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The potential of 5′ nuclease PCR for detecting a single-base polymorphism in *Orthopoxvirus*

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A fluorogenic 5′ nuclease PCR assay was evaluated for its ability to specifically detect and differentiate DNA of two *Orthopoxvirus* species. A pair of consensus primers that target a DNA segment of the *Orthopoxvirus* haemagglutinin gene, and two oligonucleotide probes, each labelled with a different fluorescent reporter dye and the same quencher dye, were used in a single-tube assay. The assay is based on the 5′→3′ nuclease activity of *AmpliTaq* DNA polymerase that cleaves a fluorescein-labelled hybridized probe. Probe cleavage generates specific fluorescent signals whose intensity can be quantified by fluorometry. After evaluating the effects of various annealing temperatures and probe concentrations and normalizing the emission intensities of the reporter dyes, it was possible to detect and differentiate monkeypox and vaccinia virus DNAs on the basis of a single-base polymorphism. The sensitivity of the 5′ nuclease PCR assay is comparable to the sensitivity of ethidium bromide-stained gels, but the assay provides higher specificity and virtually eliminates the need for laborious post-PCR processing. © 1997 Academic Press Limited

KEYWORDS: *Orthopoxvirus*, PCR primers, fluorogenic probe, 5′ nuclease assay, haemagglutinin.

INTRODUCTION

The genus *Orthopoxvirus* (family: Poxviridae) includes 10 species, four of which are known to infect humans. Smallpox, caused by *Orthopoxvirus* variola virus, has been declared eradicated since 1979. Other genus members such as cowpox, monkeypox, and the vaccinia subspecies buffalopox viruses can infect humans by natural transmission,[1–3] and smallpox vaccine, vaccinia virus, may cause fatal disease in vaccinated immunocompromised patients. The factors that influence pathogenicity and virulence in orthopoxviruses have not yet been clearly defined; however, recent studies indicate that relatively minor modifications in sequences coding for particular viral proteins may relate to virulence.[4] Furthermore, the prototype poxvirus vaccinia virus, which had been used for many years as a vaccine against smallpox, is now widely used as an expression vector for a variety of genes from numerous infectious agents and holds promise as a recombinant vaccine for human and veterinary use. Because of rapid societal, environmental and technological changes, it is not unreasonable to assume that orthopoxviruses may re-emerge as natural, incidental or deliberate health threats. The recent occurrence of buffalopox virus in India, and emergence of other disease pathogens worldwide, indicate the need for utilization of advanced diagnostic technology. Therefore, the purpose of this study was to investigate the potential of the

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newly developed fluorogenic 5′ nuclease PCR assay[5,6] to detect and differentiate two orthopoxviruses (monkeypox and vaccinia) as a prelude to expansion of the method toward laboratory diagnosis of the 10 *Orthopoxvirus* spp.

The 5′ nuclease PCR assay is based on the 5′→3′ nuclease activity of *Taq* polymerase that cleaves an oligonucleotide probe doubly-labelled with fluorescein reporter and quencher dyes.[6] The probe cleavage occurs during PCR thermal cycling, only when the probe is hybridized to a target sequence within the specific amplicon. Probe cleavage causes an increase in fluorescence emission intensity from the reporter dye, which can be measured with a fluorometer. By using consensus oligonucleotide primers derived from highly conserved regions of the *Orthopoxvirus* haemagglutinin (HA) gene, and monkeypox virus- and vaccinia virus-specific probes, each labelled with a different fluorescein reporter dye with different emission spectra, it was possible to differentiate DNAs from these two viruses in a single-tube assay.

