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Identification of Pan-Orthopoxvirus, Monkeypox-specific and Smallpox-specific DNAs by Real-time PCR Assay

Szu-Cheng Kuo*, and Yu-Ming Wang

Institute of Prevention Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China

Human pathogenic orthopoxvirus (OPV) including variola, monkeypox, vaccinia and cowpox is an important viral genus for human infection. Sharing highly similar viral protein sequences, the genus OPV poses difficulty to diagnosis using serological tests. Thus, molecular detection is an important method for quick detection and identification of orthopoxvirus infection. In this study, two novel targeting regions of VAR1 and VAR2 located on late 16-Kda putative membrane protein (*LPMP*) and 39-kDa core protein (*CP*) genes have been identified for developing PCR assays for OPV. SYBR green- and probe-based real-time PCR assays have been established for detecting and/or typing DNA of OPV. Two pan-OPV SYBR green-based PCR assays targeting *LPMP* and *CP* genes show high sensitivity in detecting pan-OPV DNAs in levels of 1 copy DNA/reaction or 10 pfu/ml. A pan-OPV probe-based real-time PCR assay targeting on *LPMP* gene also exhibits high sensitivity in detecting pan-OPV DNAs in levels of 1 copy DNA/reaction or 10 pfu/ml. Monkeypox- and smallpox-specific probe-based real-time PCR assays targeting *CP* genes can sensitively and accurately detect both important DNAs of human OPV, namely monkeypox and variola in the level of 10 copies and 1 copy DNA per reaction, respectively. This study provides alternatives for PCR detection of orthopoxvirus, monkeypox and smallpox DNA, which would be valuable for clinical diagnosis of human pathogenic orthopoxvirus infections and route laboratories.

Key words: orthopoxvirus, monkeypox, variola, real-time PCR

INTRODUCTION

Variola virus (VARV), monkeypox virus (MPXV), cowpox virus (CPXV) and vaccinia virus (VACV), classified into the genus orthopoxvirus (OPV) of the family Poxviridae, are pathogenic for humans, while other OPV including camelpox virus (CMXV), horsepox virus (HPXV), rabbitpox virus (RPXV) and ectromelia virus (ECTV) are non-pathogenic. VARV is the etiological agent of smallpox which has been eradicated. However, smallpox existing in particular laboratories remains a concern and the threat of smallpox in the world is not totally removed. Increasing human cases of MPXV, in Africa and USA,[1] CPXV in Europe[2] and VACV in India and Brazil[3-5] have been documented. Of note, MPXV infection is the most important human OPV infection since the eradication of smallpox in the 1970s.[6] VARV and MPXV can progress into lethal outcome in human infections. However, VARV infection shows a mortality rate exceeding 30% in unvaccinated individuals.[7] Immunocompromised individuals are more susceptible to CPXV and VACV infections with lethal outcome in some cases.[8] Importance of epidemiology, virology, ecology and public health of MPXV have been summarized.[9]

Sequence similarities provide immunological cross-reactivity and protection, but hamper the serological diagnosis on typing of OPV.[10,11] The immunity against smallpox decreased gradually in human population after cessation of anti-smallpox vaccination since 1980. Thus, the increasing susceptibility of OPV infection among human population led to the emergence of zoonotic OPV infections in recent years.[12] Early manifestations of four human pathogenic OPV share a common vesiculo-pustular rash illness on skin. Immunological cross-reactivity among OPV infections led to species-diagnostic difficulty using serological tests. Thus, quick species identification must rely on molecular diagnostics. Conventional PCR following restriction fragment length polymorphism assay has been employed for typing OPV species.[13,14] Microarray technology has also been employed for de-

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*Corresponding author: Szu-Cheng Kuo, Institute of Prevention Medicine, National Defense Medical Center, No. 172, Ta Po Road, Sanhsia, New Taipei 237, Taiwan, Republic of China. Tel: +886-2-81777038 ext 19946; Fax: +886-2-26736984; E-mail: szucheng@ndmctsgh.edu.tw

Table 1 Primer and probe sequence

assay	target gene	amplicon size	primer and probe		
			name	sequence	location position in MPXV or VARV
VAR1 pan-OPV SYBR green-based real-time assay	LPMP	163 bp in MPXV	VAR1-S1	CTGGTGTAGAGATAGCCGA	82469-82487 (MPXV, DQ011157.1)
			VAR1-A1	ATGGCTTCCGATTGGATTAC	82631-82612 (MPXV, DQ011157.1)
VAR2 pan-OPV SYBR green-based real-time assay	CP	202 bp in MPXV	VAR2-S1	GTCAACGCTGGAAGGAGTG	113449-113467 (MPXV, DQ011157.1)
			VAR2-A1	TCCAACCGCTACAACCAAC	113650-11363 (MPXV, DQ011157.1)
Pan-OPV probe-based real-time PCR assay	LPMP	362 bp in MPXV	VAR1-S1	CTGGTGTAGAGATAGCCGA	82469-82487 (MPXV, DQ011157.1)
			VAR1-A2	ATGCGTTGTTACCAGCCTC	82830-82812 (MPXV, DQ011157.1)
			Pan-OPV probe	FAM-GTAGCTACTCGTTTGGAATCACAAACATT-BBQ	82692-82720 (MPXV, DQ011157.1)
MPV specific probe-based real-time PCR assay	CP	217 bp in MPXV	VAR2-S1	GTCAACGCTGGAAGGAGTG	113449-113467 (MPXV, DQ011157.1)
			VAR2-A3	CCAGCAGACAGCCTATCC	113665-113648 (MPXV, DQ011157.1)
			MPXV-specific probe	LC640-CTCCTGTACTAAAACCACGWCAACAAACT-BBQ	113563- 113535 (MPXV, DQ011157.1)
VARV specific probe-based real-time PCR assay	CP	188 bp in VARV	VAR2-S1	GTCAACGCTGGAAGGAGTG	105276-105294 (VARV, DQ437582)
			VAR2-A3	CCAGCAGACAGCCTATCC	105462-105445 (VARV, DQ437582)
			VARV-specific probe	YAK-CGGTGTAGGTACCTGCAGTGGAGTTA–BBQ	105395-105420 (VARV, DQ437582)

