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Species-specific identification of variola, monkeypox, cowpox, and vaccinia viruses by multiplex real-time PCR assay

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A method of one-stage rapid identification of variola (VARV), monkeypox (MPXV), cowpox (CPXV), and vaccinia (VACV) viruses, pathogenic for humans, utilizing multiplex real-time TaqMan PCR (MuRT-PCR) assay was developed. Four pairs of oligonucleotide primers and four hybridization probes with various fluorescent dyes and the corresponding fluorescence quenchers were concurrently used for MuRT-PCR assay. The hybridization probe specific for the VARV sequence contained FAM/BHQ1 as a dye/quencher pair; MPXV-specific, TAMRA/BHQ2; CPXV-specific, JOE/BHQ1; VACV-specific, Cy5/BHQ3. The specificity and sensitivity of the developed method were assessed by analyzing DNA of 29 strains belonging to six orthopoxvirus species as well as the DNA samples isolated from archive clinical specimens of human smallpox cases and experimental specimens isolated from CPXV-infected mice and MPXV-infected marmot.

1. Introduction

The genus *Orthopoxvirus* of the family *Poxviridae* contains four viral species pathogenic for humans, namely, variola virus (VARV), monkeypox virus (MPXV), cowpox virus (CPXV), and vaccinia virus (VACV). These orthopoxviruses are immunologically cross-reactive and cross protective, so that infection with any member of this genus, provides protection against an infection with any other member (Shchelkunov et al., 2005a,b).

Thirty years ago, the 33rd World Health Assembly declared the global eradication of smallpox and recommended that vaccination against smallpox be discontinued (Pennington, 2003). Thus, worldwide, almost all individuals below 30 years of age have no immunity against both smallpox (caused by VARV) and any other human orthopoxvirus infection. Older people, that had been vaccinated against smallpox or had recovered from smallpox, display a weakened immunity against orthopoxvirus infections (Gallwitz et al., 2003). As each year passes an increasing proportion of the world's population becomes susceptible to orthopoxvirus infections. Presumably, this is why the number of reported outbreaks of the human diseases caused by the zoonotic viruses MPXV (Learned et al., 2003; Levine et al., 2007; Parker et al., 2007; Reed et al., 2004; Rimoin et al., 2010), CPXV (Amer et al., 2001; Baxby et al., 1994; Blackford et al., 1993; Campe et al., 2009; Carletti et al., 2009; Honlinger et al., 2005; Marenikova et al., 1996; Ninove et al., 2009; Pelkonen et al., 2003; Stewart et al., 2000; Tryland et al., 1998), and VACV-like viruses (Abrahao et al., 2009; Bhanuprakash et al., 2009; Damaso et al., 2000; de Souza Trindade et al., 2007; Silva-Fernandes et al., 2009; Singh et al., 2006; Zafar et al., 2007) is increasing in several countries. The possibility of emergence of a VARV-like virus as a result of natural evolution of the existing zoonotic orthopoxviruses has been raised (Michaeli, 2002; Shchelkunov, 2009; Shchelkunov et al., 2005a,b) as well as the potential use of VARV as a bioterrorism agent (Bray and Buller, 2004; Henderson, 1999).

A multicomponent strategy of surveillance and containment is recommended in the case of a smallpox outbreak (Fenner et al., 1988). A critical component of this strategy is the early detection of a smallpox case. A delay in detection is likely to have a considerable impact on the overall control strategy.

Despite the clinically apparent manifestations of orthopoxvirus infections (skin lesions), they can be misdiagnosed. Orthopoxvirus infection with generalized rash are often diagnosed as chickenpox (varicella) or herpes simplex infection. Large solitary skin lesions caused by cowpox are often diagnosed as anthrax, if unusually located; eye lesions are frequently assigned to phlegmon. On the other hand, diseases caused by parapoxviruses, including milker's nodules, are often taken for cowpox (Shchelkunov et al., 2005a,b). In immunocompromised individuals, human cowpox may cause a generalized skin eruption (Blackford et al., 1993; Pelkonen et al., 2003) with lethal outcome in some cases (Czerny et al., 1991).

