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Real-Time PCR System for Detection of Orthopoxviruses and Simultaneous Identification of Smallpox Virus

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A screening assay for real-time LightCycler (Roche Applied Science, Mannheim, Germany) PCR identification of smallpox virus DNA was developed and compiled in a kit system under good manufacturing practice conditions with standardized reagents. In search of a sequence region unique to smallpox virus, the nucleotide sequence of the 14-kDa fusion protein gene of each of 14 variola virus isolates of the Russian World Health Organization smallpox virus repository was determined and compared to published sequences. PCR primers were designed to detect all Eurasian-African species of the genus *Orthopoxvirus*. A single nucleotide mismatch resulting in a unique amino acid substitution in smallpox virus was used to design a hybridization probe pair with a specific sensor probe that allows reliable differentiation of smallpox virus from other orthopoxviruses by melting-curve analysis. The applicability was demonstrated by successful amplification of 120 strains belonging to the orthopoxvirus species variola, vaccinia, camelpox, mousepox, cowpox, and monkeypox virus. The melting temperatures (T_ms) determined for 46 strains of variola virus (T_ms, 55.9 to 57.8°C) differed significantly ($P = 0.005$) from those obtained for 11 strains of vaccinia virus (T_ms, 61.7 to 62.7°C), 15 strains of monkeypox virus (T_ms, 61.9 to 62.2°C), 40 strains of cowpox virus (T_ms, 61.3 to 63.7°C), 8 strains of mousepox virus (T_m, 61.9°C), and 8 strains of camelpox virus (T_ms, 64.0 to 65.0°C). As most of the smallpox virus samples were derived from infected cell cultures and tissues, smallpox virus DNA could be detected in a background of human DNA. By applying probit regression analysis, the analytical sensitivity was determined to be 4 copies of smallpox virus target DNA per sample. The DNAs of several human herpesviruses as well as poxviruses other than orthopoxviruses were not detected by this method. The assay proved to be a reliable technique for the detection of orthopoxviruses, with the advantage that it can simultaneously identify variola virus.

The genus *Orthopoxvirus*, family *Poxviridae*, comprises morphologically and antigenically closely related viruses, including variola virus (VAR; smallpox virus) and several pathogens of veterinary and zoonotic importance (21). Orthopoxviruses (OPVs) are allocated into 11 species, 8 Eurasian-African species (VAR, monkeypox virus [MPX], vaccinia virus [VAC], cowpox virus [CPX], camelpox virus [CML], ectromelia, taterapox, and Uasin Gishu disease viruses) and 3 North American species (raccoon poxvirus [RCN], volepox virus [VPX], and skunkpox virus [SKN]). The most notorious member is VAR, the causative agent of smallpox (14, 22). As a result of international collaboration under the World Health Organization (WHO) eradication program, smallpox was declared eradicated in 1980. Nevertheless, VAR is considered a potential threat agent or bioterrorist weapon: it is naturally transmissible by large respiratory droplets and experimentally by the aerosol route, it causes high rates of high morbidity and mortality, and much of the human population is now susceptible because of the cessation of routine smallpox vaccination (7). Therefore, the identification of a single suspected case of smallpox must be treated as an international health emergency. A potentially confusing diagnosis is monkeypox, the agent of which is zoonotic and endemic to the African rain forest and which can cause a disease indistinguishable from smallpox (16). Recently, several mild cases of human monkeypox occurred in the United States due to transmission from infected prairie dogs which had become infected at pet stores handling imported exotic African rodent species (1). Two other orthopoxvirus species, namely, CPX and VAC, can cause localized skin lesions in humans; and generalized infections are possible in immunocompromised hosts. Historically, differentiation of OPVs into single species has been achieved by biological means, such as pock morphology on the chorioallantoic membrane or ceiling growth temperature, because serological tests proved to be of limited value (3). DNA maps and several other molecular biologic features, including full genome sequencing, have been applied and have provided definitive information for virus classification (4, 24, 25, 26). Various PCR methods have been used to identify and subtype the available OPVs by using consensus primers combined with restriction cleavage and/or sequencing of amplicons (6, 10, 13, 18, 23) or oligonucleotide-based microchip technology (11, 12, 20). Today, real-time PCR is even more efficient because it combines amplification and detection of target DNA in one vessel, thereby eliminating any time-consuming post-PCR procedures and potentially limiting

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possible contamination events (5, 8, 9). Because of the serious consequences of a diagnosis of smallpox or even the consequences of a misdiagnosis, there is a need to be able to unambiguously and reliably identify smallpox and to differentiate it from other similar clinical entities. In the first international quality assessment study on the rapid detection of viral agents of bioterrorism, only 6 of 22 laboratories provided good results for OPV diagnostic PCR regarding sensitivity and specificity (21a). One option for the design of a PCR assay is to identify VAR-specific insertions or deletions. However, it has been shown that sequences previously considered VAR specific (10) are conserved in some cowpox virus strains (15). In addition, a VAR-specific assay would require VAR DNA as a positive control, which might lead to serious consequences if cross-contamination were to occur. Studies of OPV species to date have shown that unique sequences are very rare, but species-specific single nucleotide polymorphisms are observed in many genes. Therefore, the rationale of the assay design described here is based on a VAR-specific single nucleotide polymorphism that results in an amino acid substitution. This real-time PCR assay was validated by using a large number of different specimens ($n = 123$) containing OPV DNA, including VAR DNA. On the basis of melting-curve analysis, lesions or specimen containing VAR could be clearly differentiated from those containing other OPVs.