tection and specific typing of OPV.[15] PCR combining electrospray ionization-mass spectrometry was used as a typing tool of OPV.[16] In recent years, real-time PCR has been shown to be applicable as a specific and sensitive detection method of OPV infection.[17-19] The loop-mediated isothermal amplification technology has been used for animal OPV diagnosis.[20-22] In this study, VAR1 and VAR2 target regions mapped onto late 16-Kda putative membrane protein (*LPMP*) and 39-kDa core protein (*CP*) genes were selected for primer and probe designs. Two pan-OPV SYBR green-based, one pan-OPV probe-based, one MPXV-specific probe-based, and one VARV-specific probe-based real-time PCR assays have been established for rapid detection and typing of OPV DNA. Two sensitive pan-OPV SYBR green-based PCR assays provide inexpensive alternatives for route laboratories.

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MATERIALS AND METHODS

Sequence alignment, primer and probe design

To determine locations of primers and probes on OPV genome, 50 genomic DNA sequences of OPV including VARV, MPXV, CPXV, VACV, CMXV, HPXV, RPXV and ECTV were extracted from GenBank (http://www.ncbi.nih.gov). Segmented viral DNA sequences were aligned using the Lasergene suite. According to criteria of consensus flanking region and diverse internal region, two target sequences located on 82469-82830 and113448-113665 of MPXV (DQ011157.1) were mapped. The hydrolysis probes for pan-OPV, MPXV-specific and VARV-specific contain the dye/quencher pair of FAM/BBQ, LC640/BBQ, YAK/BBQ respectively. Sequence specificity of specific probes was confirmed by NCBI BLAST. Primers and probes were synthesized from MWG and Roche, respectively. The detailed information of primers and probes of real-time PCR assay are displayed in Table 2.

Positive control and nucleic acid extraction

The plasmids pSVARV, pSMPXV, pSCPXV and pSVACV contain two synthetic target DNAs of VARV, MPXV, CPXV and VACV, respectively. The above synthetic fragments were inserted into XbaI/EcoRI sites on PUC57 plasmid, and transformed into *E. coli* strain DH5α. Single colonies harboring an individual target sequence were grown overnight in Luria Bertani (LB)/ampicillin broth at 37 ^{o}C. Control plasmids were purified using a QIAprep Spin Midi/Maxiprep Kit (Qiagen, Hilden, Germany). The concentration of plasmids were determined using an UD800 spectrophotometer (BECMAN CULTER, US). The number of copies of a template is determined using the calculator provided by a website.[23] Viral DNAs of Vaccinia virus LC strain, MVA strain and WR strain were extracted from the respective viruses according to standard procedures using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). To determine the LOD (limit of detection) of serial diluted viral DNA, 3 ul viral DNA per reaction was tested.

Viral culture and Plaque assay

Table 2 The control plasmid of pSVARV, pSMPXV, pSCPXV and pSVACV contains a concatenate VAR1 (in black) and VAR2 (in blue) regions from VARV (Variola virus strain Syria 1972 V72-199, DQ437592.1; 73940-74290, 104943-105145), MPXV (Monkeypox virus strain USA_2003_039, DQ011157.1; 82469-82830, 113448-113665), CPXV (Cowpox virus strain Germany 91-3, DQ437593.1; 98723-99090, 129719-129969) and VACV(Vaccinia virus Ankara strain chorioallantois vaccinia virus Ankara, AM501482.1; 82970-83358, 113966 to 114183) respectively. The above synthetic fragments were inserted into XbaI/EcoRI sites on PUC57, and transformed into *E. coli* strain DH5α.