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Human monkeypox is always clinically problematical, because it is difficult to distinguish from smallpox (Nalca et al., 2005; Shchelkunov et al., 2005a,b).

In order to distinguish between these infections, particularly if smallpox is suspected, the main requirements for laboratory diagnostic tests are rapid results with high sensitivity and specificity. The most recent advance in the rapid diagnosis of orthopoxvirus infection has been provided by real-time polymerase chain reaction (PCR) assay. However, only genus-specific identification tests or tests to differentiate one of the four orthopoxvirus species pathogenic for humans (VARV; Aitichou et al., 2008; Fedele et al., 2006; Ibrahim et al., 2003; Kulesh et al., 2004a,b; Nitsche et al., 2004; Olson et al., 2004; Putkuri et al., 2009; MPXV, Kulesh et al., 2004a,b; Li et al., 2006; CPXV, Gavrilova et al., 2010; or VACV-like viruses, de Souza Trindade et al., 2008; Nitsche et al., 2005) have been designed so far using this method. It is evident that concurrent differentiation of VARV, MPXV, CPXV, and VACV in a single reaction mixture would be a more universal test which could find a wide application. A similar approach has been proposed using a multiplex PCR with subsequent electrophoresis of DNA amplification products (Shchelkunov et al., 2005a,b). In this work, a method for identification and species-specific differentiation of all four orthopoxvirus species pathogenic for humans in one reaction mixture of a multiplex real time TaqMan PCR assay is described.

2. Materials and methods

2.1. Viruses and DNA samples

The virus DNA specimens used in this work are listed in Table 1. All manipulations with the samples containing VARV were performed in a specialized high-level biocontainment laboratory certified for this type of work by the Russian control agency and WHO representatives as described earlier (Shchelkunov et al., 2005a,b). In addition, human DNA preparation (Medigen, Russia) was used. Sensitivity of the assay was determined using CPXV strain GRI-90 and VACV strain LIVP samples purified in sucrose density gradient.

Table 1
The list of virus strains whose DNA was used in multiplex real-time PCR.

Virus (family/genus/species)	Strain	Source of DNA
Poxviridae		
Orthopoxvirus		
Variola virus	Brazil 128	1
	Brazil 131	1
	Butler	1
	Congo 9	1
	India 4a	1
	India 71	1
	India 378	1
	Kuwait	1
	M-Gavr-60	1
	Ngami	1
	Rw-18	1
	13/62	1
	65/58	1
Monkeypox virus	CDC#v79-I-005	3
	CDC#v97-I-004	3
Cowpox virus	GRI-90	1
	OPV-Claus	2
	OPV-89/3	2
	OPV-89/4	2
	OPV-90/2	2
	OPV-90/5	2
	OPV-98/5	2
	88-Lunge	2
Vaccinia virus	Elstree 3399	1
	LIVP	1
	Western Reserve	1
Ectromelia virus	K1/2	1
	MP-2	2
Camelpox virus	CP-5	2
Leporipoxvirus		
Myxoma virus	Lausanne	4
Avipoxvirus		
Fowlpox virus	FP9	5
Herpesviridae		
Simplexvirus		
Human herpesvirus 1	HF	6
Human herpesvirus 2	MS	6
Varicellovirus		
Human herpesvirus 3	VZV No4	6

Notes: (1) Virus DNAs were isolated from the strains deposited with the collection of the SRC VB Vector. Other viral DNAs were received; (2) from H. Meyer, Munich, Germany; (3) from J.J. Esposito, Atlanta, United States; (4) from G. McFadden, London, Canada; (5) from M. Skinner, Newbury, UK; (6) from M.A. Susloparov, SRC VB Vector.