MATERIALS AND METHODS

Viruses and DNA extraction. Table 1 summarizes the designations, origins, and years of isolation of the OPV strains used. Seventy-four strains belonging to the OPV species MPX, VAC, CPX, CML, or mousepox virus (ECT) were grown on an African Green Monkey kidney cell line, MA 104. In addition, DNA from 46 VAR strains or scabs and from one strain each of RCN, SKN, and VPX had been prepared at the WHO smallpox repository at the Centers for Disease Control and Prevention, Atlanta, Ga. (8) with an Aquapure Genomic DNA Isolation kit (Bio-Rad). The concentrations of the VAR DNAs used in the test panel ranged from 100 fg per μl to 1 ng per μl and included total viral and cellular DNAs from cell lysates and crust material as well as purified viral DNAs. Nucleic acids of species other than VAR were extracted either from infected tissue culture cells or from purified preparations by using conventional chemistry (QiaAmp DNA Mini kit; Qiagen, Hilden Germany) or as described earlier (12, 18). Parapoxvirus strain D 1701, avipoxvirus strain HP-1, and tanapoxvirus TP-1, as well as one strain each of herpes simplex virus types 1 and 2, Epstein-Barr virus, varicella-zoster virus, and cytomegalovirus, were also included.

Sequencing and sequence alignment. The 14-kDa fusion protein gene sequences of 14 VAR strains which are part of the collection held at the WHO smallpox repository at the State Research Center of Virology and Biotechnology ("Vector"), Koltsovo, Russia, were determined following amplification of a 602-bp fragment containing the entire gene. The sequences of the following strains are deposited in GenBank under accession nos. AY223482 to AY223495, respectively (origins or years of isolation, or both, are given in parentheses): TajBarin (Pakistan, 1970), India 378 (India), India 164 (India), M-Sur-60 (Russia, 1960), M-Sok-60 (Russia, 1960), M-N-60 (Russia, 1960), M-A-60 (Russia, 1960), M-Bl-60 (Russia, 1960), Aslam (Pakistan, 1970), Khateen (Pakistan, 1970), 6/58 (Pakistan, 1958), Butler (United Kingdom, 1952), Brazil 128 (Brazil), and Brazil 131 (Brazil). The sequences of Aslam, India 164, India 378, and Khateen was determined from DNA extracted from scabs, whereas the others were derived from infected cell culture material. Sequences of the 14-kDa fusion protein genes of OPVs were downloaded from GenBank (www.ncbi.nlm.nih.gov) and aligned by use of the Mac Vector software package (Accelrys Inc., Cambridge, United Kingdom).

Primer and probe design. Oligonucleotides were designed with Primer Express software supplied by Applied Biosystems (Darmstadt, Germany). Primer and probe sequences were checked against those in the GenBank and EMBL databases by use of the BLAST (www.ncbi.nlm.nih.gov/BLAST) and FASTA (www.ebi.ac.uk/fasta33/) algorithms.

LightCycler PCR. The LightCycler instrument (Roche Diagnostics, Mannheim, Germany) was used to amplify a 110-bp region of the 14-kDa fusion protein gene. During amplification the fluorescence was continuously monitored at the annealing step of PCR. Fluorescence was generated by specific hybridization of two oligonucleotides inside the developing PCR fragment. Different from conventional hybridization probe assays, the anchor probe contained LC 640 fluorophore (TIB Molbiol, Berlin, Germany) at the 5′ end and a fluorescein sensor probe at the 3′ end. The sensor probe was additionally covalently linked to a minor groove binder (MGB; Applied Biosystems) and a dark quencher at the 5′ end. This design allows a much higher discrimination power in melting-curve analysis compared to that obtained with conventional hybridization pairs. PCR was performed in a total volume of 20 μl of a mixture containing 2 μl of $MgSO_4$ solution (50 mM) and 13 μl of a PCR master mixture containing *Taq* polymerase reaction buffer, a deoxynucleoside triphosphate mixture, bovine serum albumin (Artus, Hamburg, Germany), and 5 pmol each of two forward primers (Table 2), two reverse primers, the anchor probe, and the sensor probe (Aplera, Darmstadt, Germany). After distribution of 15 μl of the master mixture, 5 μl of template DNA solution was added to each glass capillary (Roche Diagnostics), centrifuged, and placed in the LightCycler sample carousel. LightCycler amplification involved a first denaturation at 95°C for 10 s, followed by amplification of the target DNA for 45 cycles (95°C for 5 s, 55°C for 15 s, and 72°C for 15 s) with a temperature transition rate of 20°C/s. All PCR runs included appropriate negative controls to exclude the possibility of DNA contamination and positive controls to ensure high PCR performance. In some experiments, 10 ng of human genomic DNA was added to the master mixture.

Melting-curve analysis. Differentiation of VAR from all other members of the genus *Orthopoxvirus* was achieved by melting-curve analysis. This feature makes use of the different melting behaviors of oligonucleotides, which results in specific shifts of the melting temperature (T_m) due to a single nucleotide mismatch. The melting-curve analysis was performed by denaturation at 95°C for 10 s, followed by a cooling step at 40°C for 20 s and heating to 80°C at a linear temperature transition rate of 0.1°C/s. For improved determination of T_m, the first derivative of the initial melting curve was plotted against the temperature.

Positive control. A positive control (TL-1), consisting of linearized plasmid DNA (pcr Skript; Stratagene, La Jolla, Calif.) with the 14-kDa fusion protein gene of VAR Bangladesh synthesized in vitro, was generated by overlap extension amplification with 80-bp oligonucleotides (Metabion, Munich, Germany). The DNA concentration was quantified by photometric and fluorescence photometric methods. TL-1 was used as a high- and a low-copy-number control (10,000 and 100 copies, respectively) to facilitate initial evaluation and optimization of the assay.

Statistical analysis. To determine the analytical sensitivity of the assay, we used purified and photometrically quantified VAC (strain Copenhagen) and VAR (strain Variolator 4) DNA preparations. The DNA was diluted in Tris-EDTA (or water for VAR) and was tested in 24 replicates with DNA concentrations of 100, 20, 4, 0.8, and 0.16 fg as inputs. A probit analysis as a model of nonlinear regression was done with a commercial software suite (SPSS, version 11.0, for Mac OSX; SPSS, Inc., Chicago, Ill.). The software determines a continuous 95% confidence interval of the probability of achieving a positive result at any given input DNA concentration within the concentration range of the experiment.