pSVARV	ctggtgtagagatagccgatagatctgttatttcagtcgctgattaatcaattagtagagatgagataagaacataataatatatcttatatctcgtttagaaaaaagct tatattaaaatagctaacgctagtaatccaatcggaagccatttgatatctataatagggtatctaatttcctgattcagatagcggacagctatattctctgtagctac tcgtttggaatcacaaacattatttacatctaatttactatctgtaatggaaacgtttcccaatgaaatggtacaatcagatacattacatcttgatatatttttttttaaaga ggctggtaacaacgcatggatccgtcaacgctggaaggagtgaactcggttgtgtattatcatctgccgacaaccaatcaaaaagcttagacatatccgatgatg tattagtttgttgttgtcgtggttttggtgtaggagccggtgtggtacctgcagtggagttatatgaatagttggttgtagcggttggataggctgtctgctgg
pSMPXV	ctggagtagagatagccgatagatctgttatttcagtcgctgattaatcaattagtagagatgagagataagaccattataataatcaataatatatcttatatctgttta gaaaaatgctaatattaaaaaagcttacgctagtaatccaatcggaagccatttgatatctataatagggtatctaatttcctgattcagatagcgtacggctatattct cggtagctactcgtttggaatcacaaacattatttacatctaatttactatctgtaatggaaacgtttcccaatggaatggtacaatcagatacattacatcttgatatatt ttttttaaagaggctggtaacaacgcatggatccgtcaacgctggaaggagtgtactcgccggcgcatcagtatctgtagacaaccaatcaaaaagcttagaca tatcggatgatgtattagtttgttgacgtggttttagtacaggagcagtactactaggtagaagaataggagccggtgtaggtgtcggaactgggtgtggagttata tgaatagttggttgtagcggttgggtaggctgtctgctgg
pSCPXV	ctggagtagagatagccgatagatctgttatttcagtcgctgattaatcaattaatagagatgagataagaacattataataarcaataatatatcttatatcttatatct cgtttagaaaaatgccaatattaaaatagctaacgctagtaatccaatcggaagccatttgatatctataatagggtatctaatttcctgattcagatagcggacggc tatattctcagtagctactcgtttggaatcacaaacattatttacatctaatttgctatctgtaatggaggcgtttcccaatgaaatggtacaatccgatacattgcatctt gatatatttttttttaaagaggctggtaacaacgcatggatccgtcaacgctggaaggagtgaactcggttgtgtattatcatctgccgacagccaatcaaaaagct taggcatatccgatgatgtattagtttgttgtcgtggttttggtgtaggagcagtactactaggtgtaagaataggagcagtactactaggtgtcagaataggagcc ggtgtaggtgtcggaatcggaaccggctgtggagttatatgaatagttggttgtagcggttggataggctgtctgctgg
pSVACV	ctggagtagagatagccgatagatctgttatttcagtcgctgattaatcaattagtagagatgagataagaacattataataatcaataatatattttatatcttatatctt atatcttatatcttatatcttgtttagaaaaaagcttatattaaaatagctaacgctagtaatccaatcggaagccatttgatatctataatagggtatctaatttcctgattt aaatagcggacagctatattctcggtagctactcgtttggaatcacaaacattatttacatctaatttactatctgtaatggaaacgtttcccaatgaaatggtacaatc cgatacattgcattttgttatattttttttttaaagaggctggtaacaacgcatggatccgtcaacgctggaaggagtgaactcgccggcgcatcagtatcttcagaca gccaatcaaaaagcttagacatatcagatgatgtattagtttgttgtcgtggttttggtgtaggagcagtactactaggtagaagaataggagccggtgtagctgtt ggaaccggctgtggagttatatgaatagttggttgtagcggttggataggctgtctgctgg

Vero cells were infected with Vaccinia virus at a multiplicity of infection (M.O.I.) of one and then cultured for two days in Dulbecco's Modified Eagles' Medium (DMEM) (Invitrogen-GIBCO, Carlsbad, CA, USA) with 5% serum. For plaque assay, 2×10^5 Vero cells cultured in DMEM with 5% serum were seeded in each well of the 6-well plate. Overnight, 0.4 ml DMEM containing 10-fold diluted virus was transferred to a well at 37 ^{o}C for 1 h. Cells were overlaid with mixture (1xDMEM, 2% serum and 1% SeaPlaque agarose) and cultured at 37 ^{o}C in a CO_2 incubator. 2 days post-infection, the number of plaque was counted after crystal violet staining.

PCR reaction, SYBR Green I detection and 5' nuclease PCR assay

In conventional PCR reaction, the reaction mixture (50 μl) contains 10 ul of Phusion High-Fidelity DNA Polymerase (New England Biolabs) 5X reaction buffer, 0.5 pmol of each primer, 0.5 ul Phusion DNA Polymerase and DNA template (10^7 copies plasmid /reaction or in 10^6 pfu viral genomic DNA / reaction) obtained under the following mode: activation, 95 ^{o}C for 15 min; amplification, 40 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C, 10 s at 72 ^{o}C; and the final stage of 10 min at 72 ^{o}C on MJ Research PTC 200 Thermal Cycler. PCR products were separated with 3%MetaPhor® agarose electrophoresis, and then stained with 0.5 μg ethidium bromide/ml in conventional PCR product. The UVP AutoChemi Image System was used for capturing and processing images of PCR products. In SYBR Green I detection, the reaction mixture (10 ul) contains 5 ul of 2x QuantiTect SYBR Green PCR kit master mix (Qiagen, Hilden, Germany), 0.5 pmol of each primer and DNA template obtained under the following mode: activation, 95 ^{o}C for 15 min;