2.2. Primer and probe design

To select oligonucleotide primers and fluorescently labeled hybridization probes, the currently available DNA genomic sequences of 85 orthopoxvirus (OPV) strains were aligned. The DNA nucleotide sequences of 84 OPV strains were extracted from the GenBank (http://www.ncbi.nih.gov) and the sequence of ectromelia virus strain Naval was obtained from http://www.sanger.ac.uk. The nucleotide sequences were aligned using the programs BioEdit v.7.0 and Muscle v.3.6. Species-specific regions were found for each OPV species. Within these regions, the primers and corresponding hybridization probes were selected. The criteria for selecting the primers were specific DNA amplification of each OPV species (VARV, MPXV, CPXV, or VACV) independently of the presence of other poxvirus DNA and the length of the produced amplicon not exceeding 250 bp. The potential oligonucleotide primers and hybridization probes were analyzed by the program Oligo v.6.31 and tested for the absence of homology to the nucleotide sequences of the other viruses and the human genome with the help of the program BLAST. The primer pairs and hybridization probes were also tested for the absence of mutual homology. All the oligonucleotides used in the work were synthesized in an ABI 3400 DNA/RNA Synthesizer (DNA sintez, Russia). Fluorescent dyes and the corresponding fluorescence quenchers were introduced into each hybridization probe. The hybridization probe specific for VARV sequence contained the dye/quencher pair FAM/BHQ1; the MPXV-specific, TAMRA/BHQ2; CPXV-specific, JOE/BHQ1; VACV-specific, Cy5/BHQ3.

2.3. Positive controls

The plasmids pVARV-A38R, pMPXV-B7R, pCPXV-D11L, and pVACV-B10R, containing fragments of VARV ORF A38R (Shchelkunov et al., 1993), MPXV ORF B7R (Shchelkunov et al., 2001), CPXV ORF D11L (Shchelkunov et al., 1998), and VACV ORF B10R (Goebel et al., 1990), respectively, were constructed. Virus DNA fragments were obtained by PCR, inserted into the vector pBluescript II SK (+) (Stratagene, USA), and transformed into *E. coli* strain Dh5αF′. Recombinant plasmids were isolated using a QIAprep Spin Miniprep Kit (QIAGEN, Germany). The presence of the target insert in the selected hybrid plasmids was verified by restriction analysis, PCR assay, and sequencing. The concentration of plasmids was determined spectrophotometrically in an Ultrospec 3000 pro (Biochrom, UK) spectrophotometer.

2.4. 5′ nuclease PCR assay

The PCR assay with cleavage of the 5′-terminal label (TaqMan assay) was conducted in a Real-Time PCR System 7500 (Applied

Table 2
Oligonucleotide primers and hybridization probes used in multiplex real-time PCR.

Virus	Viral gene	Oligonucleotide name	Oligonucleotide sequence
VARV	A38R	VARV_A38R_probe	FAM-5′-CGTTGATGGACACCACGTTTGTTATTA-3′-BHQ1
		VARV_A38R_forward	5′-TCTGTACTATGTGTTAAAAGATTCTACAA-3′
		VARV_A38R_reverse	5′-AATGTATCTGTTATAGTCAGCATACCC-3′
MPXV	B7R	MPXV_B7R_probe	TAMRA-5′-TGAATGAATGCGATACTGTATGTGTGGG-3′-BHQ2
		MPXV_B7R_forward	5′-ACGTGTTAAACAATGGGTGATG-3′
		MPXV_B7R_reverse	5′-AACATTTCCATGAATCGTAGTCC-3′
CPXV	D11L	CPXV_D11L_probe	JOE-5′-CCACAATCAGGATCTGTAAAGCGAGC-3′-BHQ1
		CPXV_D11L_forward	5′-AAAACTCTCCACTTTCCATCTTCT-3′
		CPXV_D11L_reverse	5′-GCATTCAGATACGGATACTGATTC-3′
VACV	B10R	VACV_B10R_probe	Cy5-5′-CAATGTGTCCGCTGTTTCCGTTAATAAT-3′-BHQ3
		VACV_B10R_forward	5′-GGCAATGGATTCAGGGATATAC-3′
		VACV_B10R_reverse	5′-ATTTATGAATAATCCGCCAGTTAC-3′