RESULTS AND DISCUSSION

The nucleotide sequences of the 14-kDa fusion protein genes of 14 VAR strains from the Russian repository were determined and aligned to sequences of various OPVs deposited in GenBank. All VAR sequences showed a stable mismatch at nucleotide position 231 of the 14-kDa fusion protein gene (A27L) compared to the sequences of all other OPV strains. This results in a change from amino acid Glu-77 to Asp. This unique change was selected for by designing a hybridization probe pair that would discriminate a single nucleotide mismatch by T_m. Conventional hybridization probe pairs showed a maximum discrimination power of 4°C; thus, we decided to use a new approach and selected a relatively short sensor probe (the probe which hybridizes to the mutated site) with a covalently linked MGB molecule (Table 2). MGB-linked oligonucleotides are more sensitive to nucleotide mis-

TABLE 1. Designations and origins of OPV strains and isolates investigated

Species	Strain	Isolated	Origin	Host
VAR	Minnesota124	1939	United States	Human
VAR	Yamada	1946	Japan	Human
VAR	Hinden	1946	United Kingdom	Human
VAR	Harvey	1946	United Kingdom	Human
VAR	Lee	1947	Korea	Human
VAR	Juba	1947	Sudan	Human
VAR	Rumbec	1947	Sudan	Human
VAR	Higgins	1948	United Kingdom	Human
VAR	Horn	<1948	China	Human
VAR	Harper	<1951	Japan	Human
VAR	Stillwell	<1951	Japan	Human
VAR	Butler	1952	United Kingdom	Human
VAR	Kali Mathu	1953	India	Human
VAR	New Delhi	1953	India	Human
VAR	Herrlich[a]	1958	India	Human
VAR	Kudano[a]	1961	Nigeria	Human
VAR	7124	1964	India	Human
VAR	7125[b]	1964	India	Human
VAR	SAF65-102	1965	RSA[c]	Human
VAR	SAF65-103	1965	RSA	Human
VAR	Garcia	1965	Brazil	Human
VAR	Hembula	1965	Tanzania	Human
VAR	V66-39	1966	Brazil	Human
VAR	K1629	1966	Kuwait	Human
VAR	V68-59	1968	Benin	Human
VAR	Lahore	1969	Pakistan	Human
VAR	V68-258	1969	Sierra Leone	Human
VAR	Variolator 4[b]	1970	Afghanistan	Human
VAR	Congo	1970	Congo	Human
VAR	V70-222	1970	Indonesia	Human
VAR	V70-228	1970	Indonesia	Human
VAR	V72-119	1972	Syria	Human
VAR	V72-143	1972	Botswana	Human
VAR	ETH72-16	1972	Ethiopia	Human
VAR	ETH72-17	1972	Ethiopia	Human
VAR	V73-225	1973	Botswana	Human
VAR	Nepal 73	1973	Nepal	Human
VAR	Nur Islam	1974	Bangladesh	Human
VAR	Shahzaman	1974	Bangladesh	Human
VAR	Parvin[a]	1974	Bangladesh	Human
VAR	Mannan[a]	1974	Bangladesh	Human
VAR	Solaiman	1974	Bangladesh	Human
VAR	Bangladesh	1975	Bangladesh	Human
VAR	V77-1252	1977	Somalia	Human
VAR	Heidelberg		Germany	Human
VAR	Iran 2602		Iran	Human
MPX	Copenhagen	1958	Denmark	Monkey
MPX	AP-5	1961		Monkey
MPX	AP-2	1965	The Netherlands	Anteater
MPX	AP-4	1965	The Netherlands	Monkey
MPX	AP-6	1966		Monkey
MPX	Gabon	1986	Gabon	Human
MPX	V97-I-003	1997	Zaire	Human
MPX	INRB 41	2000	Zaire	Human
MPX	MSF#2	2001	Zaire	Human
MPX	MSF#6	2001	Zaire	Human
MPX	MSF#10	2001	Zaire	Human
MPX	009/01	2001	Zaire	Human
MPX	038/01	2001	Zaire	Human
MPX	169/02	2002	Zaire	Human
MPX	180/02	2002	Zaire	Human
VAC	Uetrecht	1941	The Netherlands	Rabbit
VAC	MVA			
VAC	Tian Tian		China	
VAC	Wyeth		United States	
VAC	CVA		Turkey	
VAC	IHD			
VAC	Levaditi			
VAC	Bern			
VAC	Elstree			
VAC	EP Marina	1961	Germany	Elephant
VAC	BP-1			Buffalo
CPX	Brighton red	1938	United Kingdom	Human
CPX	OPV 85 Hamburg	1985	Germany	Human
CPX	EP-1	1971	Germany	Elephant
CPX	EP-2	1975	Germany	Elephant
CPX	EP-3	1977	Germany	Elephant
CPX	Rat Moscow	1977	Russia	Rat
CPX	EP-Riems	1980	Germany	Elephant
CPX	EP-4	1980	Germany	Elephant
CPX	Catpox 5	1982	United Kingdom	Cheetah
CPX	Catpox 3	1983	United Kingdom	Cat
CPX	EP-5	1988	Germany	Elephant
CPX	OPV 88H	1988	Germany	Cat
CPX	OPV 89-1	1989	Germany	Cat
CPX	OPV 89-2	1989	Germany	Cat
CPX	OPV 89-3	1989	Germany	Cat
CPX	OPV 89-4	1989	Germany	Cat
CPX	OPV 89-5	1989	Germany	Cat
CPX	OPV 90-1	1990	Germany	Cat
CPX	OPV 90-2	1990	Germany	Human
CPX	OPV 90-3	1990	Germany	Cat
CPX	OPV 90-4	1990	Germany	Dog
CPX	OPV 90-5	1990	Germany	Cat
CPX	Sweden I	1990	Sweden	Human
CPX	Sweden II	1990	Sweden	Human
CPX	OPV 91-1	1991	Germany	Cat
CPX	OPV 91-3	1991	Germany	Human
CPX	OPV 91-4	1991	Germany	Cow
CPX	Nw man	1995	Norway	Human
CPX	Biber	1997	Germany	Beaver
CPX	EP-6	1998	Germany	Elephant
CPX	OPV 98-1	1998	Germany	Human
CPX	OPV 98-2	1998	Germany	Human
CPX	OPV 98-3	1998	Germany	Cat
CPX	OPV 98-4	1998	Germany	Human
CPX	OPV 98-5	1998	Germany	Horse
CPX	Finland	2000	Finland	Human
CPX	427	2000	United Kingdom	Cat
CPX	428	2000	United Kingdom	Cat
CPX	780	2000	United Kingdom	Cat
CPX	Sweden III	2001	Sweden	Human
CML	CP-1	1972	Iran	Camel
CML	Saudi	1986	Saudi Arabia	Camel
CML	Mauretania	1988	Mauretania	Camel
CML	Niger	1988	Niger	Camel
CML	CP-5	1992	Dubai	Camel
CML	CP-14	1993	Dubai	Camel
CML	CP-17	1993	Dubai	Camel
CML	CP202/95 H	1995	Saudi Arabia	Camel
ECT	MP-1	1983	Germany	Mouse
ECT	Moscow		Russia	Mouse
ECT	MP-2	1983	Germany	Mouse
ECT	MP-3		Germany	Mouse
ECT	MP-4		Germany	Mouse
ECT	Silberfuchs	1992	Czech Republic	Fox
ECT	MP-33221	1995	United States	Mouse
ECT	MP-4619	1995	United States	Mouse
SKN	Skunkpox		United States	Skunk
RCN	Herman		United States	Raccoon
VPX	Volepox		United States	Vole

