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Rapid Detection and Differentiation of Human Pathogenic Orthopox Viruses by a Fluorescence Resonance Energy Transfer Real-Time PCR Assay

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Background: **The orthopox viruses that are pathogenic for humans include variola major virus (VAR), monkeypox virus (MPV), cowpox virus (CPV), and to a lesser extent, camelpox virus (CML) and vaccinia virus (VAC). PCR is a powerful tool to detect and differentiate orthopox viruses, and real-time PCR has the further advantages of rapid turnaround time, low risk of contamination, capability of strain differentiation, and use of multiplexed probes.**

Methods: **We used real-time PCR with fluorescence resonance energy transfer technology to simultaneously detect and differentiate VAR, MPV, CPV/VAC, and CML. An internal control generated by cloning and mutating the PCR target gene facilitated monitoring of PCR inhibition in each individual test reaction.**

Results: **Strain differentiation results showed little interassay variability (CV, 0.4–0.6%), and the test was 100-fold more sensitive than virus culture on Vero cells. Low copy numbers of DNA could be detected with ≥95% probability (235–849 genome copies/mL of plasma).**

Conclusions: **The real-time PCR assay can detect and differentiate human pathogenic orthopox viruses. The use of an internal control qualifies the assay for high sample throughput, as is likely to be needed in situations of suspected acts of biological terrorism, e.g., use of VAR.**

Orthopox viruses are antigenetically closely related vertebrate viruses, including variola major virus (VAR),[1] monkeypox virus (MPV), camelpox virus (CML), vaccinia virus (VAC), cowpox virus (CPV), and six other species not pathogenic for humans. Orthopox viruses are among the largest of all known viruses (140–450 nm; brick-shaped) and contain a 130- to 300-kbp genome of linear double-stranded DNA *(1)*. VAR and MPV are of special interest.

Smallpox was eradicated in 1979 but regained attention recently because of the potential use of its causative virus, VAR, as a bioterrorism agent. The virus is highly contagious, environmentally stable, and has fatality rates of 20–50% *(2)*. Human monkeypox is a similar but distinct disease that until recently occurred only in Central/West Africa. Person-to-person transmission of its causative agent, MPV, is less efficient, and the fatality rate is estimated to be 1–10% *(3)*. By mid-2003, MPV had been introduced in North America as the result of importation of rodents *(4)*. VAC was used worldwide as a vaccine to eradicate smallpox. Adverse events such as vaccinia necrosum or postvaccination encephalitis are rare, except in immunosuppressed persons *(5)*.

CPV is endemic in Europe and Asia. Infection in humans is self-limiting and localized. Severe courses have been described in immunosuppressed patients *(6)*.

CML can cause systemic and economically important disease in camels. It is still poorly understood, and infection in humans is rare.

In light of the serious public health concern, reliable tools for the detection and differentiation of orthopox

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[1] Nonstandard abbreviations: VAR, variola major virus; MPV, monkeypox virus; CML, camelpox virus; VAC, vaccinia virus; CPV, cowpox virus; FRET, fluorescence resonance energy transfer; HA, hemagglutinin gene; ENIVD, European Network for the Diagnosis of Imported Viral Diseases; and geq, genome equivalents.

viruses are needed. Once an orthopox virus is detected, it is important that the other human pathogenic species be reliably differentiated from VAR. Orthopox viruses are traditionally detected by electron microscopy, which does not allow differentiation among relevant species. Differentiation can be achieved by analyzing the morphology of pocks on infected chicken egg chorioallantoic membranes or the ceiling growth temperature of isolates. Serologic differentiation is also possible, as well as DNA endonuclease cleavage *(7)*. All of these procedures are lengthy and require experienced handling in highly specialized laboratories. PCR allows primarily detection and simultaneous genotyping of orthopox viruses *(8)*. However, a major drawback of conventional PCR assays is the laborious and time-consuming post-PCR processing. This problem can be overcome by real-time PCR technology, which allows detection of PCR products during the reaction by use of fluorescent DNA probes. With fluorescence resonance energy transfer (FRET) probes *(9, 10)*, real-time PCR is also capable of differentiating strains *(11)*. Recently, real-time assays for orthopox viruses have been described that target the hemagglutinin (HA) gene and differentiate VAR from the other orthopox viruses *(12, 13)*.