Biosystems, USA) device. The reaction mixture (25 μl) for a monoplex real-time PCR variant contained 2.5 μl 10 Ч TaqMan® Buffer A (Applied Biosystems, USA), 200 μM dNTP, 5 mM $MgCl_2$, 300 nM of each primer, 250 nM hybridization probe, 0.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems, USA), and 1 μl of analyzed DNA solution. The reaction mixture (25 μl) for a multiplex real-time PCR contained 2.5 μl 10 Ч TaqMan® Buffer A, 200 μM dNTP, 5 mM $MgCl_2$, four primer pairs (300 nM each), four hybridization probes (250 nM each), 0.5 U AmpliTaq Gold® DNA polymerase, and 1 μl of analyzed DNA solution. The PCR with recording of fluorescence intensity was performed according to the following protocol: 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 58 °C. The specimen is considered negative if there is no signal of fluorescence during 40 cycles.

3. Results

Selection of species-specific oligonucleotide primers and hybridization probes was performed by aligning the genomic DNA nucleotide sequences of 85 OPV strains belonging to six species: VARV, MPXV, CPXV, VACV, ectromelia virus (ECTV) and camelpox virus (CMLV). Based on these data, the species-specific regions in the genomes of these viruses were detected. For VARV, this was the *A38R* gene region (according to the VARV-IND strain classification; Shchelkunov et al., 1993); for MPXV, gene *B7R* (according to the MPXV-ZAI strain classification; Shchelkunov et al., 2001); for CPXV, *D11L* (according to the CPXV-GRI strain classification; Shchelkunov et al., 1998); for VACV, *B10R* (according to the VACV-COP strain classification; Goebel et al., 1990).

For each selected region, the oligonucleotide primers and the corresponding hybridization probes were calculated using the program Oligo (Table 2). Fig. 1 shows the localization of primers and hybridization probes in a selected species-specific region on the example of VARV genome. Each species-specific hybridization probe contained various fluorescent dyes, which provided for recording the amplification of the target virus DNA at a certain wavelength.

Initially, each primer pair and the corresponding hybridization probe were tested for specificity in a monoplex format using a limited set of orthopoxvirus strains. For this purpose, DNAs of the VARV strains Ngami, 65/58, Butler, and Congo 9; MPXV strains CDC#v79-I-005 and CDC#v97-I-004; CPXV strains GRI-90 and OPV-Claus; VACV strains Elstree 3399 and Western Reserve were used (Table 1). An extended panel of unrelated poxviruses, namely, rabbit myxoma virus (genus *Leporipoxvirus*) and fowlpox virus (genus *Avipoxvirus*), as well as human genomic DNA and the DNAs of herpes simplex (HHV-1 and HHV-2) and varicella (HHV-3) viruses (Table 1) were used as negative controls. In each of the four real-time PCR variants, amplification of the orthopoxvirus DNA samples of only one species was observed (data not shown).

The analytical specificity in a multiplex format was determined using a panel of DNA specimens of 29 strains belonging to six orthopoxvirus species (Table 1). Various VARV, MPXV, CPXV, and VACV strains were successfully identified in the multiplex real-time PCR (Fig. 2). The used mixture of primers gave no products in the PCR with DNA of unrelated poxviruses, namely, rabbit myxoma virus (genus *Leporipoxvirus*) and fowlpox virus (genus *Avipoxvirus*), as well as with human genomic DNA and the DNAs of the human exanthemic pathogens, such as herpes simplex (HHV-1 and HHV-2) and varicella (HHV-3) viruses (Table 1). Thus, the analytical specificity of the multiplex real-time PCR assay in the conducted experiments equaled 100%.

The diagnostic specificity was verified using 20 whole blood preparations of healthy humans. After isolation, the blood total DNA specimens were analyzed in the designed multiplex real-time PCR system, and none of the specimens gave a positive result.

The analytical sensitivity was assessed using the recombinant plasmids pVARV-A38R, pMPXV-B7R, pCPXV-D11L, and pVACV-B10R and the DNA samples isolated from the CPXV strain GRI-90 and VACV strain LIVP purified in sucrose density gradient. Tenfold dilutions in three independent replicates were prepared for each plasmid. The minimal detectable in MuRT-PCR assay plasmid quantity for VARV and MPXV was 20 copies per reaction; for CPXV, 50 copies per reaction; for VACV, 70 copies per reaction.