[a] The strain was analyzed by use of scab-derived DNA.
[b] The strain was analyzed by use of purified viral DNA.
[c] RSA, Republic of South Africa.

TABLE 2. Designations and sequences of primers and probes

Primer or probe designation	Sequence[a]
Pox fw 14kd 1	5′-CCG XCC AGT CTG AAC ATC AAT C-3′
Pox fw 14kd 2	5′-CCG ACC AGT CTG XAC ATC AAT C-3′
Pox RT 14kd 1	5′-ACA AAT XGA AAA GTG TTG TAA ACG CAA-3′
Pox RT 14kd 2	5′-ACA AAT AGA AAA GTG TTG TAA ACX CAA-3′
Anchor probe	5′-CAG AGA TAT CAT AGC CGC TCT TAG AGT TTC AGC GTG
Pox Anc	ATT T-3′-LC 640
Sensor probe MGB pox 14kd	5′-FAM-CCA ACC TAA ATA GXA CTT CAT-3′-Q-MGB

[a] X, inosine; FAM, 6-carboxyfluorescein; Q, quencher.

TABLE 3. Investigation of different amounts of total DNA prepared from VAR-infected cell lysate material or scabs by LightCycler PCR combined with melting-curve analysis

VAR strain	Reaction result with[a]:			Avg T_m (°C) for all amplified products
	100 fg of DNA	10 fg of DNA	1 fg of DNA	
SAF65-102	+++ (31.29)	+++ (33.47)	++− (35.05)	56.49
SAF65-103	+++ (33.19)	+++ (34.77)	+−− (35.44)	56.74
7124	+++ (31.98)	+++ (35.58)	+++ (35.63)	56.12
Butler	+++ (31.48)	+++ (34.13)	+−− (35.56)	56.58
Congo	+++ (31.77)	+++ (33.91)	++− (35.57)	56.88
ETH72-16	+++ (31.17)	+++ (33.80)	+−− (34.97)	56.09
ETH72-17	+++ (30.13)	+++ (32.59)	+++ (34.52)	56.16
Garcia	+++ (31.83)	+++ (34.28)	+−− (35.93)	56.42
Harper	+++ (30.68)	+++ (33.39)	+++ (34.98)	56.80
Harvey	+++ (30.48)	+++ (33.75)	++− (35.15)	56.27
Heidelberg	+++ (31.88)	+++ (34.05)	++− (35.95)	57.08
Higgins	+++ (31.48)	+++ (34.60)	++− (35.37)	56.98
Hinden	+++ (30.80)	+++ (33.46)	++− (35.10)	56.23
Horn	+++ (30.19)	+++ (33.33)	−−−	56.56
Iran 2602	+++ (30.84)	+++ (33.39)	+++ (33.54)	56.86
Juba	+++ (31.18)	+++ (33.53)	++− (35.98)	56.74
K1629	+++ (32.31)	+++ (34.47)	++− (35.33)	56.86
Kali Mathu	+++ (31.02)	+++ (33.77)	+++ (35.76)	55.95
Hembula	+++ (31.30)	+++ (33.37)	++− (35.35)	56.98
Lee	+++ (30.35)	+++ (33.13)	+−− (36.01)	56.14
Minnesota 124	+++ (32.37)	+++ (35.44)	++− (36.30)	57.66
New Delhi	+++ (32.42)	+++ (34.36)	+−− (35.70)	55.95
Nur Islam	+++ (30.92)	+++ (33.68)	++− (34.83)	56.23
Lahore	+++ (30.60)	+++ (33.42)	+++ (35.42)	56.84
Rumbec	+++ (30.86)	+++ (33.56)	+−− (34.63)	56.77
Shahzaman	+++ (29.02)	+++ (32.06)	+++ (34.33)	56.06
Solaiman	+++ (31.54)	+++ (33.37)	+−− (34.15)	56.90
Stillwell	+++ (29.51)	+++ (32.03)	++− (34.23)	56.68
V66-39	+++ (30.44)	+++ (33.44)	+−− (36.26)	56.42
V68-258	+++ (31.43)	+++ (34.75)	++−(35.77)	56.70
V68-59	+++ (30.41)	+++ (33.58)	++− (34.55)	56.52
V70-222	+++ (32.81)	+++ (34.40)	−−−	57.12
V70-228	+++ (32.01)	+++ (33.80)	+−− (35.20)	57.18
V72-119	+++ (30.87)	+++ (34.14)	++− (35.11)	56.70
V72-143	+++ (30.90)	+++ (33.27)	+++ (35.09)	56.87
Nepal 73	+++ (31.68)	+++ (33.57)	+++ (34.44)	56.94
V73-225	+++ (31.24)	+++ (34.50)	+−− (35.12)	56.79
V77-1252	+++ (30.50)	+++ (33.24)	++− (35.30)	56.61
Variolator 4	+++ (31.80)	+++ (32.99)	+++ (35.03)	57.06
Yamada	+++ (35.42)	+++ (35.12)	+−− (34.93)	56.74
Herrlich[b]	+++ (33.50)	++− (35.46)	−−−	57.07
Mannan[b]	+++ (34.69)	++− (35.72)	−−−	56.68
Kudano[b]	+++ (34.07)	−−−	+−− (34.62)	57.14
Parvin[b]	+++ (33.50)	++− (34.35)	−−−	57.36
7125[c]	+++ (30.58)	+++ (32.48)	+++ (34.66)	56.24
Bangladesh[c]	+++ (29.06)	+++ (32.71)	+++ (35.43)	57.82
Variolator 4[c]	+++ (28.88)	+++ (31.88)	+++ (34.91)	57.62

