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PCR Strategy for Identification and Differentiation of Smallpox and Other Orthopoxviruses

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Rapid identification and differentiation of orthopoxviruses by PCR were achieved with primers based on genome sequences encoding the hemagglutinin (HA) protein, an infected-cell membrane antigen that distinguishes orthopoxviruses from other poxvirus genera. The initial identification step used a primer pair of consensus sequences for amplifying an HA DNA fragment from the three known North American orthopoxviruses (raccoonpox, skunkpox, and volepox viruses), and a second pair for amplifying virtually the entire HA open reading frame of the Eurasian-African orthopoxviruses (variola, vaccinia, cowpox, monkeypox, camelpox, ectromelia, and gerbilpox viruses). *Rsa*I digest electropherograms of the amplified DNAs of the former subgroup provided species differentiation, and *Taq*I digests differentiated the Eurasian-African orthopoxviruses, including vaccinia virus from the vaccinia virus subspecies buffalopox virus. Endonuclease *Hha*I digest patterns distinguished smallpox variola major viruses from alastrim variola minor viruses. For the Eurasian-African orthopoxviruses, a confirmatory step that used a set of higher-sequence-homology primers was developed to provide sensitivity to discern individual virus HA DNAs from cross-contaminated orthopoxvirus DNA samples; *Taq*I and *Hha*I digestions of the individual amplified HA DNAs confirmed virus identity. Finally, a set of primers and modified PCR conditions were developed on the basis of base sequence differences within the HA genes of the 10 species, which enabled production of a single DNA fragment of a particular size that indicated the specific species.

The orthopoxviruses are morphologically large, antigenically closely related vertebrate viruses that include the now eradicated smallpox variola virus, the smallpox vaccine vaccinia virus, and several animal pathogens of veterinary economic and public health zoonotic importance. Orthopoxviruses contain a covalently closed, double-stranded genome DNA with a length of approximately 200 kbp and 35% G+C content and with large regions that cross-hybridize between members of the genus (2, 12, 15, 18, 20, 31, 35).

Identification and differentiation of orthopoxvirus species and strains have been achieved by a variety of immunologic and biologic methods, including virus neutralization; hemagglutination inhibition and other serologic assays; determination of plaque or pock morphology, reproductive ceiling temperature in cell cultures or on chicken embryo chorioallantoic membranes, and lethality or infectivity for various animals or selected tissues of animals; and the ability of infected cells to hemadsorb or hemagglutinate chicken erythrocytes (7, 14, 15, 18, 19, 36–38). Analysis of virus proteins by polyacrylamide gel electrophoresis has also enabled genus, species, and strain differentiation (1, 16, 24, 40); however, determinations of genome DNA endonuclease cleavage profiles, DNA restriction maps, and nucleotide sequences have become the most definitive methods for poxvirus classification (12, 17, 22, 27, 31–33).

A relatively simple, rapid and accurate detection and differential diagnostic method would be very useful for the identification and control of sometimes devastating orthopoxvirus infections. The goal of the present study, therefore, was to develop a strategy to identify orthopoxviruses by PCR methods, as has been done for several other viruses (3). Recently, PCR has been used to distinguish five orthopoxvirus species on the basis of sequences coding for the acidophilic inclusion proteins of various sizes (34); however, the extreme hypervariability of interruptions and deletions in the gene region encoding the inclusion body protein (5, 27, 33) in different species and strains might preclude the effectiveness and accuracy of this method.

In the present study, we examined orthopoxvirus identification and differentiation on the basis of sequences encoding the orthopoxvirus hemagglutinin (HA) protein, an infected-cell surface antigen that distinguishes orthopoxviruses from all other poxviruses (20, 36–38, 46, 48). Recently, we examined the base sequences encoding the HA protein for 50 different orthopoxviruses (26) and noted a phylogenetic relationship that corresponded with differentiation by DNA restriction mapping (12, 21, 28, 31). In the present report, we describe PCR strategies developed with a series of oligonucleotide primer pairs designed to amplify sequences of the HA open reading frame to differentiate orthopoxvirus species. We have successfully used the strategies to identify virus DNA in clinical materials and infected cell cultures and chicken embryo chorioallantoic membranes.