Sensitivity of the assay on full-sized viral DNAs was analyzed with the help of purified in sucrose density gradient preparations of CPXV strain GRI-90 and VACV strain LIVP with known physical titers determined by electronic microscopy. Viral DNAs were isolated using a QIAamp DNA Mini Kit (QIAGEN, Germany). The experiments demonstrated a reproducible detection of approximately 20 copies of the genomic CPXV DNAs and 90 copies of the genomic VACV DNAs.

Applicability of the designed assay to diagnosing purposes was tested using clinical and experimental samples. Archive clinical specimens obtained from human cases during the smallpox eradication program in 1970–1975 and stored in the Russian collection for over 35 years (Table 1) and scabs from human skin lesions formed after vaccination with VACV strain LIVP in 2000 were used. DNAs from these clinical samples were isolated as described (Shchelkunov et al., 2005a,b). The designed assay successfully detected VARV or VACV in these specimens.

The experimental specimens were obtained after intranasal infection of mice with CPXV strain GRI-90 (Gavrilova et al., 2010) or marmot with MPXV strain CDC#v79-I-005 (unpublished data). The blood samples were taken from the animals during 10 days after infection. DNAs were isolated by method described previously (Fedele et al., 2006). CPXV or MPXV were successfully detected in

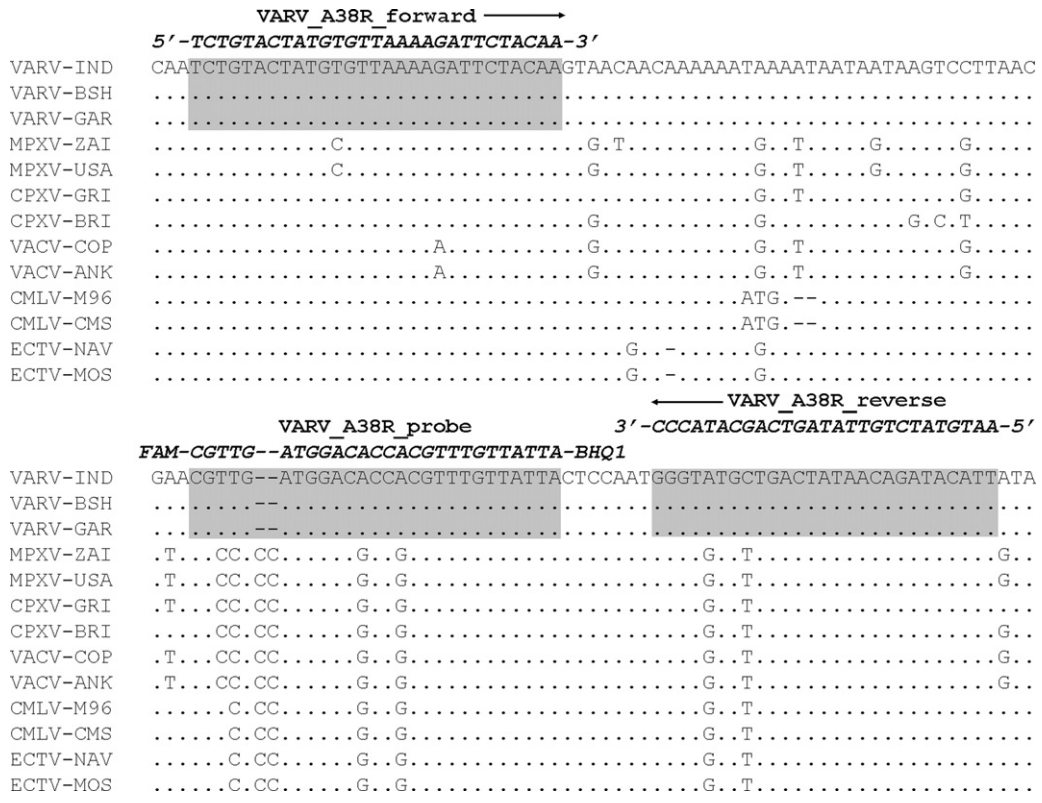

Fig. 1. Comparison of the ORF A38R nucleotide sequence of VARV strains India-1967 (VARV-IND), Garcia-1966 (VARV-GAR), and Bangladesh-1975 (VARV-BSH) with the corresponding regions of MPXV strains Zaire-96-I-16 (MPXV-ZAI) and USA_2003_039 (MPXV-USA); CPXV strains GRI-90 (CPXV-GRI) and Brighton Red (CPXV-BRI); VACV strains Copenhagen (VACV-COP), and Ankara (VACV-ANK); CMLV strains CMS (CMLV-CMS) and M-96 (CMLV-M96); ECTV strains Naval (ECTV-NAV) and Moscow (ECTV-MOS). The identical nucleotides in the compared sequences of virus genomes relative to the VARV-IND sequence are denoted with dots and the deletions, with dashes. The nucleotide positions in analyzed DNA segment are shown to the left and right of nucleotide sequence. The sequences of oligonucleotide primers VARV_A38R_forward and VARV_A38R_reverse and hybridization probe VARV_A38R_probe for fluorescence detection of VARV DNA are shown in boldfaced italic.

these samples. Nonspecific detection was not observed in any of these assays.

4. Discussion