MATERIALS AND METHODS

Viruses and DNA purification

The viruses used, Copenhagen monkeypox virus (MPV), Copenhagen vaccinia virus (VAC), Somalia camelpox virus (CML), and Brighton cowpox virus (CPV), were propagated in Vero cell culture maintained in Eagle's Minimum Essential Medium that contained Earle's salts, 5% fetal bovine serum and 0·1% gentamicin. The origins of the stock viruses have been documented elsewhere.[7] Infected Vero cell monolayers in T75 flasks were harvested after severe cytopathic effect was apparent, cells were pelleted by centrifugation, and total DNA was extracted from the virus-infected cell pellets using Easy DNA method (Invitrogen). Infectious virus titres were determined by plaque assay. The numbers of pfu ml^{-1} in proportions of MPV, VAC, CML, and CPV were $5{\cdot}6 \times 10^6$, $4{\cdot}4 \times 10^6$, $1{\cdot}4 \times 10^6$, and 1×10^6, respectively.

Primers and probes

The consensus oligonucleotide primers OPOXHAU1 (161197/5′-ACCAATACTTTTGTTACTAAT-3′/161217) and OPOXHAL1 (161471/5′-CAGCAGTCAATGATT-TA-3′/161455) were designed from the sequences of the haemagglutinin gene of MPV and VAC viruses. The oligonucleotide probes T-MPVHA1 (161266/5′-TET-TGATGATGCAACTATATCATGTA-TAMRA-p-3′/161288) and F-VACHA1 (161280/5′-FAM-TATCAT-GTAATCGAAAATAATA-TAMRA-p-3′/161301) were synthesized (Applied Biosystems Division, Perkin-Elmer) with the reporter dyes 6-carboxy-4,7,2′,7′-tetrachlorofluorescein (TET) and 6-carboxyfluorescein (FAM) phosphoramidites, respectively, at the 5′ ends. The quencher dye, 6-carboxytetramethyrhodamine (TAMRA) and a phosphate (p) were linked to the 3′ ends of the probes to prevent probe extension during amplification. The starting and ending nucleotide positions encompassing the annealing sites of the primers and probes are indicated based on the published VAC sequence (GenBank accession No. M35027). Primers and probes were designed with the aid of Oligo 5.0 software (National Biosciences).

5′ nuclease PCR assay

The reagents for the 5′ nuclease PCR assay were purchased from Perkin-Elmer. Typical reactions were performed in a 50 μl volume that contained 1 μl of viral DNA (approximately 30 ng), 5 μl of PCR buffer (10 mM Tris–HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$), 1 μl of dNTP mix (200 μM each), 1 μM each), 1 μl (300 nM) of each primer, 1 μl (200 nM) of each probe and 0·5 μl (2·5 U) of *AmpliTaq* DNA polymerase. Thermal cycling was performed in PTC-100 cycler (MJ Research Inc.) for 2 min at 94°C, followed by 40 cycles, each for 15 s at 94°C, 15 s at 44°C, and 1 min at 72°C. After amplification, 40 μl of each reaction were transferred to individual wells of a white 96-well microtitre plate and fluorescence of each reaction mixture were measured at 518 nm (FAM), 538 nm (TET) and 582 nm (TAMRA) using a LS-50B luminescence spectrometer (Perkin-Elmer). Where indicated, 10 μl of each reaction were analysed by agarose gel electrophoresis and ethidium bromide staining.

Data analysis

Data acquisition and analysis were performed with a microcomputer running EXCEL software (Microsoft Corp.) as described.[8,9] Briefly, the reporter/quencher ratios in each sample (R/Q+), and in three no-template negative controls (R/Q−) were calculated. The mean R/Q− value was subtracted from the R/Q+ of each sample to obtain ΔRQ. The ΔRQ of each sample was then compared with a threshold limit, which was the product of the standard deviation of the mean R/Q− and the critical value of two-tailed *t*-distribution at the 99% confidence limit. Fluorescence data in