MATERIALS AND METHODS

Viruses and DNA preparation. The origins of the viruses used in this study have been described elsewhere (4, 8, 10, 12, 17, 20, 22, 25, 26, 28, 30–33, 39). DNA was also prepared from cowpox virus CPV-58 (25), which was kindly provided by S. Dales and Y. Ichihashi, and from ectromelia virus Moscow (ATCC 1374), which was kindly provided by R. M. L. Buller. Genome DNA preparations of the vaccinia virus subspecies buffalopox virus, strains 81 and 3906 (8), were kindly provided by K. R. Dumbell, and DNAs of several different isolates of camelpox virus and cowpox virus (34) were a generous gift from H. Meyer.

The preparation of viral DNA from purified virions and from lysates of infected cells has been described before (11, 13, 14, 28, 29). DNA from several different clinical samples from smallpox or monkeypox virus infections was pre-

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TABLE 1. Consensus sequence primer pairs for amplifying fragments within the HA open reading frame of Eurasian-African or North American orthopoxvirus subgroups

Primer name (orientation)	Sequence[a]	% G+C content	Annealing location[b]	Product size (bp)
North American				
NACP1 (5′→3′)	5′ A**C**G A**T**G T**C**G TAT ACT TTG AT 3′	35	191–210 (RCN, VPX) 194–213 (SKP)	580–658
NACP2 (3′←5′)	5′ GAA AC**A** A**CT** CCA AAT ATC TC 3′	35	823–842 (RCN) 832–851 (SKP) 751–770 (VPX)	
Eurasian-African				
EACP1 (5′→3′)	5′ ATG **A**CA CGA TT**G C**CA ATA C 3′	42	Start codon	846–960
EACP2 (3′←5′)	5′ CTA GAC TTT GTT **T**TC TG 3′	35	Stop codon	

[a] Nucleotides in boldface and underlined represent residues not conserved in the virus HA sequences examined (26, 41).
[b] Abbreviations of viruses are defined in the legend to Fig. 1.

pared by a procedure that used a small aliquot of clinical material (a single scab or portion of dried vesicle fluid) suspended in 90 μl of lysis solution (50 mM Tris-hydrochloride [pH 8.0], 100 mM disodium EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate) adjusted with 10 μl of proteinase K (10 mg/ml) and then digested for 10 min at 37°C. Crusted scabs were manually disrupted with a microcentrifuge tube pestle (Kontes, Inc., Vineland, N.J.). After the initial digestion, additional lysis solution (350 μl) and proteinase K (50 μl) were added and the mixture was incubated for 2 h at 37°C. The digest was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (45). Two volumes of ethanol were then added to the aqueous phase to precipitate DNA. After the precipitate was washed with 70% ethanol, the DNA was air dried and dissolved in H_2O.

Primers. Oligonucleotide primers, described in Results, were synthesized by β-cyanoethyl phosphoramidite chemistry (47) by using a model 380B synthesizer (Applied Biosystems, Inc., Foster City, Calif.). Primers were designed with the aid of the Oligonucleotide Selection Program (23) and by direct inspection of multiply aligned HA sequences (26) of 50 different orthopoxvirus species and strains.