The cessation of vaccination against smallpox after this disease was eradicated has led over the last 30 years to the situation when the major part of population on the globe has no immunity not only to smallpox, but also to other orthopoxvirus infections caused by the zoonotic viruses, such as MPXV, CPXV, and VACV. This is the reason why human infections caused by these viruses have recently become ever more frequent (Abrahao et al., 2009; Campe et al., 2009; Carletti et al., 2009; de Souza Trindade et al., 2007; Levine et al., 2007; Ninove et al., 2009; Parker et al., 2007; Rimoin et al., 2010; Silva-Fernandes et al., 2009; Singh et al., 2006; Zafar et al., 2007). An increasing abundance in human population of earlier relatively mild infections like monkeypox, cowpox, and buffalopox can enhance an accelerated evolution of zoonotic orthopoxviruses towards a more pathogenic and epidemiologically dangerous for humans VARV-like variant (Shchelkunov, 2009). Correspondingly, a rapid and reliable diagnosing of VARV, MPXV, CPXV, and VACV has become considerably more important.

Several approaches have been recently proposed for diagnosing orthopoxviruses: microarray assay (Fitzgibbon and Sagripanti, 2006; Laassri et al., 2003; Lapa et al., 2002; Ryabinin et al., 2006), PCR in combination with mass spectrometry (Grant et al., 2010; Eshoo et al., 2009), standard PCR (Humer et al., 2008; Shchelkunov et al., 2005a,b), and real-time PCR (Olson et al., 2004; Putkuri et al., 2009; Schroeder and Nitsche, 2009). Currently, real-time PCR is the most appropriate of these approaches for rapid clinical diagnosing, as it combines a relative simplicity of analysis and a high sensitivity.

TaqMan assay (Mackey et al., 2002) undoubtedly occupies a central position among the formats of real-time PCR. This approach to orthopoxvirus diagnosis was first applied to VARV (Ibrahim et al., 1997). So far, several TaqMan assay-based methods for detection of orthopoxvirus DNA have been developed (Aitichou et al., 2008; de Souza Trindade et al., 2008; Gavrilova et al., 2010; Ibrahim et al., 2003; Kulesh et al., 2004a,b; Nitsche et al., 2005; Scaramozzino et al., 2007). However, all these methods are intended for detecting orthopoxvirus DNA with subsequent differential detection of only one of the four orthopoxviruses pathogenic for humans. In this work, a method that makes it possible to differentiate four species of the orthopoxviruses pathogenic for humans concurrently with their detection was proposed. This considerably accelerates the assay. The multiplex format also provides for unification of the analytical procedure, since the same protocol and reaction conditions are used for all pathogens.