In this study we present an assay that uses FRET real-time technology for the detection, differentiation, and quantification of VAR, MPV, CPV/VAC, and CML in a single PCR run. The assay contains an internal inhibition control to detect the inhibitory effect that some clinical samples have on PCR, making it suitable for high-throughput application when large numbers of samples have to be tested.

Material and Methods

CELL CULTURE AND VIRUS STRAINS

MPV was grown on subconfluent Vero cells under 5% CO_2 in DMEM under biosafety level 4 conditions. VAC, CPV, and CML stock solutions were kindly provided by Dr. Hermann Meyer, Institute of Microbiology of the German Armed Forces (Munich, Germany).

PROFICIENCY STUDY SAMPLE PANEL

A panel of 15 lyophilized plasma samples to which gamma-irradiated, heat-inactivated orthopox viruses (MPV, CPV, VAC, CML, and ectromelia virus) had been added was provided by the European Network for the Diagnosis of Imported Viral Diseases (ENIVD; Berlin, Germany). Viral concentration in the samples after resuspension in water ranged from 5×10^7 to 5×10^1 genome copies/mL.

PLAQUE ASSAY

MPV was grown for 3 days on subconfluent Vero cells overlaid with 10 g/L methylcellulose in medium solution in 24-well cell culture dishes. The overlay was removed, and cells were stained with methylene blue to visualize plaques.

NUCLEIC ACID EXTRACTION

For DNA extraction, we used a QIAamp Viral RNA Mini Kit (specified by the manufacturer, Qiagen, as also for use on DNA viruses) according to the manufacturer's instructions.

PLASMID GENOTYPE STANDARDS

We ligated a 275-bp insert of the HA gene of MPV, CML, CPV, and VAC into plasmid vectors and cloned it in *Escherichia coli* by means of a pCR 2.1-TOPO TA cloning reagent set (Invitrogen). Plasmids were purified with a Nucleo Spin Plasmid reagent set (Macherey Nagel) and sequenced with BigDye terminator cycle sequencing chemistry (Applied Biosystems) on an automatic ABI 377 DNA sequencer (Applied Biosystems). Plasmids were quantified spectrophotometrically. To obtain a genotype standard for VAR, we introduced the required point mutation into the MPV standard plasmid by means of a QuickChange site-directed mutagenesis reagent set (Stratagene). The recommendations of the manufacturer were followed.

INTERNAL CONTROL PLASMID CONSTRUCT

The plasmid genotype standard for VAC was used to construct an internal control plasmid. Gene splicing by overlap extension was carried out with primers IcS (5′-atcgttcgttgagcgattagcagtattttgataattataccaaggataaaatatc-3′) and IcAs (5′-ctgctaatcgctcaacgaacgataaagaataatggaattgggctccttataccaag-3′) to introduce an alternative probe-binding site at nucleotide positions 178–221. The resulting constructs of the correct length were cloned by means of a pCR 2.1-TOPO TA cloning reagent set (Invitrogen) and processed as described above.

OLIGONUCLEOTIDE DESIGN

Sequence alignments were generated with default settings using the BLAST algorithm as provided by the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). GenBank accession number X65516 (VAR) served as the query sequence. Primers were chosen to facilitate amplification of all sequences in the alignment without base mismatches. Dual FRET probes were chosen within the 275-bp amplicon to allow differentiation of VAR, MPV, CML, and VAC/CPV based on sequence polymorphisms at the site of hybridization of the downstream probe (Fig. 1). The probe was homologous for VAC/CPV and contained one mismatch for MPV, two for VAR, and three for CML.