[a] Each plus or minus sign represents the result of a single reaction with 100, 10, and 1 fg of total viral and cellular DNA, respectively, derived from virus-infected cell lysates or scab material. The average cycle in which the fluorescence crossed the threshold (average C_t is presented in parentheses.

[b] The strain was analyzed by use of scab-derived DNA.

[c] DNA was derived from purified virus.

matches, and thus, the power of discrimination of a site with a single nucleotide polymorphism is increased. Since the probe was designed to be specific for Eurasian-African OPV species other than VAR, such as VAC, MPX, and CPX, primer-probe hybrids specific for these species should melt at temperatures higher than those for hybrids specific for VAR. In order to prove this assumption, a total of 123 OPVs (listed in Table 1) were investigated. Amplification was seen for all 46 VAR strains (Table 3) and 82 other isolates belonging to the Eurasian-African OPV species, whereas the DNAs of three strains representing the North American OPV species (RCN, SKN, and VPX) were not amplified. The results of melting-curve analyses of one LightCycler run for VAR and VAC are shown in Fig. 1. The results of all analyses are summarized in Table 4. A clear discrimination of smallpox virus is possible: all 46 VAR strains displayed lower T_ms (T_ms, 55.9 to 57.8°C) than the 11 VAC strains (T_ms, 61.7 to 62.7°C), the 15 MPX strains (T_ms, 61.9 to 62.2°C), the 40 CPX strains (T_ms, 61.3 to 63.7°C), the 8 ECT strains (T_m, 61.9°C), and the 8 CML strains (T_ms, 64.0 to 65.0°C). Melting point differences between OPV species were statistically analyzed by one-way analysis of variance (with five different post hoc tests). The differences in the T_ms between VAR and the other OPV species were significant ($P = 0.005$). Although no amplification of VPX was observed, 1 ng of genomic VPX DNA was sufficient to cross-react with the probe and produce a melting curve of a much lower temperature (T_m, 46.09°C) than those for all other OPVs tested (Table 4). Furthermore, the primers and probes did not cross-react with the DNA of parapoxvirus, avipoxvirus, or tanapoxvirus strains or with the DNA of human herpesviruses (cytomegalovirus, Epstein-Barr virus, herpes simplex virus types 1 and 2, and varicella-zoster virus).

The analytical sensitivity of the assay was determined by probit regression analysis with purified VAR DNA and 24 replicate amplification reactions. Amplification was positive in all 24 replicate reactions containing 100, 20, and 4 fg of VAR input DNA. When 0.8 and 0.16 fg of VAR DNA was input, amplification resulted in 23 and 16 positive reactions, respectively. Figure 2 shows the results of the probit analysis for VAR. The concentration at which 95% of results are expected to be positive was calculated to be 0.7 fg of DNA. On the basis of the number of VAR base pairs (ca. 186 kbp), this corresponds to about 4 genome copies per assay. By using the same statistical approach with purified VAC DNA, the sensitivity was calculated to be about 6 genome copies (data not shown). However, these results were achieved with purified DNA from only two OPV species. Crude viral-cellular lysates derived