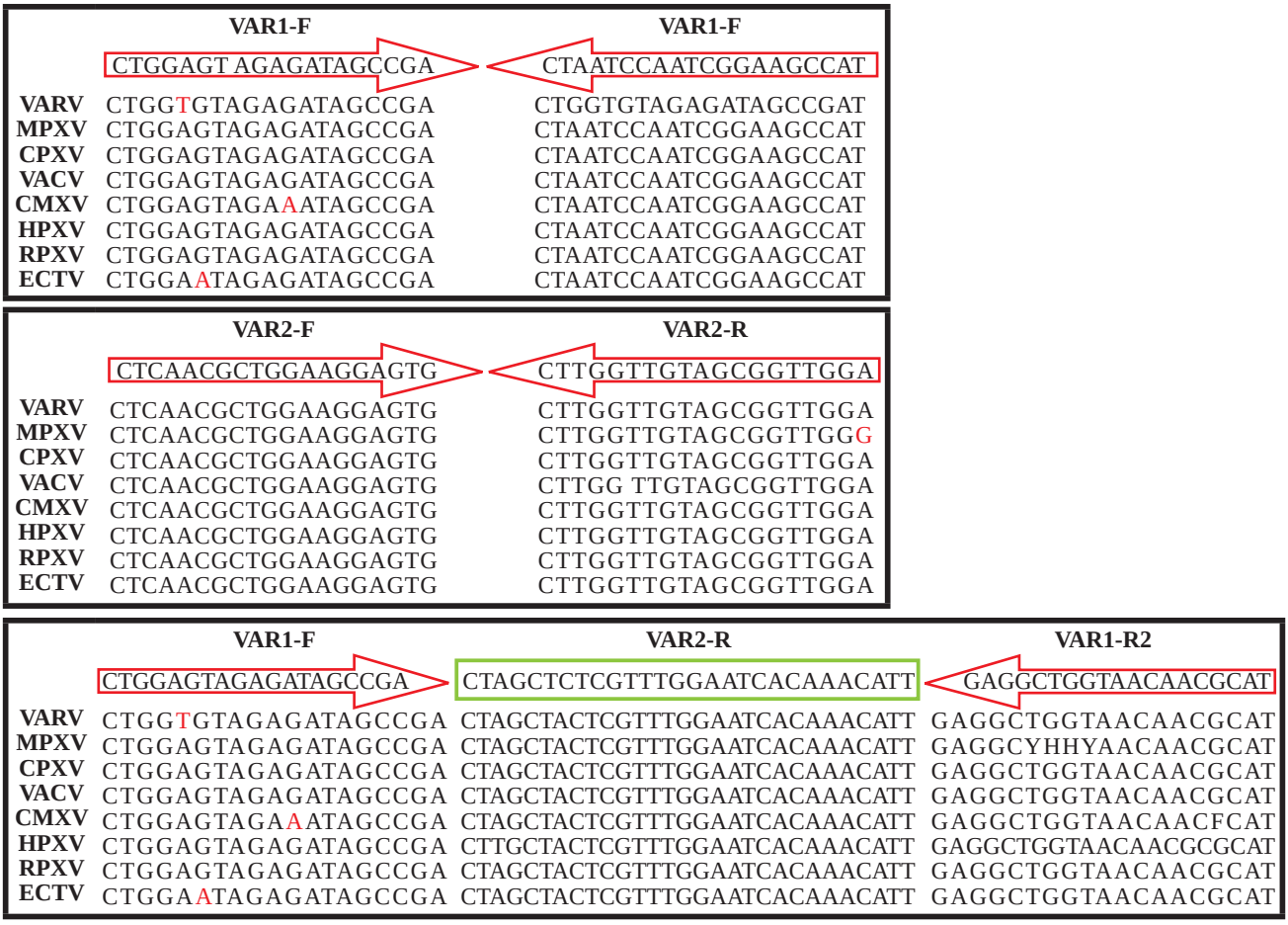

Fig. 1 Primer and probe sequence alignment in real-time PCR assays of pan-OPV. Conserved oligonucleotide sequences of VAR1 and VAR2 pan-OPV SYBR green-based real-time assays as well as pan-OPV probe-based real-time PCR assay were displayed in top, middle and bottom panel, respectively. Primer sequences were shown inside the red arrows. The highly conserved sequence of pan-OPV probe was shown in the green box. Abbreviations of OPV and GenBank accession numbers held by the National Center for Biotechnology Information Database are as follows: VARV, variola virus (DQ437582); MPXV, Monkeypox virus (DQ011157); CPXV, Cowpox virus (DQ437593); Vaccinia virus, VACV (AF095689); CMXV, Camelpox virus (AY009089); HPXV, Horsepox virus (DQ792504); RPXV, Rabbitpox virus (AY484669); ECTV, Ectromelia virus (NC004105). Red letters indicate mismatch with primer.

amplification, 45 cycles of 15 s at 95 °C, 25 s at 55 °C and 20 s at 68 °C. Subsequent melting curve analysis was conducted with 5 s at 95 °C, 15 s at heating to 65 °C with a ramping rate of 0.1 °C/s. In 5' nuclease PCR assay, the 10-ul reaction mixture contains 5 ul of QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany), 0.5 pmol of each primer, 2.5 pmol of each hybridization probe and DNA template obtained using the following mode: activation, 95 °C for 15 min; amplification, 45 cycles of 15 s at 95 °C, 20 s at 54 °C, and 25 s at 68 °C; and the final stage of 5 s at 40 °C. In order to mimic the normal condition, 10 ng genomic DNAs of human and monkey per ml existed in the serial dilution buffer. The threshold cycle (*Ct*) was defined using the default run mode. LOD was determined from serial dilutions of control plasmid or viral DNA. Roche LightCycler Version 2.0 with Lightcycler Relative Quantification Software Version 4.05 and LightCycler 480 Instrument II with software of version 1.5.0 SP3 were used for real-time PCR performance and analysis.

RESULTS

The aim of this study was to apply real-time PCR assay for detection of OPV as well as typing of VARV and MPXV. According to sequence analysis of 50 OPV, two target regions mapped on *LPMP* and *CP* genes were