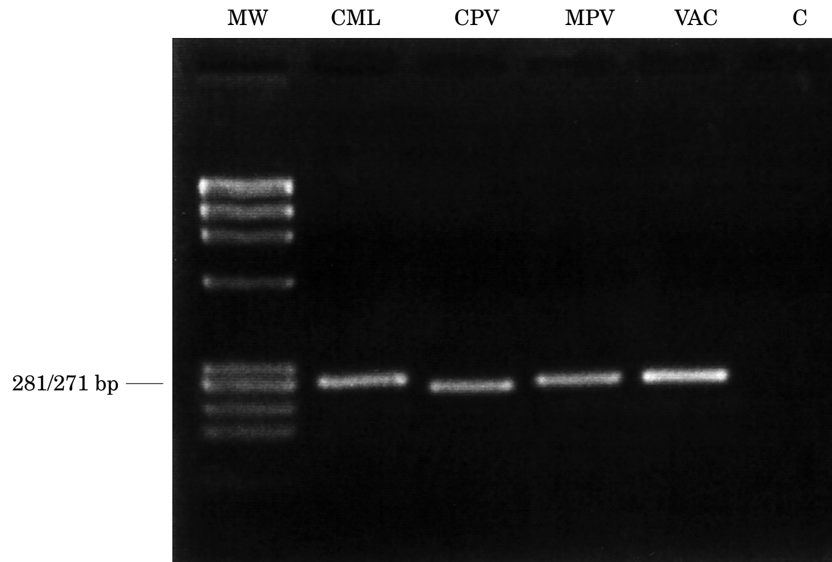

Fig. 1. Agarose gel electrophoresis of PCR products from vaccinia (VAC), monkeypox (MPV), cowpox (CPV) and camelpox (CML) viruses. MW: DNA molecular weight marker *Phi*X *Hae*III (Gibco BRL, Gaithersburg, MD).

the mixed probe assay was normalized by multicomponent analysis using an EXCEL software macro (Perkin Elmer).

RESULTS

Our strategy was to amplify orthopoxviruses DNA using *Orthopoxvirus* genus-specific PCR primers and concomitantly identify species using specific fluorogenic probes. The consensus primers (OPXHAU1 and OPXHAL1) were selected from the HA gene coding sequences after multiple alignment of HA sequences of 32 different strains (Ropp *et al.*, unpublished) representing 10 different *Orthopoxvirus* spp. from diverse geographic areas. When these primers were tested with MPV, VAC, CPV and CML DNAs, amplicons corresponding to predicted-size fragments (266–281 bp) were detected by agarose gel electrophoresis as shown in Fig. 1.

The overall efficacy of the 5′ nuclease PCR assay is a function of the efficiency with which the reporter dye is released in the reaction mixture as a result of probe cleavage. The release of dye from the annealed probe is influenced by a number of factors, including the annealing temperature, probe concentration and number of initial target molecules. In order to develop the present assay, these parameters were examined. The TET-labelled MPV-specific probe (T-MPVHA1) and the FAM-labelled VAC-specific probe (F-VACHA1) were evaluated with homologous MPV and VAC DNA templates at four annealing temperatures (44, 48, 52 and 56°C), four probe concentrations (20, 10, 5 and 2·5 pmol per reaction) and three DNA template concentrations (30 ng, 300 pg and 3 pg). The results were analysed by comparing the ΔRQ values with the threshold as previously described (see Materials and Methods). The highest ΔRQ values were obtained at 44°C annealing temperature and 5 or 2·5 pmoles of homologous probes (Fig. 2). The results were consistent at the three different DNA template concentrations of MPV or VAC. At 48°C annealing temperature, ΔRQ values were still significantly higher than the threshold at all template concentrations; however, at 52°C, the ΔRQ values were significantly higher than the threshold only when 30 ng DNA templates were used (not shown). At 56°C annealing temperature, the ΔRQ values were less than or equal to the threshold, and were considered negative at all template concentrations (not shown). Probe concentrations between 5 and 0·65 pmoles were also tested at 44°C annealing temperature and no significant improvement in the ΔRQ values were obtained (not shown).