Reaction mixtures. Standard PCR mixtures (9, 42–44) used 0.5 μg of each oligonucleotide of the primer pairs described in Tables 1 to 3 plus 50 ng of template DNA. Reactions were in a volume of 100 μl of a solution that contained 50 mM KCl; 10 mM Tris-hydrochloride (pH 8.3); 2.5 mM $MgCl_2$; 200 μM (each) dATP, dCTP, dGTP, and dTTP; and 2.5 U of DNA polymerase (PCR Core kit; Boehringer-Mannheim Biochemicals, Indianapolis, Ind.; and GeneAmp PCR Reagent Kit with *AmpliTaq* DNA polymerase; Perkin-Elmer Cetus Corp., Norwalk, Conn.). Reaction mixtures that used oligonucleotides described in Table 4 contained titration-optimized concentrations of deoxynucleoside triphosphate (dNTP), $MgCl_2$, and primer as described in Table 5. Reaction mixtures were cycled 25 times through denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and polymerization at 72°C for 3 min (model 9600 thermal cycler; Perkin-Elmer Cetus). Samples were stored at 4°C until analyzed by agarose gel electrophoresis, either directly or after restriction endonuclease digestion. Restriction digestions were done by adding 5 U of either *Taq*I, *Rsa*I, or *Hha*I (New England Biolabs, Inc., Beverly, Mass.) to 30 μl from the completed amplification reactions and then incubating the mixtures at 37°C (*Hha*I and *Rsa*I) or 65°C (*Taq*I) for 1 to 2 h. DNA products (15 μl undigested or 30 μl digested) were resolved by submerged gel electrophoresis in 3% NuSieve–genetic technology grade (GTG) agarose containing 1% SeaKem–GTG agarose (FMC Corp., Marine Colloids Div., Rockland, Maine); TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM disodium EDTA) was used. *Msp*I-digested pBR322 DNA and *Hae*III-digested ϕX174 DNA (New England Biolabs, Inc.) were used as DNA fragment size markers. Gels were stained in ethidium bromide, and DNA was visualized with a transilluminator (Fotodyne, Inc., Hartland, Wis.).

Sequencing. To verify the virus identity of amplified DNAs, the sequential order of nucleotides was determined by direct sequencing of PCR products, and then the sequences determined were compared with the original sequences (26). For this procedure, amplified DNAs were purified (Wizard PCR Preps DNA purification system; Primage, Inc., Madison, Wis.). Subsequently, 1 μg of each purified PCR product was used as a template in separate 20-μl mixtures that contained 3.2 pmol of oligonucleotide primer, 4 μl of 5× sequence buffer, 1 μl (each) of DyeDeoxy nucleotide terminator, 1 μl of dNTP mixture, and 4 U of *Taq* DNA polymerase (*Taq* DyeDeoxy-Terminator cycle sequencing kit; Applied Biosystems, Inc.). Mixtures were then heated through 25 cycles at 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. Sequence reaction products were then purified by centrifugation (Centri-Sep columns; Princeton Separations, Inc., Adelphia, N.J.) and dried in vacuo (Savant Instruments, Inc., Hicksville, N.Y.). The residue was resuspended in 4 μl of a 5:1 mixture of deionized formamide and 50 mM EDTA, heated at 95°C for 2 min, and then cooled on ice. Samples were loaded onto a 6% polyacrylamide gel to resolve the DNA sequences by electrophoresis (model 373A automated DNA sequencer; Applied Biosystems, Inc.). Chromatographic data from the sequencer were used for sequence assembly with the Staden package (49). The Genetics Computer Group Fasta and Pileup programs (6) were used for sequence alignments.

RESULTS

Identification with consensus sequence primers and endonuclease digestion. After multiply aligning the HA open reading frame nucleotides (26), we designed primer pairs based on

TABLE 2. Specific sequence primers for biased amplification of the HA open reading frame of particular Eurasian-African orthopoxviruses