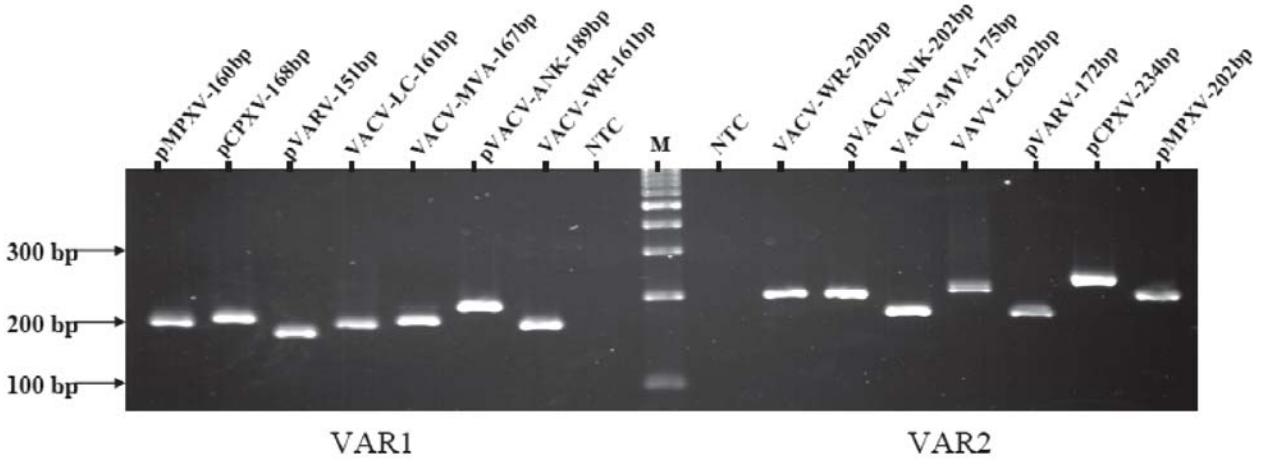

Fig. 2 Electrophoresis analysis of VAR1 and VAR2 PCR products. VAR1 (left) and VAR2 (right) PCR products from PCR reaction were separated with 3%MetaPhor® agarose electrophoresis. OPV abbreviations following an expected size of PCR product were displayed on the top. NTC stands for no template controls. The size of 100-bp DNA marker (M) was indicated on the left. Templates of pMPXV-160 bp, pCPXV, pVARV and pVACV-ANK (Vaccinia virus ANKARA strain) were from control plasmid in 107 copies/reaction. Templates of VACV-LC (Vaccinia virus LC strain), VACV-MVA (Vaccinia virus MVA strain) and VACV-WR (Vaccinia virus WR strain) were from viral genomic DNA in 10^6 pfu / reaction.

suitable for design of primers and probes (Table1). In the absence of authentic variola, monkeypox, vaccinia virus Ankara strain and cowpox viral DNAs, artificial control DNAs containing two target regions were generated by gene synthesis. The target sequence of pSVARV, pSMPXV, pSCPXV and pSVACV contains a concatenate VAR1 and VAR2 regions from VARV, MPXV, CPXV and VACV, respectively (Table 2). These control plasmids were applied for initial evaluation and optimization of assays. The detailed information about target gene, amplicon size and oligonucleotide sequences of PCR assays are summarized in Table 1.

1. Detection of pan-OPV DNA by VAR1 and VAR2 SYBR green-based real-time PCR assays

Two common OPV assays were designed for detection of pan-orthopoxvirus DNAs. The highly conserved primer sequences targeting *LPMP* and *CP* genes among OPV (Figure 1, top and middle panels) indicate that these two primer-pairs have potential to amplify pan-OPV's DNAs by PCR reaction. Electrophoresis analysis of VAR1 and VAR2 PCR products amplified from templates of pSMPXV, pSCPXV, pSVARV, pSVACV or viral DNAs of VACV reveals an expected-size of PCR product in individual reaction. No DNA signal was observed in NTC, indicating the absence of primer dimer under this reaction condition (Figure 2). Although VARV presented a single base mismatch (base 5, A→T) in VAR1 forward primer and MPXV presented (base 19, A→G) in VAR2 reverse primer (Figure 1), amplification was observed for all OPXV samples tested including MPXV, CPXV, VARV and some VACV strains described. Even though ECTV, CMXV, HPXV and RPXV DNA were not tested in this study, sequence alignments reveal that the above non-human OPV should be amplified by the generic reaction just as human OPV (Figure 2). In altogether, above reaction conditions were suitable for further development of SYBR green-based real-time PCR assay.

On the VAR1 SYBR green-based real-time PCR assay (Figure 3A, left), the forward primer has a single internal mismatch with VARV (base 5, A→T), CMXV (base 11, G→A) and ECTV (base 6, G→A). However, amplification of 10-fold serial dilutions of pSMPXV revealed a linear detection range from 10^7 to 10^0 copies per reaction, with an efficiency of 1.84, indicating an efficient PCR reaction. As for the VAR2 SYBR green-based real-time PCR assay (Figure 3A, right), the reverse primer has a single internal mismatch with MPXV (base 19, A→G). Amplification of 10-fold serial dilutions of pSMPXV, which is a single internal mismatch with reverse primer, also revealed a linear detection range from 10^7 to 10^0 copies per reaction, with an efficiency of 1.9, indicating an efficient PCR reaction. Two assays showed an ability to detect 1 copy of plasmid per reaction. The sensitivity and specificity of SYBR green-based real-time PCR as-