CONVENTIONAL PCR

A PCR protocol for amplification of the gene coding for the 32-kDa adsorption protein via open reading frame D8L of all orthopox viruses was adapted previously and optimized in our laboratory. The reaction volume of 25 μL contained 1× PCR buffer II, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), 3 mM $MgCl_2$, 200 μM each of the deoxynucleotide triphosphates, 400 nM

Virus strain	Oligonucleotide name, position	Accession No.
	OPS2, 17–40 / OPP1, 145–177 / OPP2-640, 179–199 / OPPAs4, 271–291	
	TACTTTTGTTACTAATATCATTAG//GCTTGGTATAAGGAGCCCAATTCXATTATTCTT//TAGCTGCTAAAAGCGACGTCT//AGCAGTCAATGATTTAATTGT	
Cowpox	----------//----------Y---------//----------//----------	Z99048
Vaccinia	----------//----------Y---------//----------//----------	Z99051
Camelpox	----------//----------//-----TC------T-------//----------	AF438165
Monkeypox	----------//----------//------C-------------//----------	AF380138
Variola major	----------//----------//------C------T-------//----------	X65516

Fig. 1. Aligned binding regions of oligonucleotides within the HA gene of orthopox viruses.

Representative virus strains are listed in the *left column*, and corresponding GenBank accession numbers are listed in the *right column*. The antisense primer sequence is given in coding sense, i.e., reverse complementary to the oligonucleotide actually used. A *dash* represents the same base as in the headline sequence. *Double slashes* represent residues that are not used for oligonucleotide hybridization. *X* represents an abasic ribose phosphate spacer nucleotide. Positions of oligonucleotides refer to GenBank accession no. X65516.

primer D8L forward [5′-atgccgcaacaactatctcct-3′ *(14)*], and 200 nM primer D8L reverse [5′-ctagttttgtttttctcgcgaa-3′ *(14)*]. Thermal cycling included 95 °C for 15 min, followed by 45 cycles of 95 °C for 20 s, 56 °C for 10 s, and 2 °C for 60 s. Products were visualized on agarose gels.

REAL-TIME PCR

Each 30-μL reaction contained 5 μL of DNA, 3.3 mM $MgCl_2$, 1× Platinum Taq polymerase reaction buffer (Invitrogen), 40 ng/μL bovine serum albumin (Sigma), 200 μM each of the deoxynucleotide triphosphates, 1 μM primer OPS3 (5′-tacttttgttactaatatcattag-3′), 1 μM primer OPAs4 (5′-agcagtcaatgatttaattgt-3′), 0.03 μM probe OPP1 (5′-gcttggtataaggagcccaattcXattattctt-3′, where X is a ribose phosphate spacer nucleotide), 0.2 μM each of probes OPP2-640 (5′-tagctgctaaaagcgacgtct-3′) and OPP2-705 (5′-atcgttcgttgagcgattagcag-3′), and 1 U of Platinum Taq DNA polymerase (Invitrogen). Probe OPP1 was 3′-labeled with fluorescein. Probes OPP2-640 and -705 were 3′-phosphorylated and 5′-labeled with the LCred640 and LCred705 dyes (Roche), respectively. Sealed LightCycler glass capillaries were used for amplification. Cycling conditions in a Roche LightCycler (Ver. 3.5) were as follows: 94 °C for 6 min and 40 cycles of 94 °C for 5 s, 51 °C for 10 s, and 72 °C for 15 s. Fluorescence was measured once per cycle at the end of the 51 °C segment.

A color compensation file was generated for all fluorescence measurements to compensate for the spectral overlap of the emission spectra between channels 2 and 3 of the LightCycler, as recommended by the manufacturer. The virus amplification signal was detected on fluorescence channel F2/Back-F1, and the internal control was detected on channel F3/Back-F1. Subsequent melting curve analysis involved an initial denaturation at 95 °C for 10 s, followed by an increase from 40 °C to 90 °C at a rate of 0.1 °C/s with continuous measurement of fluorescence. Melting points were calculated by the LightCycler operation software in a three-step procedure; the measured fluorescence signal was first plotted against the temperature to obtain a melting curve. The negative first derivative was then calculated to yield a maximum at the point of the strongest slope, termed the melting peak. Finally the maximum of a curve fit to the melting peak was recorded as the melting point.

STATISTICAL ANALYSIS

We examined different input concentrations of orthopox virus plasmid DNA to calculate the predicted proportion of positive results in replicate tests, using probit analysis as a nonlinear regression model. The Statgraphics plus (Ver. 5.0) software package was used for probit analysis.