An important part of the work was the selection of the species-specific regions to be used for selecting the primers and hybridization probes. This stage determines the reliability of subsequent assays. When selecting species-specific genomic loci for orthopoxviruses, the terminal variable regions of the virus genome are of special interest (Shchelkunov et al., 2005a,b). Comparison of the nucleotide sequences of orthopoxvirus genomes demonstrates that these particular regions contain species-specific and strain-specific differences, which can be used for differential diagnosis. The species-specific regions selected in this work are localized to the terminal regions of the virus genomes. Within each of these regions, the primers and corresponding species-specific hybridiza-

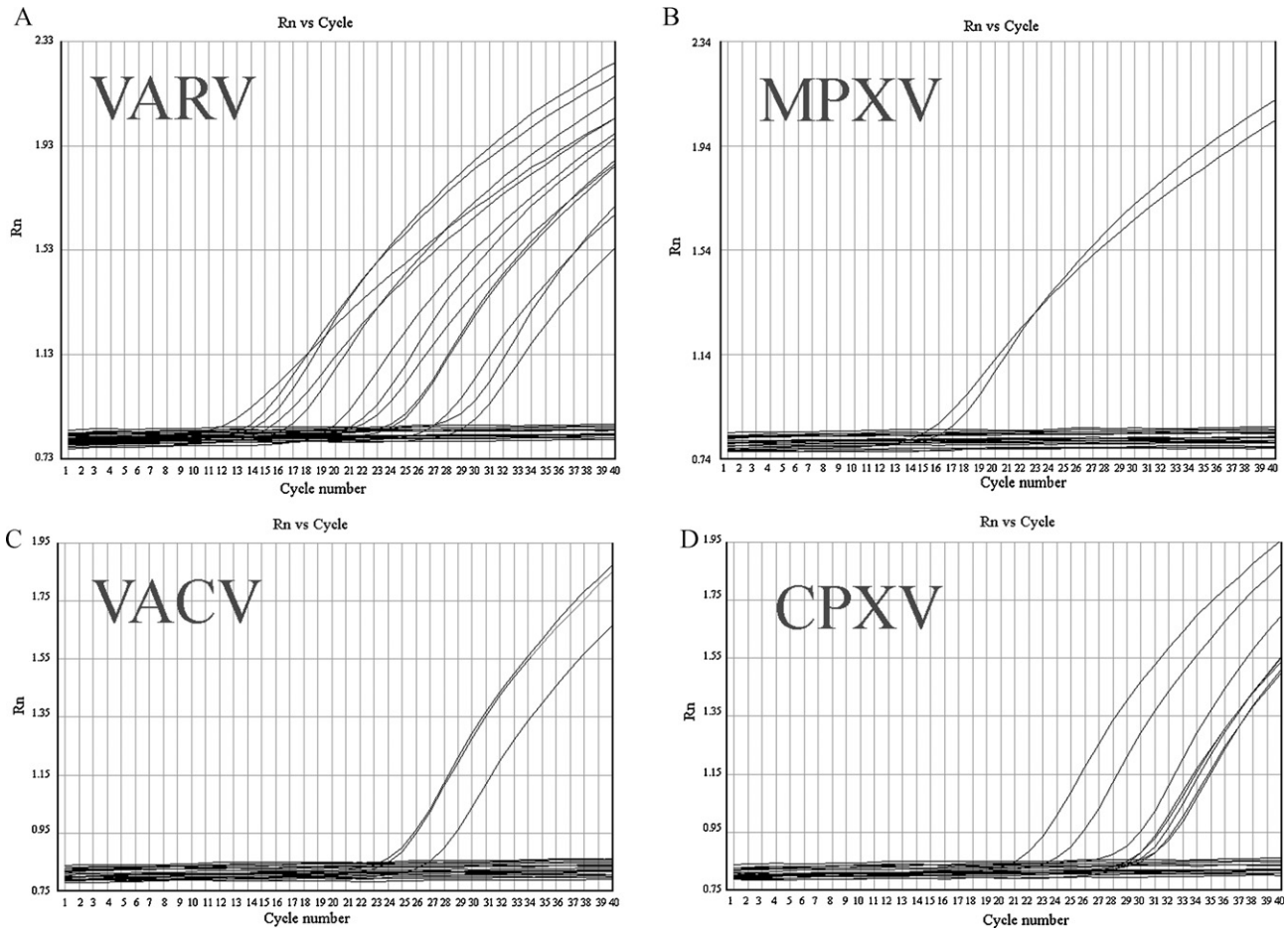

Fig. 2. The dependences of fluorescence signal on the number of cycles in multiplex real-time PCR. The data were obtained using a Real-Time PCR System 7500 (Applied Biosystems) device with the oligonucleotide primers and hybridization probes calculated for species-specific identification of orthopoxviruses (Table 2) and are shown for each optical channel used for fluorescence detection: (A) the signal of FAM dye conjugated with VARV-specific hybridization probe, 518 nm; (B) the signal of TAMRA conjugated with MPXV-specific hybridization probe, 580 nm; (C) the signal of Cy5 conjugated with VACV-specific hybridization probe, 643 nm; (D) the signal of JOE conjugated with CPXV-specific hybridization probe, 548 nm. The amplicons produced from DNAs of 29 strains belonging to six orthopoxvirus species and several control DNAs (Table 1) were analyzed simultaneously for 4 optical channels noted above. The lines above the horizontal band correspond to positive results and reflect accumulation level of the PCR products characteristic of each analyzed orthopoxvirus species (A–D) and the lines below the horizontal band, to the negative results for the remaining poxvirus and herpesvirus species. Cycle Number, the number of cycles in real-time PCR and Rn, the value of fluorescence signal.

tion probes were selected. The diagnostic robustness of the assay can be inferred by noting that the target loci were selected following the analysis of the genomes of 85 orthopoxvirus strains belonging to several species. The selection procedure for real-time PCR primers was similar to that used for a standard PCR; the main difference being the smaller length of the resulting amplicons, which do not exceed 250 bp. The hybridization probes were selected so that their melting temperature would exceed the melting temperature for primers by 8–10 °C (Mackey et al., 2002).