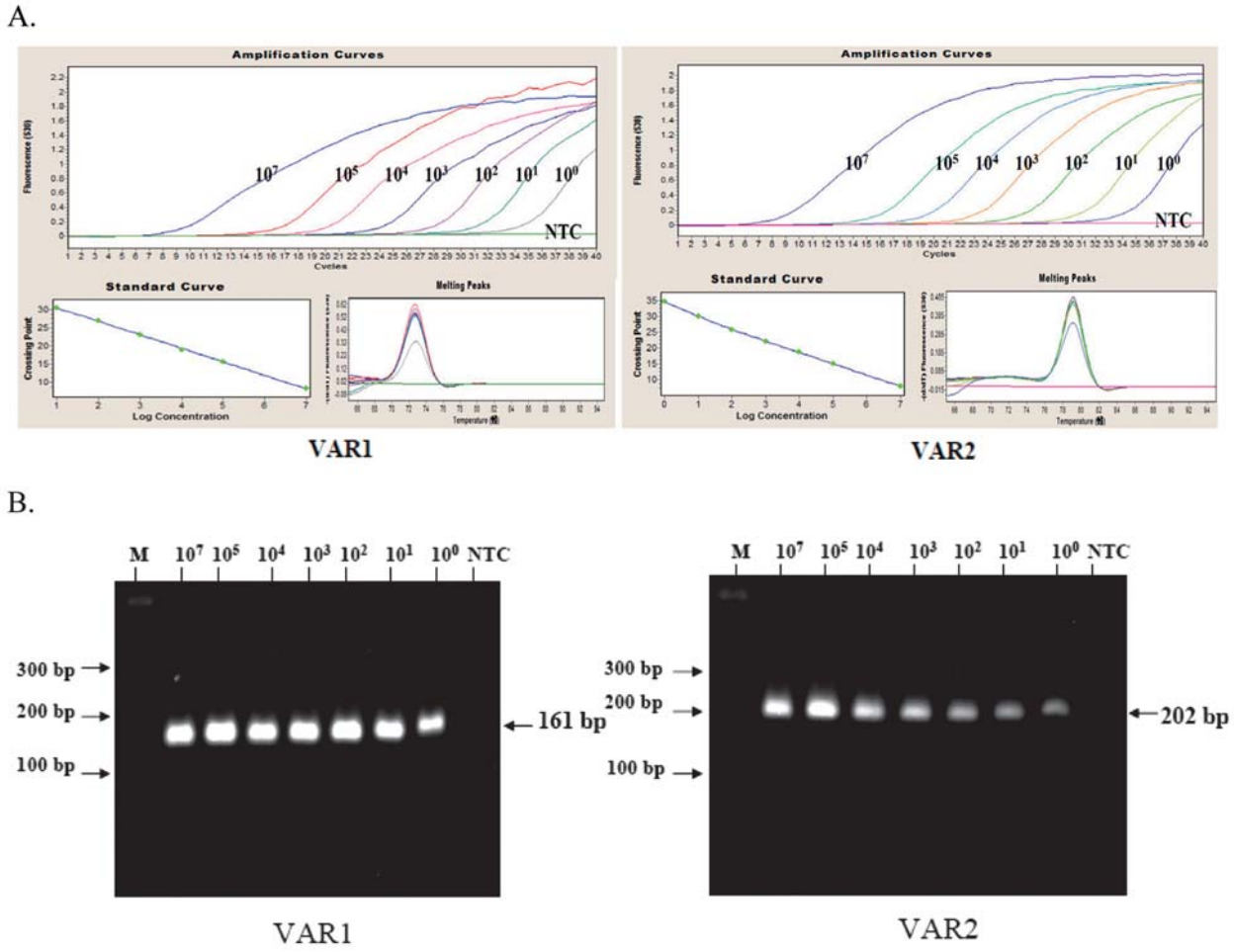

Fig. 3 LOD of VAR1 and VAR2 SYBR green-based real-time PCR assays expressed in number of copies. **A.** VAR1 (left) and VAR2 (right) SYBR green-based real-time PCR assays reveal the sensitivity of OPV detection. Amplification of 10-fold serial dilutions of pSMPXV was shown a linear detection range from 10^7 to 10^0 copies per reaction mixture in the standard curve analysis. The efficient PCR reaction was supported by PCR efficiency of 1.84 and 1.9. NTC stands for no template controls. Melting analysis detects uniform melting peaks. **B.** Electrophoresis analysis of VAR1 and VAR2 PCR products of serial-diluted pSMPXV. VAR1 (left) and VAR2 (right) PCR products from the above SYBR green-based real-time PCR assays were separated with 3%MetaPhor® agarose electrophoresis. The number of DNA copies per reaction was displayed on the top. The size of DNA marker (M) was indicated on the left. The expected size of PCR products were indicated on the right.

says were verified with electrophoresis analysis (Figure 3B). Similar results were obtained from pSVACV which matches perfectly with primers (data not shown). No specific signals were observed in any of the negative controls. However, melting analysis results reveal that it was not easy to identify OPV species by melting temperature of amplified products (data not shown). PCR sensitivities on the viral DNA level of vaccinia virus LC strain were also evaluated. As shown in Fig. 4, the assays can detect virus as low as 10 plaque-forming units (pfu)/ml (0.03 pfu/reaction). In conclusion, VAR1 and VAR2 SYBR green-based real-time PCR assays exhibit a high sensitivity in detecting pan-OPV DNA in levels of 1 copy DNA/reaction or 10 pfu/ml.

2. Detection of pan-OPV, monkeypox-specific and smallpox-specific DNA by probe-based real-time PCR assay

In order to develop a more specific real-time PCR assay, a pan-OPV probe-based real-time PCR assay was developed. Sequence alignment reveals most OPV includ-

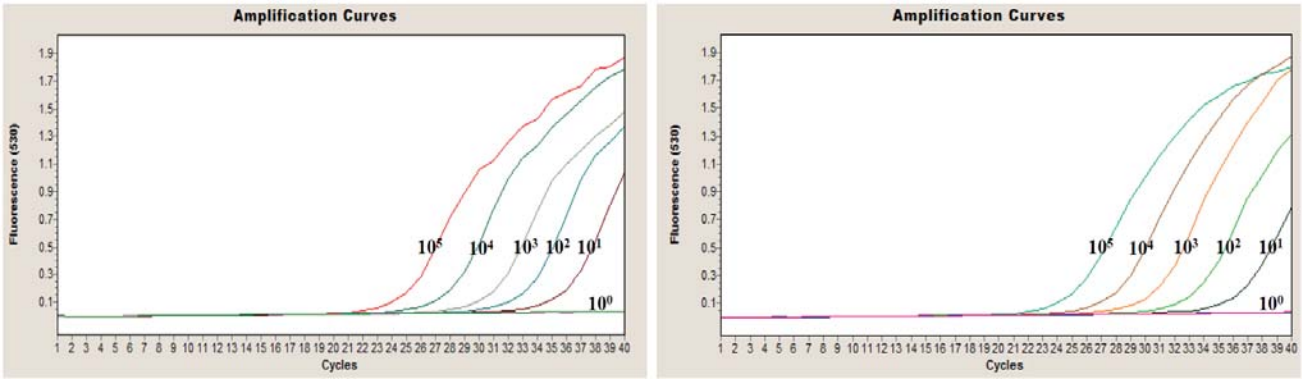

Fig. 4 LOD of VAR1 and VAR2 SYBR green-based real-time PCR assay expressed in viral DNA of Vaccinia virus LC strain. Viral DNA was purified from serial diluted culture medium of Vaccinia virus LC strain. Amplification of 10-fold serial dilutions of viral DNA from Vaccinia virus LC strain revealed a linear detection range from 10^5 to101 pfu/ml per reaction.

