
Herpes simplex virus type 2	G	5×10^6 genomes	Neg	Neg
Human genomic DNA		500 pg	Neg	Neg
Klebsiella pneumoniae		5×10^3 CFU	Neg	Neg
Orf		500 pg	Neg	Neg
Peptostreptococcus anaerobius		5×10^3 CFU	Neg	Neg
Propionibacterium acnes		5×10^3 CFU	Neg	Neg
Pseudomonas aeruginosa		5×10^3 CFU	Neg	Neg
Rickettsia prowazekii	breinl	ND	Neg	Neg
Rickettsia sibirica		ND	Neg	Neg
Staphylococcus aureus	Strain 1	5×10^3 CFU	Neg	Neg
S. aureus	Strain 2	5×10^3 CFU	Neg	Neg
Staphylococcus epidermidis	Strain 1	5×10^3 CFU	Neg	Neg
S. epidermidis	Strain 2	5×10^3 CFU	Neg	Neg
S. epidermidis	Strain 3	5×10^3 CFU	Neg	Neg
Streptococcus gallylyticus		5×10^3 CFU	Neg	Neg
Streptococcus pyogenes		5×10^3 CFU	Neg	Neg
Varicella-zoster virus	pOKA	5×10^9 genomes	Neg	Neg
Varicella-zoster virus	Webster	4×10^{10} genomes	Neg	Neg

[a] Each sample was tested in triplicate. ND, the concentration of DNA could not be determined, refer to BEIResources; Neg, C_T value was not produced.
[b] Bacterial strains were part of the skin flora panel, which was a gift from the CDC Bioterrorism Rapid Response and Advanced Technology Laboratory.

with the smallest amount of VARV-SLN68_258 DNA (0.1 pg/μl, 10× LOD). These C_T values were 4.5 to 5 C_T values later than those of the other three specimens spiked with 0.1 pg/μl of VARV-SLN68_258 DNA. Further PCR analysis of specimens 4 and 35 failed to yield positive results for a control assay designed to detect human DNA (data not shown), suggesting that these two clinical sample remainders were compromised, possibly containing DNA-degrading components or inhibitors to PCR. With the exception of the one negative A36R reaction for specimen 35, the operators achieved similar assay results with the spiked specimen panel (Table 7). Despite these potential compromised clinical sample remainders, both the A4L and the A36R assay remained sensitive and specific in testing spiked samples, our best measure to ensure their utility within a clinical diagnostic setting.

The newly designed diagnostic assays (A4L and A36R) underwent extensive testing with a wide-ranging exclusivity panel to ensure that cross-reactivity with the pathogens most likely to be confused with smallpox did not occur. The assays appeared extremely sensitive (LOD = 50 fg), both analytically and within spiked clinical samples. In a public health setting, these assays could provide rapid diagnosis of smallpox disease, with results taking less than 4 h from receipt of the sample. Ideally, diagnostic assays are most informative when laboratorians work closely with clinicians and epidemiologists to identify patients with the highest suspicion of smallpox. Confidence in the identification of an eradicated disease, such as smallpox, is increased when multiple assay signatures are used to identify the pathogen and are linked to clinical manifestations. In order to improve confidence in a negative assay result, the sample should be validated for the presence of human DNA and the absence of PCR inhibitors. Both assays (A4L and A36R) show great promise as variola virus-specific diagnostic assays. However, orthopoxviruses are continually being

TABLE 7 Sensitivities of A4L and A36R assays within clinical sample matrixes[a]

Specimen	Strain	Concn of DNA (pg/μl)	Avg positive C_T value for:	
			A4L	A36R
Control	CHN48 horn	0.01	35.3	35.3
3	CHN48 horn	0.1	32.8	33.9
18	CHN48 horn	0.1	33.0	34.4
19	CHN48 horn	0.1	33.3	34.1
32	CHN48 horn	0.1	33.0	34.1
7	CHN48 horn	1	28.1	29.5
12	CHN48 horn	1	28.6	29.7
27	CHN48 horn	1	29.2	30.2
33	CHN48 horn	1	28.7	29.9
5	CHN48 horn	100	21.2	22.5
14	CHN48 horn	100	21.8	23.2
21	CHN48 horn	100	21.2	22.5
28	CHN48 horn	100	21.4	23.1
8	IND64 vel5	0.1	30.2	31.4
13	IND64 vel5	0.1	30.4	31.1
24	IND64 vel5	0.1	31.7	32.6
29	IND64 vel5	0.1	30.9	31.8
4	IND64 vel5	1	Neg	Neg
16	IND64 vel5	1	26.9	27.9
23	IND64 vel5	1	27.1	27.9
36	IND64 vel5	1	26.7	27.5
1	IND64 vel5	100	19.0	20.1
17	IND64 vel5	100	19.3	20.5
22	IND64 vel5	100	19.3	20.4
30	IND64 vel5	100	19.2	20.2
2	SLN68 258	0.1	33.6	35.0
15	SLN68 258	0.1	34.0	35.0
26	SLN68 258	0.1	33.7	34.8
35	SLN68 258	0.1	38.8	39.1[b]
6	SLN68 258	1	29.3	30.1
11	SLN68 258	1	29.9	31.6
25	SLN68 258	1	29.6	30.3
31	SLN68 258	1	30.3	30.6
9	SLN68 258	100	22.4	23.7
10	SLN68 258	100	21.7	23.1
20	SLN68 258	100	22.3	23.7
34	SLN68 258	100	22.4	23.5

[a] Clinical sample remainders were spiked with variola virus DNA at various concentrations as indicated. DNA was extracted and tested in duplicate by two operators. The average cycle when fluorescence crossed the threshold is shown.
[b] One of two replicates was positive.

discovered or identified. In the late 2000s, the full genomic sequences of new strains of cowpox viruses from Europe became available (6). Phylogenetic analysis illustrated that these included previously unknown lineages, suggesting the need to include additional cowpox virus strains in the exclusivity panel for assay validations. Two assays previously thought to be specific for variola virus (see the supplemental material) cross-reacted, with various degrees of sensitivity, with three of the five additional strains of cowpox virus that were screened (Table 1). Since cowpox viruses actively cause disease in humans, commonly in Europe (6), this dramatically increased the potential for clinical misdiagnosis. While bioinformatics analysis predicts that the A4L and A36R assays remain specific for variola virus, it will be important to remain vigilant in reassessing the specificity as other closely related viruses are identified.

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