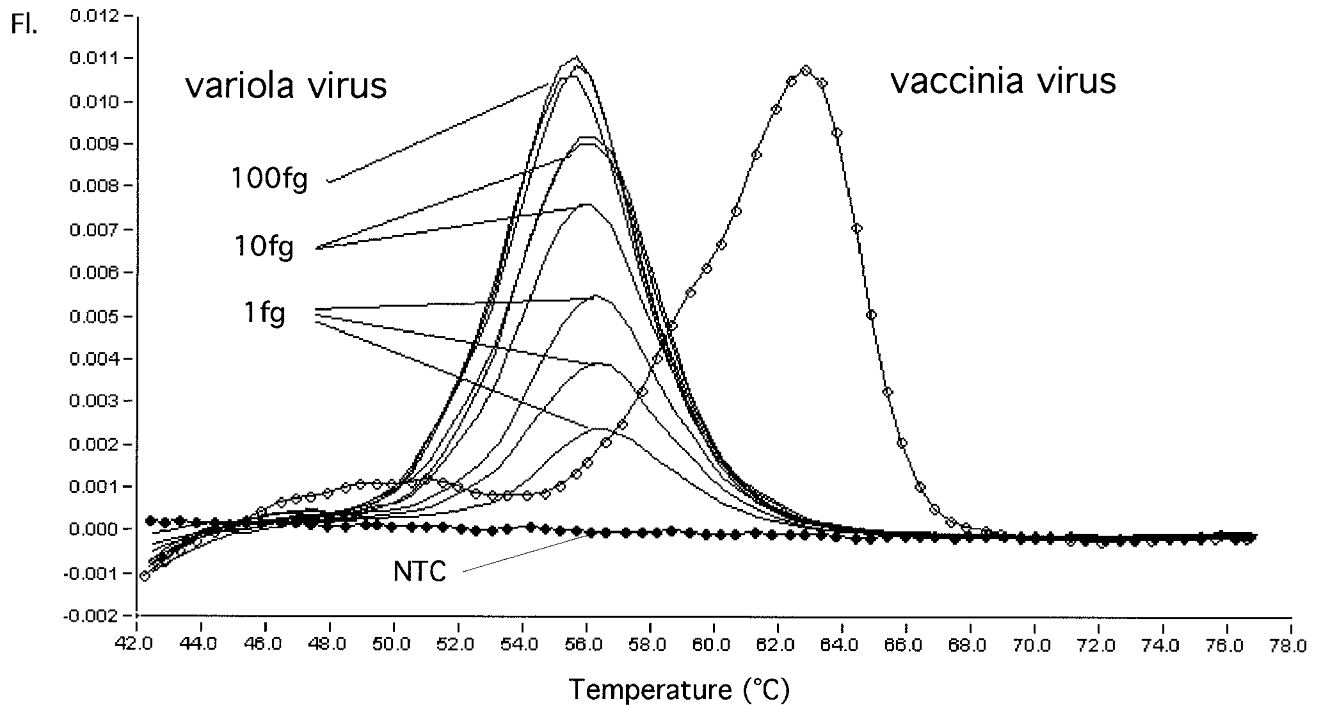

FIG. 1. Melting curves generated after LightCycler amplification of 100, 10, and 1 fg of DNA prepared from VAR (strain Kali Mathu)-infected cell culture material. Each DNA concentration was run in triplicate. The melting curve after amplification of 100 fg of VAC (strain MVA) is also given. Fl, fluorescence.

from infected cell cultures may more faithfully correspond to the nucleic acid material present in a clinical specimen, as both viral and host cell DNA are present in samples of DNA extracted directly from infected tissue cultures. In these samples (a total of 40 samples were examined; Table 3) the assay was able to detect VAR DNA in samples in which as little as 10 fg of total input nucleic acid was present. Investigation of four archival human smallpox specimens (Table 3) yielded positive amplification with an input nucleic acid concentration of 100 fg per reaction mixture. Given that 10^6 pock-forming units can be isolated from skin lesions by day 2 of the smallpox rash (3) and that a greater number of viral particles are present than can be quantified as pock-forming units, at a minimum, 200 pg of VAR DNA can be extracted per skin lesion by day 2 of the rash. This amount of viral DNA exceeds the detection limit of the assay by several orders of magnitude and is certainly sufficient to diagnose smallpox at this stage.

The linearity of the assay was established by using a dilution series of purified VAR DNA from both the Variolator 4 and the Bangladesh strains. The cycle at which the fluorescence crossed the threshold increased in a linear manner from 1 ng to 1 fg of VAR DNA (Table 5). The T_ms varied slightly, depending on the concentration of viral DNA, and increased up to 1.3°C over a 10^6-fold dilution series. Limiting amounts (1 ng to 1 fg) of purified VAR DNA (strain Bangladesh) were amplified in the presence of 10 ng of purified human genomic DNA/reaction mixture. This did not inhibit the detection of VAR DNA, nor did it affect the VAR T_m (Table 5).

All reagents used in this assay were produced under good manufacturing practice conditions and were assembled into a kit in order to standardize OPV diagnostics. The kit contains an internal control, which consists of 100 copies of a 400-bp artificial DNA fragment and which is detected in the F3 channel of the LightCycler instrument. The internal control is designed in a way that the amplification efficiency of the internal control reaction is generally weaker than that of the specific amplification assay. Thus, problems with DNA extraction or

TABLE 4. Comparison of T_ms of different OPV species

OPV species	No. of positive amplifications/no. of strains tested	Avg T_m (°C)[b]
OPVs		
VAR	46/46	56.71 (55.9–57.8)
MPX	15/15	62.09 (61.9–62.2)
VAC	11/11	62.03 (61.7–62.7)
CPX	40/40	62.26 (61.3–63.7)
CML	8/8	64.25 (64.0–65.0)
ECT	8/8	61.9
RCN	0/1	
VPX	0/1	46.09[a]
SKN	0/1	
Total OPVs	120/123	
Other viruses		
Human herpes viruses	0/5	
Parapoxvirus	0/1	
Tanapoxvirus	0/1	
Avipoxvirus	0/1	

[a] No amplification was detected; however, the probe did cross-react with VPX genomic DNA at a much lower efficiency.
[b] Values in parentheses are ranges.