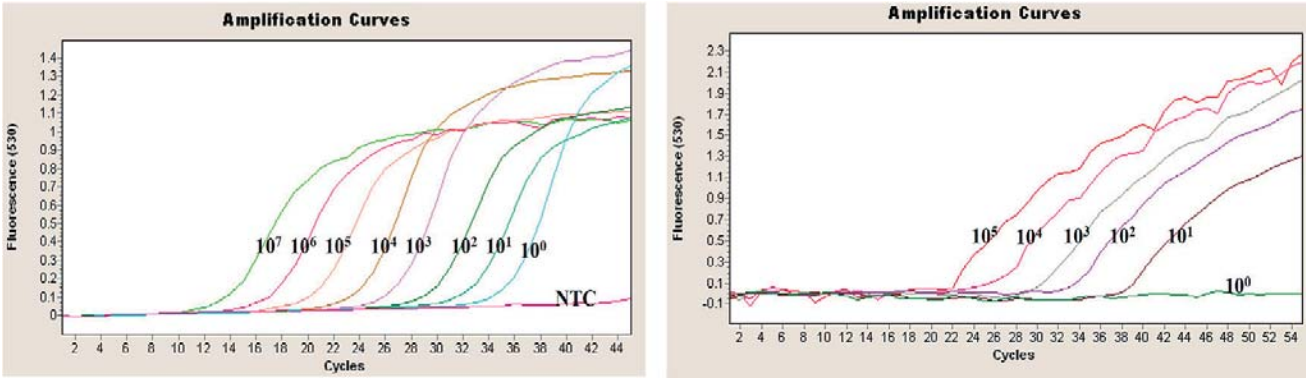

Fig. 5 LOD of VAR1 pan-OPV probe-based real-time PCR assay expressed in plasmid DNA and viral DNA of VACV LC strain. Amplification of 10-fold serial dilutions of pSVARV revealed a linear detection range from 10^7 to 10^0 copies per reaction mixture(left). NTC stands for no template control. Amplification of 10-fold serial dilutions of viral DNA from Vaccinia virus LC strain revealed a linear detection range from 10^5 to 10^1 pfu/ml per reaction mixture (right).

ing VARV, MPXV, CPXV, VACV, ECTV, CMXV, HPXV and RPXV match perfectly with sequences of primers and probe (Figure 1). Amplification of 10-fold serial dilutions of pSVARV, which is a single internal mismatch with forward primer, revealed that the sensitivity of this assay is 1 copy of plasmid per reaction (Figure 5, left). PCR efficiency of 1.98 indicates efficient PCR reaction. Similar results were obtained from pSVACV which is a perfect match with primers (data not shown). No specific signals were observed in any of the negative controls. PCR sensitivities were also evaluated in terms of VACV viral DNA level (Figure 5, right). The results show that this assay can also detect virus as low as 10 pfu / ml (0.03 pfu / reaction).

Both VARV and MPXV are the most important human pathogenic OPV. Next, MPXV-specific and VAR-specific probe-based real-time PCR assays targeting VAR2 region were developed. Species-specific probes combined with highly conserved primer-pair provide the specificity of OPV typing reactions using probe-based real-time PCR assay. Amplification of 10-fold serial dilutions (from 10^6 to 10^0) of self-DNA control plasmid revealed an efficient PCR reaction (Figure 6). Result shows that MPXV-specific and VAR-specific probe-based real-time PCR assays exhibit the LOD in level of 10 copies and 1 copy DNA/reaction, respectively. PCR efficiency of 1.92 and 2.0 indicate efficient PCR reactions (data not shown). No cross-reactivity was proved by insignificant florescent signals detected from reactions of 10^7 copies of other OPV control plasmids of pSVACV, pSCPXV, pSVARV