Primer	Preference[a]	Sequence	% G+C content	Annealing location
5′→3′				
G-VRB	VAC, RPV, BFL	5′ ATG ACA CGA TTA CCA ATA C 3′	36	Start codon to residue 19
G-ECG	ECT, CML, GBL	5′ ATG GCA CGA TTG TCA ATA C 3′	42	Start codon to residue 19
G-CPV	CPV	5′ ATG ACA CGA TTG CCA ATA C 3′	42	Start codon to residue 19
G-VAR	VAR	5′ ATG ACA CGA TTG TCA ATA C 3′	36	Start codon to residue 19
G-MPV	MPV	5′ ATG ACA CAA TTA CCA ATA C 3′	31	Start codon to residue 19
3′←5′				
G-CGV	CPV, GBL, VAR	5′ CTA GAC TTT GTT TTC TG 3′	35	Proximal to stop codon
G-EMVRB	ECT, MPV, VAC, RPV, BFL	5′ CTA GAC TTT GTT CTC TG 3′	41	Proximal to stop codon
G-CML	CML	5′ TGT TTT GTA TTT ACG TGA AC 3′	30	926–945 (near stop codon)

[a] Abbreviations of viruses are defined in the legend to Fig. 1.

TABLE 3. Primer pairs used to amplify selected species

Species to amplify[a]	Primer	
	5′→3′	3′←5′
VAR	G-VAR	G-CGV
MPV	G-MPV	G-EMVRB
VAC	G-VRB	G-EMVRB
CPV	G-CPV	G-CGV
GBL	G-ECG	G-CGV
ECT	G-ECG	G-EMVRB
CML	G-ECG	G-CML

[a] Abbreviations of viruses are defined in the legend to Fig. 1.

a consensus at or near the ends of the sequences. Table 1 shows the consensus sequence primers that enabled amplification of an HA DNA fragment either from the genome of North American orthopoxviruses (primer pair NACP1 and NACP2) or the Eurasian-African orthopoxviruses (primer pair EACP1 and EACP2). Table 1 also shows the G+C contents of the primers, the nucleotide locations at which primers anneal within the HA open reading frame sequences, and the sizes of PCR products expected.

Initially, individual reaction mixtures containing genome DNA of a known sample of a North American or Eurasian-African orthopoxvirus were examined. Figure 1A, lanes 13 to 15, shows that, depending on the virus, DNA fragments of the expected size (Table 1) were produced, which differentiated the three known North American orthopoxviruses. Figure 1B shows the cleavage products of the PCR DNA after *Rsa*I digestion enhances resolution of the differences between North American orthopoxviruses. Figure 1 shows that no fragment is produced with Eurasian-African virus DNAs in amplifications primed with NACP1 and NACP2.

Figure 2A (lanes 1 to 12) shows the fragments amplified from individual genome DNA templates of known Eurasian-African orthopoxviruses from PCRs with consensus sequence primer EACP1 paired with EACP2; the amplified DNAs were of the expected size (Table 1). Figure 2B shows that digestion with *Taq*I produces a distinctive electrophoretic pattern suitable for differentiation of Eurasian-African orthopoxvirus species. In addition, the vaccinia virus subspecies buffalopox virus and cowpox virus strains Brighton and 58 were noted to produce distinctive *Taq*I digest electrophoresis patterns. We note here that separate amplifications from genome DNA with EACP1 and EACP2 followed by *Taq*I digestion enabled identification of other viruses in our collection (41), including a buffalopox virus, an ectromelia virus, a vaccinia virus, and four different isolates of variola virus. The results in Fig. 1 and 2 indicate that the protocol of consensus primers followed by *Taq*I digestion is generally suitable for orthopoxvirus identification at the species level.

As Fig. 2B shows, *Taq*I digestion did not differentiate smallpox variola major virus from alastrim variola minor viruses or vaccinia virus from the vaccinia virus subspecies rabbitpox virus. As also shown in Fig. 2B and as we have observed in separate experiments (41), DNAs of 20 different monkeypox virus isolates from humans or monkeys from different African countries could not be differentiated below the species level by using the protocol of consensus primer PCR and *Taq*I digestion. We note here that differentiation below the species level, including distinguishing Zaire monkeypox virus from other

TABLE 4. Species-specific primers within the HA open reading frame of orthopoxviruses