Results

After suitable primers and probes had been selected, they were first tested with virus stock solutions and plasmid genotype standards containing the PCR target sequences from each respective virus's HA gene (Fig. 2). Melting peaks were congruent among MPV, CPV/VAC, and CML genomic DNA and the respective plasmid constructs, indicating that only the desired target region was amplified and detected. [For VAR, only a plasmid genotype standard could be used because of safety regulations *(15)*.] Because the FRET probes were completely homologous to VAC/CPV, the melting point of the 275-bp amplicon was the highest of all viruses (64.6 °C). The one mismatched base in the probe binding site of MPV

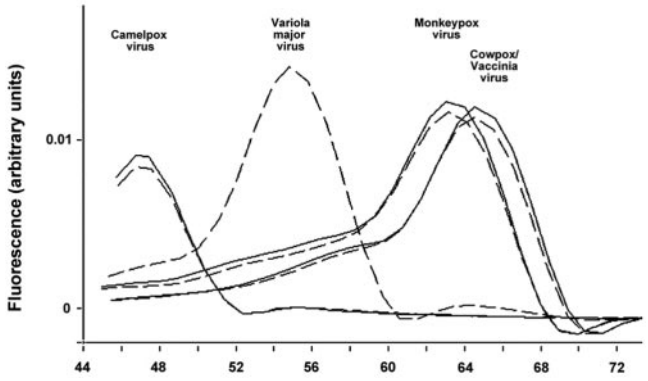

Fig. 2. Melting peak analysis of PCR products from four different orthopox genotypes, as depicted.

The melting peaks result from plotting the negative first derivative of the measured fluorescence emission (*y axis*) at a given temperature along the *x axis*. *Solid lines* represent melting peaks of DNA amplified from virus stock solutions; *dashed lines* represent melting peaks of amplification products from plasmids containing the corresponding viruses' PCR target sequences. For the VAR plasmid, the probe binding site in a 275-bp PCR product derived from MPV was mutagenized to contain the sequence present in VAR genomic DNA at these sites. Genomic VAR DNA was not available because of safety regulations (see text).

produced a melting point of 63.2 °C, the two mismatches in VAR shifted the melting point to 54.9 °C, and the three mismatches in CML gave a melting point of 47.0 °C. The interassay variability (CV) in strain differentiation was determined from the melting points observed for each plasmid in 10 independent test runs. CV were 0.4–0.6% for all genotypes. Box-plot analysis of the data indicated that the four possible melting points could be reliably differentiated, and there were no outlying data points (Fig. 3). VAR, for which differentiation is particularly important, had a particularly distinct melting point (~8 °C difference from CML and MPV).

Because our assay was intended for diagnostic use in a high-throughput setting, an internal control reaction was implemented that allowed in-tube detection of PCR inhibition without additional controls. A plasmid was constructed that contained the same primer binding sites as the virus but was detected by a heterologous downstream FRET probe (OPP2-705). The binding site for OPP2-705 had been introduced into the VAR plasmid by gene splicing and overlap extension, thereby removing the VAR probe binding site. After the concentration of the probe had been adjusted, 1 copy of the internal control plasmid could be amplified occasionally, and 20 copies were always detected without affecting the efficiency of amplification of the virus (data not shown).

To determine the limit of detection of our PCR compared with virus culture, we prepared serial 10-fold dilutions of a quantified MPV stock solution ranging from 15 000 to 0.015 plaque-forming units/mL in negative human plasma and subjected it to the plaque assay and real-time PCR. Dilution endpoints of 15 and 0.15 plaque-forming units/mL, respectively, were detectable. This corresponds to a ratio of DNA to infectious virus particles of ~100:1. To determine the limit of detection in a statistically precise way, we added plasmid genotype standards in five different concentrations to human plasma, and plasma containing each concentration was amplified in five replicate reactions ($5 \times 5 = 25$ reactions/genotype). Before extraction, the internal control plasmid was added to the lysis buffer at a concentration corresponding to 20 copies/PCR reaction. The observed proportions of positive results for each concentration for each genotype were subjected to probit regression analysis (Fig. 4). The following virus genome-equivalents (geq)/mL of plasma were calculated to be detectable at a ≥95% chance: VAR, 849 geq/mL; MPV, 779 geq/mL; VAC, 843 geq/mL; and CML, 235 geq/mL. These detection limits correspond to 2.74–9.88 copies/PCR vial.