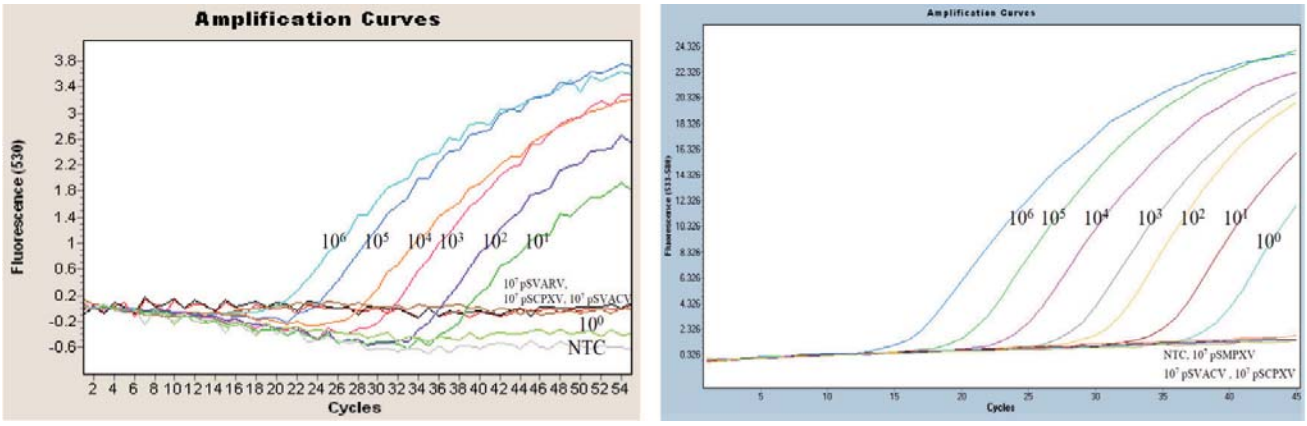

Fig. 6 LOD and specificity of MPXV-specific and VARV-specific probe-based real-time PCR assays expressed in plasmid DNA. Amplifications of 10-fold serial dilutions of control plasmids from 10^6 to 10^0 copies per reaction mixture revealed a linear range for detection of MPXV- and VARV-specific DNAs. The LOD of MPXV-(left) and VARV-specific (right) probe-based real-time PCR assays were expressed in number of copies. Amplifications of 10^7 copies unrelated DNAs of pSVACV, pSCPXV, pSVARV and pSMPXV revealed no cross-reactivity among other OPV. NTC stands for no template controls.

or pSMPXV (Figure 6), indicating that these specific probe-based assays can discriminate the self-DNA from other OPV DNAs. Results demonstrate that MPXV-specific and VAR-specific probe-based real-time PCR assays can detect the self-DNA sensitively.

DISCUSSION

Clinical manifestations for diagnosis of smallpox or monkeypox infections in humans have been well described.[6,24] However, symptoms of human OPV infections are closely related in the early stages of disease. The safe, fast and reliable molecular tests are important for early diagnosis and prompt risk assessment. There were many viral gene targets including *HA*,[17,25-28] 14-kD protein,[29] VARV-A38R, MPXV-B7R, CPXV-D11L, VACV-*B10R*,[30] MPXV-B6R, NON-VARV-*E9L*,[31] *C3L*,[32] *vgf*,[33] *G9R*,[34] 14-kDa fusion protein,[19] *J7R*, *B9R*, *E9L* and *B10R* genes[35] designed for detecting or typing OPV. According to sequence analysis of 50 OPV sequences deposited in GenBank, two novel target genes, *LPMP* and *CP* genes, were selected for the design of OPV real-time PCR assay in this study. Plasmid DNAs containing target sequences have been widely employed to evaluate the performance of PCR assay of OPV despite the lack of authentic OPV.[17,19,25-27,30,36] In our two conventional PCR assays, a single expected-size of PCR product amplified from pSMPXV, pSCPXV, pSVARV, pSVACV or viral DNAs of VACV has been verified using agarose-gel electrophoresis analysis. The sensitivity of real-time PCR for detecting OPV DNAs was evaluated by the serial dilution of control plasmid. Different sensitivities were further confirmed by viral genomic DNA. The sequence varieties exist in context of VAR1 and VAR2 amplicons among OPV. However, the identification of OPV species cannot be achieved by melting temperature assay on VAR1 and VAR2 SYBR green-based real-time PCR assays (data not shown). Although VARV and MPXV sequences contain one base-mismatch with VAR1's forward primer and VAR2's reverse primer, respectively. VAR1 and VAR2 SYBR green-based real-time PCR assays showed high sensitivity for detection of pSVARV and pSMPXV in level of 1 copy DNA/reaction. Thus, the highly conserved primer sequences reveal that these assays have potential to detect other non-human OPV including camelpox, horsepox, rabbitpox and ectromelia virus. Owing to the existence of lots of defective viruses and degraded viral DNA in viral culture fluid, the LOD (1 copy DNA/reaction) on plasmid level is greater than that (0.03 pfu/reaction) on pfu level in pan-OPV SYBR assay.

The aim of this study was to design a simple, rapid, sensitive, inexpensive and potentially direct diagnostic real-time PCR assay. The highly sensitivity and specificity of pan-OPV probe-based real time PCR assay targeting on late 16-Kda putative membrane protein (*LPMP*) gene has high potential for application of OPV epidemiological screening. More importantly, smallpox-specific and monkeypox-specific probe-based real-time

PCR assays can easily differentiate DNA of VARV and MPXV from DNA of other OPV (Figure 6). None of the above assays yielded amplification products or fluorescence signals with human and monkey genomic DNA (Figure, NTC). However, the validation of this study will rely on testing authentic viruses of VARV and MPXV in advance. To combine VAR2 SYBR green-based real-time PCR assay with MPXV- and VARV-specific probe-based assays targeting *CP* gene, this multiplex real-time PCR assay exhibits an ability to detect pan-OPV DNA and identify VARV and MPXV DNA. Real-time PCR identification of OPV species is dependent on individual primer-probe set.[30,35-37] Thus, complicated combination assays are required for typing unknown cases. This is the first study revealing that a single VAR2 target on 39-kDa core protein (*CP*) gene has the potential for designing pan-OPV, VARV- and MPXV-specific PCR assay. The highly conserved primer-pair of VAR2-S1 and VAR2-A3 offers an ability to amplify human pathogenic OPV DNAs (data not shown). In the future, the multiplex reaction composed of a conserved primer-pair (VAR2-S1 and VAR2-A3) and three different dye-labeled probes (a pan-OPV probe, a VARV-specific probe and a MPXV-specific probe) will be developed for rapid OPV detection as well as typing of MPXV and VARV simultaneously. In summary, this study provides not only novel PCR methods for early detection and differentiation of smallpox, monkeypox and OPV DNA, but two inexpensively pan-OPV SYBR green-based PCR assays for route laboratories. The validation of this study will rely on testing authentic virus in future.

DISCLOSURE

The authors declare that they have no conflict of interest.

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