Based on these results, 44°C annealing temperature and 5 pmoles of probe were selected to test the discriminatory potential of the two fluorogenic probes in a mixed probe assay that contained either MPV DNA, VAC DNA or no-DNA templates. Three replicates for each of the DNA template concentrations (30 ng, 300 pg and 3 pg), and three replicates for the no-DNA negative control were performed. Figure 3(a) shows a scatter plot of fluorescence data. The data show seven clusters: three clusters derive from the three template concentrations for MPV, three clusters derive from the three template concentrations of VAC,

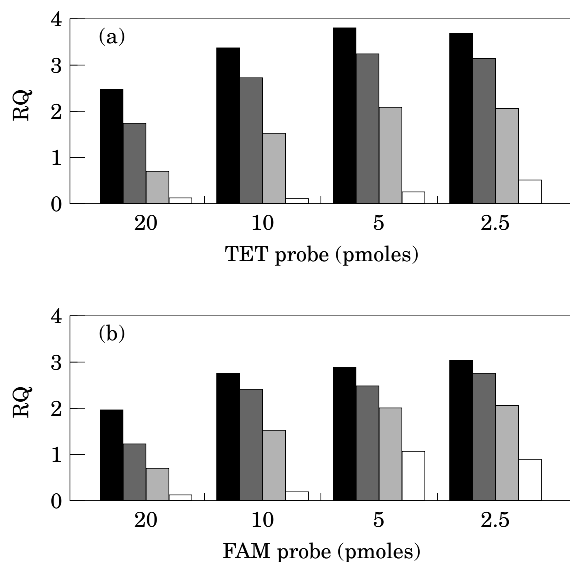

Fig. 2. The effect of probe concentration (20, 10, 5 and 2·5 pmol) at 44°C annealing temperature and 30 ng, 300 pg and 3 pg template viral DNA. (a) Results after using probe T-MPVHA1 with MPV template DNA and (b) results of F-VACHA1 with VAC DNA. Each value was calculated from the mean of three replicates. The threshold was determined from three no-template controls at 99% confidence limit. ■, 30 ng; □, 300 pg; □, 3 pg; □, threshold.

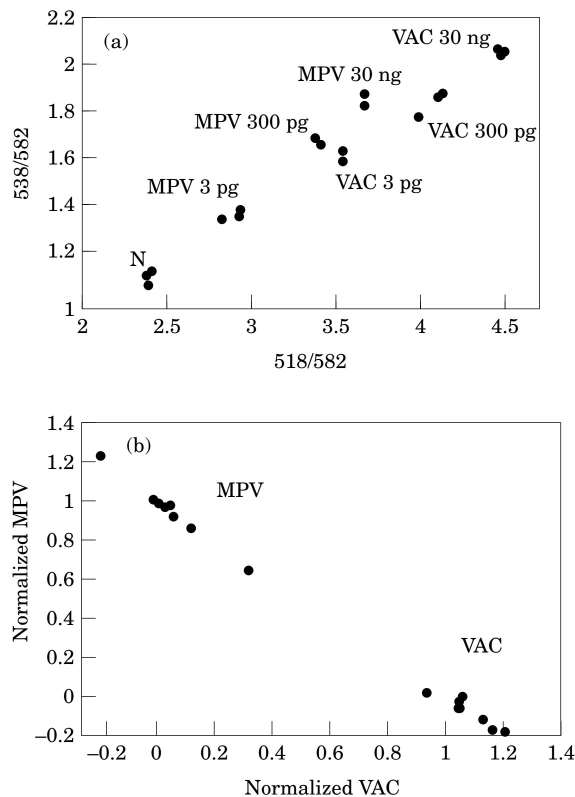

Fig. 3. Scatter plots of the fluorescence data (a) before and (b) after normalization. Three replicates using monkeypox templates DNA at three different concentrations (MPV 30 ng, MPV 300 pg, MPV 3 pg), and each of the corresponding vaccinia template concentrations (VAC 30 ng, VAC 300 pg, VAC 3 pg) and three no-template controls were assayed as described. In (a), the RQ value for each observation was calculated from the 538/582 (TET/TAMRA) and 518/582 FAM/ TAMRA) ratios. In (b), the fluorescence data were normalized by multicomponent analysis using an EXCEL macro.