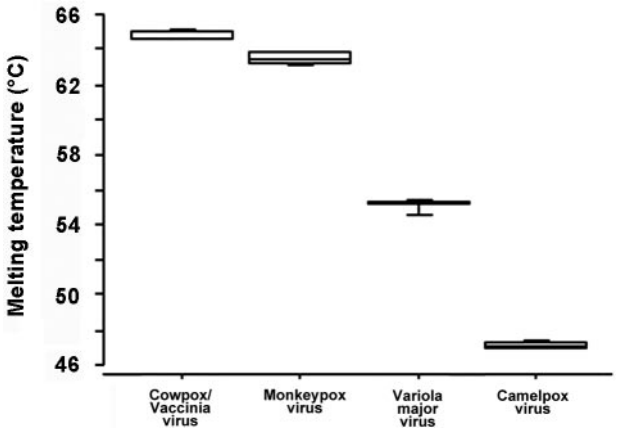

Fig. 3. Box-plot of the observed melting point temperatures (*y axis*) observed in 10 test runs for each orthopox virus genotype (*x axis*).

The *box* represents the innermost two quartiles of data points within the ranked cohort of melting points, whereas the *whiskers* delimit the range (outermost quartiles).

To mimic a real diagnostic situation, the novel assay was used in a blinded manner in a quality assurance study of the ENIVD. The sample panel contained different orthopox viruses added to human plasma. Virus concentrations ranged from 5×10^7 to 5×10^1 geq/mL, as indicated in data sheets provided by ENIVD. The real-time PCR detected virus in 14 of 15 virus-containing samples, whereas a conventional PCR assay gave only 11 of 15 correctly positive results. Results of strain differentiation in all samples were correct. The one sample that was not detected contained 3.6×10^6 copies/mL of ectromelia virus, a nonpathogenic orthopox virus for which our test had not been designed.

The specificity of our assay was confirmed by amplification of genomic DNA or RNA from 21 viral, bacterial, and fungal pathogens (Table 1) that might be present in clinical samples submitted for orthopox virus testing (skin, blood, and organs). No amplification was observed in any of these materials.

Discussion

In this report we describe a novel real-time PCR for the rapid detection and differentiation of human pathogenic orthopox viruses. Compared with conventional PCR methods, the turnaround time for the real-time PCR assay is decreased by 1–2 days, which in conventional PCR is spent on post-PCR processing and DNA sequencing. Real-time PCR technology minimizes the risk of PCR contamination *(16)*, a sensitive issue in such critical diagnostic settings. It also permits an inhibition control reaction to be performed in one tube along with the test reaction by use of multiplexed detection probes *(17, 18)*.

The HA gene chosen as the target sequence is highly specific for orthopox viruses: other pox viruses do not produce hemagglutinin *(1)*. The suitability of this target gene has already been described *(8, 12, 13)*. As in a previously published test intended only to separate VAR from other orthopox viruses *(12)*, we chose FRET probes for detection of PCR products. Our assay differentiated VAR from other orthopox viruses by a particularly distinct FRET probe melting point, making it less susceptible to interference from sample impurities. This is also reflected in the small interassay CV for the melting points. As an improvement, our test also identifies the other human pathogenic orthopox viruses. The relevance of this