The initial experimental verification using a small number of orthopoxvirus strains allowed not only to confirm the appropriateness of selected primer pairs and hybridization probes for provision of PCR specificity, but also to determine the optimal identical conditions for conducting these PCR assays. The feasibility of implementing a multiplex format with a selected set of different fluorophores was also assessed.

The specificity in a multiplex format was determined using the panel of viral DNA specimens listed in Table 1. The applicability of the assay for diagnostic purposes was tested using clinical and experimental samples. Archive clinical specimens obtained from human cases during the smallpox eradication program in 1970–1975 and stored in the Russian collection for over 35 years were used. The assay successfully detected VARV in these specimens. Experimental samples were also obtained from mice intranasally infected with CPXV (Gavrilova et al., 2010) and marmots infected with MPXV (unpublished data). Blood samples were taken from the animals during a 10 day period after infection. CPXV or MPXV were detected in the mice or marmots, respectively, in all samples taken on days 3–10 after infection.

The sensitivity of the assay was determined using the constructed recombinant plasmids pVARV-A38R, pMPXV-B7R, pCPXV-D11L, and pVACV-B10R, containing fragments of the target nucleotide sequences of VARV, MPXV, CPXV, and VACV. These tests demonstrated a high sensitivity of the designed assay. An explanation for the difference in numerical values observed for the various orthopoxvirus species could be that there were different amplification efficiencies of the primers, as has been demonstrated in several works (Polz and Cavanaugh, 1998; Wagner et al., 1994). As the assay would ultimately be used to detect the corresponding viral DNAs in clinical samples, we determined the assay sensitivity using full-sized DNA isolated from characterized virus preparations. The results of these experiments with CPXV and VACV preparations indicated that the sensitivity of the assay is 20 copies of genomic CPXV DNA and 90 copies of genomic VACV DNA.

In conclusion, we have pioneered a one-step, rapid identification method for VARV, MPXV, CPXV, and VACV utilizing multiplex real-time PCR assay.

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