and one cluster derives from the no-template negative control. The input fluorescence data used to establish the plot were then normalized using multicomponent analysis[8] to account for differences in emission intensities and unequal spectral overlap of the two fluorescent reporter dyes. As shown in Fig. 3(b), the normalized data gave two distinct clusters depending on whether MPV or VAC DNA templates were used in the reaction.

DISCUSSION

PCR-based methods to differentiate orthopoxviruses have been described.[2,7] Prior methods relied on size analysis of PCR products by agarose gel electrophoresis and restriction fragment length polymorphism. Although the methods provide high specificity, and allow species and sometimes strain differentiation, they require laborious and time-consuming post-PCR processing. In this study, the 5′ nuclease PCR assay was evaluated as a novel alternative *Orthopoxvirus* detection method. Development of the assay was initiated using monkeypox and vaccinia virus HA DNA to establish a working test model. These viruses, and other orthopoxviruses, offer a particularly interesting subject for evaluating the fluorogenic 5′ nuclease PCR assays because their genome DNAs contain numerous highly conserved regions, which differ in many cases by single-nucleotide substitutions.[1] In addition, their genome DNAs contain low (<35%) G+C content, which limits the choice of probes. Despite the limitations, the results presented here showed that the assay performed well using the PCR amplification and cycling conditions described above. Under the conditions used, the method enabled detection and simultaneous differentiation of MPV and VAC viruses if a range of 30 000 to 3 pg of template DNA was used. This result is similar to the concentrations of DNA detected by electrophoresis with ethidium bromide-stained gels. Moreover, the assay provides an additional level of specificity because of annealing of a specific, dye-labelled probe. Compared with other PCR-based

sequence detection methods, the present method offers an alternative that does not require extensive post-PCR sample processing. The only post-PCR processing was the measurement of sample fluorescence after transferring the reaction mixtures to a microtitre plate followed by data acquisition and analysis, which were automatically performed within 10–30 min using programmable software. The accuracy of the assay was demonstrated by its high specificity and reproducibility. In this study, it was possible to discriminate a single-base mismatch in two closely related viral sequences. In a study by Livak *et al.*,[8] a similar assay for human DNA was able to discriminate two alleles of the insulin gene, which differed by a single A–T base polymorphism, in 75 individuals. Their 5′ nuclease PCR assay results were in complete concordance with results using the standard genotyping endonuclease digestion assay. In another study by Bassler *et al.*,[9] employing the 5′ nuclease PCR assay for detecting the food-borne bacterium *Listeria monocytogenes*, the authors reported 'good reproducibility' over a 5-log linear range of DNA template dilutions, with detection sensitivity of about 50 bacterial cells. In this study, in experiments using 10 replicates of three different concentrations of MPV or VAC DNA templates, the coefficient of variation observed was between 4 and 11%.

In conclusion, the present study is a first step towards development of fluorogenic 5′ nuclease PCR assay for orthopoxviruses. The general assay strategy and performance were demonstrated using two orthopoxviruses. It is important to note, however, that the high, single-base specificity of the probes used in this study must be further tested against a variety of clinical isolates of various *Orthopoxvirus* DNAs to establish true efficacy and feasibility for further development. Nevertheless, the accuracy and performance simplicity observed in this and previous studies[8,9] indicate that the 5′ nuclease assays have promising diagnostic potential. Another advantage of the 5′ nuclease PCR assays is that they can be automated to allow handling of a large number of samples. The main disadvantage of such assays, however, is the cost of specialized reagents and procedures required for probe synthesis, and the detection device. Further enhancements in probe synthesis methodology, and instrument design and manufacturing may increase the general applicability of such assays.

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