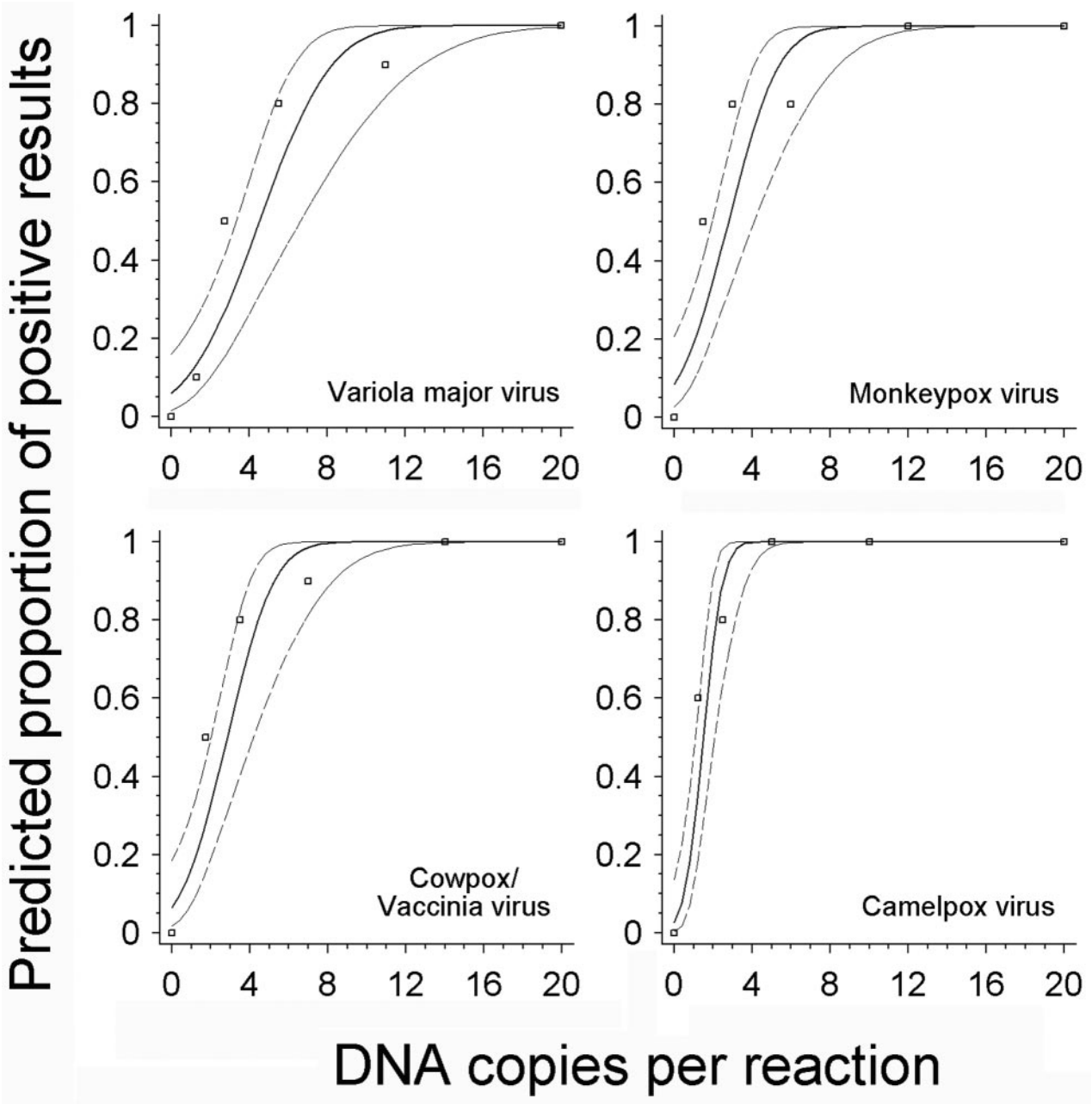

Fig. 4. Determination of PCR detection limits by probit regression analysis.

Defined amounts of plasmid genotype standards were added to negative human plasma and tested in parallel reactions. The experimentally determined fraction of positive reactions ($n_{positive}/n_{tested}$; *y axis*) at the corresponding DNA test concentration (copies/PCR reaction; *x axis*) is indicated by □. The calculated regression curves (*solid lines*) indicate the probability (*y axis*) of obtaining a positive result at any DNA concentration. The 95% confidence intervals for this probability are indicated by the *dashed lines*. The DNA concentration at which a positive result is achieved with a probability of 95% is given in the text.

feature has been illustrated by the recent introduction of a new orthopox virus (MPV) into North America *(4)*.