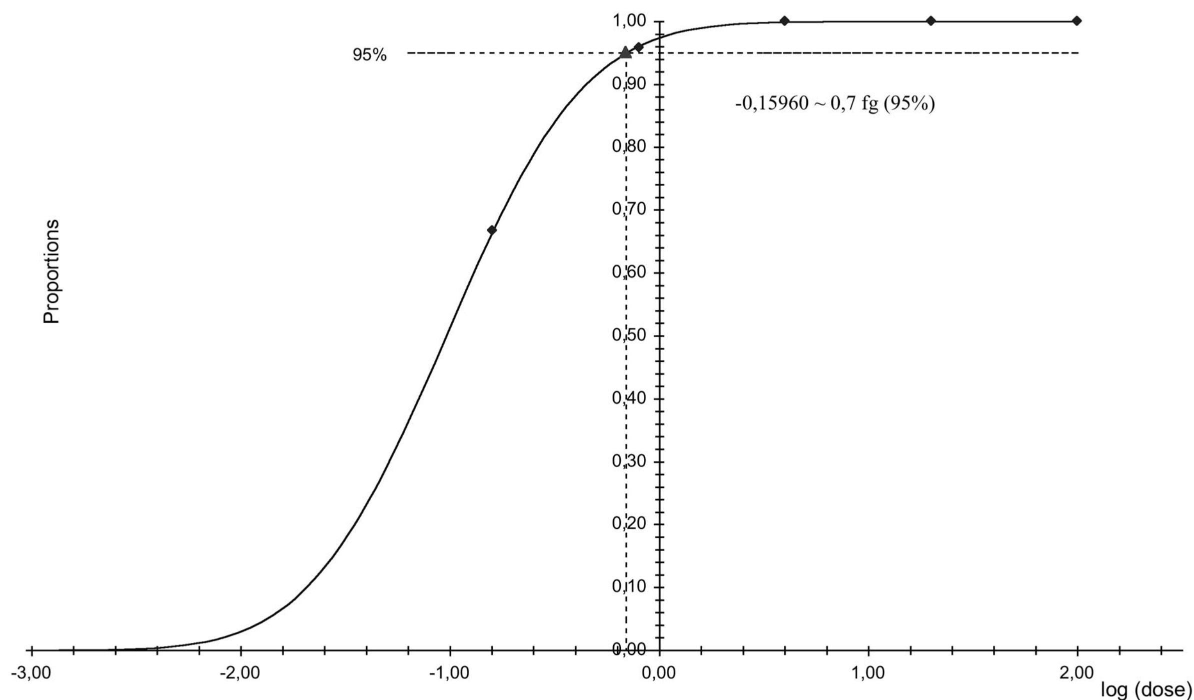

FIG. 2. Predicted proportion of positive amplification results versus the input concentration of purified VAR (strain Variolator 4) DNA, as determined by probit regression analysis. The DNA concentration at which 95% of results are expected to be positive was calculated to be 0.7 fg, which corresponds to ca. 4 copies/assay.

the presence of inhibitors would affect the internal control earlier than they would affect the specific amplification signal. Spiking experiments with known PCR inhibitors (e.g., heparin) demonstrated that this type of internal control inhibits the reaction at 1/10 of the concentration actually required to inhibit the amplification of the test target gene (data not shown). To monitor inhibition of the polymerase, the internal control was included in the master mixture used in each amplification assay. The internal control was amplified in all experiments except when a great excess of target viral DNA was present. In such instances, the internal control was not amplified due to the sequestering of resources for amplification of the viral DNA.

The assay was developed on the basis of sequences of the 14-kDa fusion protein gene. This gene, named open reading frame A27L, plays an important role in virulence and immunogenicity (2, 19). We confirmed by successful amplification of 120 Eurasian-African OPVs that A27L sequences are highly conserved and thereby useful for establishment of an OPV consensus PCR. Furthermore, we demonstrated by sequencing that the unique amino acid change (Glu-77 to Asp) of VAR is conserved in 14 different VAR strains of the Russian WHO collection. To our knowledge, this is the highest number of different VAR strains ($n = 60$) and OPVs other than VAR ($n = 74$) used to evaluate a PCR assay for the genus *Orthopoxvirus*. We stress that the screening of large OPV strain collections is essential to demonstrate the usefulness and to establish the performance characteristics of assays being developed. In a recent paper (5), the investigators stated that mismatches in the fluorescence resonance energy transfer probes used in their assay enabled discrimination of VAR from other OPVs by DNA melting-curve analysis. Due to the new OPV sequences in GenBank, the fluorescence resonance energy transfer probes also display identity to CML and some CPX strains.

TABLE 5. Investigation of different amounts of purified VAR DNA by LightCycler PCR combined with melting-curve analysis

Amt of viral DNA (input [no. of genome copies)	Avg C_t/avg T_m (°C)[a]		
	Strain Bangladesh	Bangladesh spiked with 10 ng of human genomic DNA/reaction	Strain Variolator 4
1 ng (5.7×10^6)	15.49/57.21	15.63/56.68	15.09/56.74
100 pg (5.7×10^5)	18.89/57.24	18.91/56.73	18.52/56.85
10 pg (5.7×10^4)	22.50/57.35	22.48/56.98	21.91/57.02
1 pg (5.7×10^3)	25.80/57.44	25.92/57.02	25.49/57.14
100 fg (5.7×10^2)	29.06/57.55	29.34/57.23	28.88/57.29
10 fg (5.7×10^1)	32.71/57.80	32.49/57.55	31.87/57.51
1 fg (5.7)	35.43/58.11	35.22/57.99	34.91/58.07

[a] C_t, the average cycle at which the fluorescence crossed the threshold. Values represent the means of triplicate reactions.

Analysis of such strains must be conducted to prove whether the reliable identification of VAR is still possible. In the assay described here, we included a rather large number ($n = 40$) of CPX strains, since viruses of this species displayed considerable genetic heterogeneity (17) and have a large genome (ca. 220 kbp) containing sequences previously considered VAR specific (15).

Nevertheless, we want to stress that a positive PCR result for VAR must be confirmed by amplifying other parts of the OPV genome. In this respect, the hemagglutinin gene has been the most comprehensively characterized, and a VAR-specific real-time PCR assay was recently described (8). In addition, the use of classical techniques, such as viral culture and electron microscopy, will enhance the confidence in the final diagnostic results.

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2003. Update: multistate outbreak of monkeypox, Illinois, Indiana, Kansas, Missouri, Ohio, and Wisconsin, 2003. Morb. Mortal. Wkly. Rep. **52:**642–646.
2. **Czerny, C.-P., S. Johann, L. Hölzle, and H. Meyer.** 1994. Epitope detection in the envelope of intracellular naked orthopox viruses and identification of encoding genes. Virology **200:**764–777.
3. **Damon, I. K., and J. J. Esposito.** 2003. Poxvirus infections, p. 1583–1591. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. ASM Press, Washington, D.C.
4. **Esposito, J. J., and J. C. Knight.** 1985. Orthopoxvirus DNA: comparison of restriction profiles and maps. Virology **143:**230–251.
5. **Espy, M. J., F. R. Cockerill, F. R. Meyer, M. D. Bowen, G. A. Poland, T. L. Hadfield, and T. F. Smith.** 2002. Detection of smallpox virus DNA by LightCycler PCR. J. Clin. Microbiol. **40:**1985–1988.
6. **Gavrilova, E. V., I. V. Babkin, and S. N. Shchelkunov.** 2003. A multiplex PCR assay for species-specific rapid identification of orthopoxviruses. Mol. Genet. Mikrobiol. Virusol. **1:**45–52.
7. **Henderson, D. A.** 1999. Looming threat of bioterrorism. Science **283:**1279–1289.
8. **Ibrahim, M. S., D. A. Kulesh, S. S. Saleh, I. K. Damon, J. J. Esposito, A. L. Schmaljohn, and P. B. Jahrling.** 2003. Real-time PCR assay to detect smallpox virus. J. Clin. Microbiol. **41:**3835–3839.
9. **Ibrahim, M. S., J. J. Esposito, P. B. Jahrling, and R. S. Lofts.** 1997. The potential of 5′ nuclease PCR for detecting a single-base polymorphism in orthopoxvirus. Mol. Cell. Probes **11:**143–147.
10. **Knight, J. C., R. F. Massung, and J. J. Esposito.** 1995. Polymerase chain reaction identification of smallpox virus, p. 297–302. *In* Y. Becker and G. Darai (ed.), Diagnosis of human viruses by polymerase chain reaction technology, 2nd ed. Springer-Verlag, Berlin, Germany.
11. **Laassri, M., V. Chizhikov, M. Mikheev, S. Shchelkunov, and K. Chumakov.** 2003. Detection and discrimination of orthopoxviruses using microarrays of immobilized oligonucleotides. J. Virol. Methods **112:**67–78.
12. **Lapa, S., M. Mikheev, S. Shchelkunov, V. Mikhailovich, A. Sobolev, V. Blinov, L. Babkin, A. Guskov, E. Sokunova, A. Zasedatelev, L. Sandakhchiev, and A. Mirzabekov.** 2002. Species-level identification of orthopoxviruses with an oligonucleotide microchip. J. Clin. Microbiol. **40:**753–757.
13. **Loparev, V. N., R. F. Massung, J. J. Esposito, and H. Meyer.** 2001. Detection and differentiation of Old World orthopoxviruses: restriction fragment length polymorphism of the *crmB* gene region. J. Clin. Microbiol. **39:**94–100.
14. **Marennikova, S. S., and S. N. Shchelkunov.** 1998. Orthopoxviruses pathogenic for humans. KMK Press Ltd., Moscow, Russia.
15. **Meyer, H., H. Neubauer, and M. Pfeffer.** 2002. Amplification of variola virus-specific sequences in German cowpox virus isolates. J. Vet. Med. Infect. Dis. Vet. Public Health **49:**17–19.
16. **Meyer, H., M. Perrichot, M. Stemmler, P. Emmerich, H. Schmitz, F. Varaine, R. Shungu, F. Tshioko, and P. Formenty.** 2002. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. J. Clin. Microbiol. **40:**2919–2921.
17. **Meyer, H., C. Schay, H. Mahnel, and M. Pfeffer.** 1999. Characterization of orthopoxviruses isolated from man and animals in Germany. Arch. Virol. **144:**491–501.
18. **Meyer, H., S. L. Ropp, and J. J. Esposito.** 1997. Gene for A-type inclusion body protein is useful for a polymerase chain reaction assay to differentiate orthopoxviruses. J. Virol. Methods **64:**217–221.
19. **Meyer, H., N. Osterrieder, and C.-P. Czerny.** 1994. Identification of binding sites for neutralizing monoclonal antibodies on the 14 kDa fusion protein of different orthopoxvirus species. Virology **200:**778–783.
20. **Mikheev M. V., S. A. Lapa, S. N. Shchelkunov, A. K. Chikova, V. M. Mikhailovich, A. Y. Sobolev, I. V. Babkin, D. A. Gryadunov, M. A. Bulavkina, A. A. Guskov, E. B. Sokunova, G. V. Kochneva, V. M. Blinov, L. S. Sandakhchiev, A. S. Zasedatelev, and A. D. Mirzabekov.** 2003. Species-specific identification of orthopoxviruses on oligonucleotide microchips. Voprosi Virusol. **48:**4–9.
21. **Moyer, R. W., B. M. Arif, D. N. Black, D. B. Boyle, R. M. Buller, K. R. Dumbell, J. J. Esposito, G. McFadden, B. Moss, A. A. Mercer, S. L. Ropp, D. N. Tripathy, and C. Upton.** 2000. Poxviridae, p. 137–157. *In* M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Castens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, G. J. McGeoch, C. R. Pringle, and R. B. Wickner (ed.), Virus taxonomy: classification and nomenclature of viruses. Seventh Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, Calif.

21a. **Niedrig, M., H. Schmitz, S. Becker, S. Günther, J. ter Meulen, H. Meyer, H. Ellerbrok, A. Nitsche, H. R. Gelderblom, and C. Drosten.** 2004. First international quality assurance study on rapid detection of viral agents of bioterrorism. J. Clin. Microbiol. **42:**1753–1755.

22. **Ropp, S. L., J. J. Esposito, V. N. Loparev, and G. J. Palumbo.** 1999. Poxviruses infecting humans, p. 1137–1144. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 7th ed. ASM Press, Washington, D.C.
23. **Ropp, S. L., Q. Jin, J. C. Knight, R. F. Massung, and J. J. Esposito.** 1995. Polymerase chain reaction strategy for identification and differentiation of smallpox and other orthopoxviruses. J. Clin. Microbiol. **33:**2069–2076.
24. **Shchelkunov, S. N., A. V. Totmenin, I. V. Babkin, P. F. Safronov, O. I. Ryazankina, N. A. Petrov, V. V. Gutorov, E. A. Uvarova, M. V. Mikheev, J. R. Sisler, J. J. Esposito, P. B. Jahrling, B. Moss, and L. S. Sandakhchiev.** 2001. Human monkeypox and smallpox viruses: genomic comparison. FEBS Lett. **509:**66–70.
25. **Shchelkunov, S. N., A. V. Totmenin, V. N. Loparev, P. F. Safronov, V. V. Gutorov, V. E. Chizhikov, J. C. Knight, J. M. Parsons, R. F. Massung, and J. J. Esposito.** 2000. Alastrim smallpox variola minor virus genome DNA sequences. Virology **266:**361–386.
26. **Shchelkunov, S. N., R. F. Massung, and J. J. Esposito.** 1995. Comparison of the genome DNA sequences of Bangladesh-1975 and India-1967 variola viruses. Virus Res. **36:**107–118.