The availability of plasmid genotype standards for all human pathogenic orthopox species makes it particularly easy to transfer our method to other laboratories. Moreover, in light of the new WHO safety regulations restricting work with variola virus DNA ≥500 bp in length *(15)*, the availability of an MPV-derived, mutagenized plasmid provides a practical and compatible positive control for VAR. We have shown in this and an earlier study *(11)* that such cloned DNA fragments have the same properties as genomic DNA in FRET melting point analysis.

Once established, the sensitivity of a new PCR assay must be validated to qualify it for diagnostic use. Classic virologic quantification methods, e.g., plaque assays, can be used as reference methods, and we have shown that our assay is 100-fold more sensitive than virus culture. However, the disproportion between infectious particles

Table 1. Pathogens testing nonreactive in the orthopox real-time FRET PCR assay.

Pathogen	Strain, isolate, or material
Human immunodeficiency virus type 1	NL4-3, tissue culture supernatant
Hepatitis C virus	1st International WHO NAT standard[a]
Herpes simplex virus type 1	Reference material no. 13017[b]
Human cytomegalovirus	Reference material no. 15005[b]
Epstein–Barr virus	Patient isolate[c]
Varicella zoster virus	Reference material no. 16017[b]
Hepatitis B virus	Reference material no. 11019[b]
Mycobacterium tuberculosis	In-house reference strain[d]
M. bovis	In-house reference strain[d]
M. avium	In-house reference strain[d]
M. ulcerans	In-house reference strain[d]
M. marinum	In-house reference strain[d]
Staphylococcus aureus	In-house reference strain[d]
S. epidermidis	In-house reference strain[d]
Streptococcus pneumoniae	ATCC[e] 6305
S. pyogenes	ATCC 19615
Corynebacterium diphtheriae	In-house reference strain[d]
Neisseria meningitides	In-house reference strain[d]
Haemophilus influenzae	ATCC 49247
Yersinia enterocolitica	ATCC 9610
Candida albicans	ATCC 10231

[a] Source: National Institute for Biological Standardisation and Control, Hertfordshire, UK.
[b] Source: Gesellschaft für Biotechnologische Diagnostik, Berlin, Germany.
[c] Kindly provided by Dr. N. Brattig, Bernhard Nocht Institute for Tropical Medicine.
[d] Strain collection of the Department of Bacteriology, Bernhard Nocht Institute for Tropical Medicine.
[e] ATCC, American Type Culture Collection.

and DNA copies in virus stock solutions may be problematic, making such results poorly comparable among laboratories. As an alternative approach we used quantified plasmid DNA to determine the analytical sensitivity in a statistically precise way, using probit analysis *(18, 19)*. Human plasma was chosen as a mimic for clinical samples because patients with smallpox are viremic during the preeruptive stage of the disease, a window of opportunity to prevent the spread of the disease by identification of infected patients. Use of plasma also allows more precise definition of the experimental target concentrations than do solid samples, e.g., skin crusts. The sensitivities achieved by our assay are comparable to those of commercial US Food and Drug Administration-cleared PCR assays *(20, 21)*. The good performance of our test in the international ENIVD quality-assurance study on PCR for orthopox viruses further underlines that our test is technically optimized.

In testing of patient samples, PCR inhibition may occur in a frequency of 0.34–2.1% of tests *(20, 21)*. The use of inhibition controls is critical to prevent false-negative results *(22)*. Use of an internal control that is copurified with the test samples and amplified with the same primers as the virus has been shown to be particularly efficient *(17, 18, 23)*. It reduces test costs and laboratory effort by requiring no additional reactions. This in turn qualifies the test for high sample throughput, which might occur in case of a suspected event of biological terrorism.

In conclusion, the described assay can provide support to physicians who suspect on clinical grounds an infection with pathogenic orthopox viruses. Its high analytical sensitivity and inhibition control could qualify the test for exclusion diagnostics. The availability of plasmid standards and validation data make it an easily transferable method. However, we must emphasize that a positive laboratory diagnosis of an orthopox viral infection requires further confirmation by classic techniques such as electron microscopy, serology, or viral culture, given the enormous consequences for the public health sector that some of these diseases entail.

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