Primer	Sequence	% G+C content	Annealing location[a]	Product size (bp)
VAR1	5′ TAA ATC ATT GAC TGC TAA 3′	27	276–293	
VAR2	5′ GTA GAT GGT TCA TTA TCA TTG TG 3′	34	739–761 (VAR-BSH) 754–776 (VAR-GAR)	486–501
VAC1	5′ ATG CAA CTC TAT CAT GTA A 3′	31	86–104	
VAC2	5′ CAT AAT CTA CTT TAT CAG TG 3′	30	339–358 (BFL, VAC) 338–357 (RPV)	273
CML1	5′ GCC GGT ACT TAT GTA TGT GT 3′	45	298–317	361
CML2	5′ GAT CTT CTT CTT TAT CAG TG 3′	35	639–658	
MPV1	5′ CTG ATA ATG TAG AAG AC 3′	35	518–534	406
MPV2	5′ TTG TAT TTA CGT GGG TG 3′	41	907–923	
ECT1	5′ CAT ACA GTC ACA GAC ACT GTT G 3′	45	544–565	150
ECT2	5′ GAT GCT TTC TAC AGT TGT TGG TA 3′	39	671–693	
CPV1	5′ ATG ACA CGA TTG CCA ATA CTT C 3′	40	1–22	
CPV2	5′ CTT ACT GTA GTG TAT GAG ACA GC 3′	43	607–629 (CPV-BRT) 655–677 (CPV-58)	629–677
GBL1	5′ CGT CGG TAT TCG AAA TCG CGA A 3′	50	494–515	451
GBL2	5′ GTT TTG TAT TTA CGT GAA CGG 3′	38	924–944	
RCN1	5′ GAT GAT ACG CAA TAT AAT GT	30	592–611	185
RCN2	5′ TCT ACC GTT GTT GGT ATC GAG 3′	47	756–776	
SKP1	5′ AGT TCT GCT AAT ATC GCT AG 3′	40	18–37	640
SKP2	5′ AGT GGT TGT GGG AGC AGT GG 3′	60	638–657	
VPX1	5′ CCA TCA CCA GAA GTA GTT GCA G 3′	50	586–607	269
VPX2	5′ ATA TGT GCT CCA TAT GAA CT 3′	35	835–854	

[a] Abbreviations of viruses are defined in the legend to Fig. 1.

TABLE 5. Concentrations of dNTP, $MgCl_2$, and each oligonucleotide for amplification with species-specific primers in Table 4

Primer pair	dNTP (μM)	$MgCl_2$ (mM)	Each oligonucleotide (mM)
MPV1 and -2	800	2.5	0.5
RCN1 and -2	800	2.5	0.5
VPX1 and -2	800	2.5	0.5
ECT1 and -2	800	1.5	0.5
VAR1 and -2	25	1.5	0.5
SKP1 and -2	25	1.5	0.1
GBL1 and -2	8	1.5	0.5
CPV1 and -2	8	1.5	0.25
CML1 and -2	8	1.5	0.025
VAC1 and -2	8	1.5	0.025

monkeypox virus strains, has previously been done by direct sequencing of the HA gene (26, 41) and mapping of genome DNA (12, 17, 21, 28, 31).

Differentiation of variola major virus from alastrim variola minor virus. In a comparison of the HA sequences of several different variola virus strains (26, 41), we noted the presence of a single *Hha*I site in all but alastrim variola minor viruses. It followed that production of a *Taq*I digest pattern could be used initially for identifying variola virus DNA (Fig. 2B) and that an *Hha*I pattern could then be used for differentiating alastrim virus from other variola virus strains. Figure 3 shows *Hha*I digest profiles after DNA amplification with EACP1 and EACP2 from the genome of three variola major virus strains (Bangladesh-1975, Congo-1970, and Harvey-1944) produces 738- and 204-bp fragments. Fragments of the same size were observed with an African variola minor virus strain (Somalia-1977), which further sequence analysis (41) suggests is a spontaneously attenuated African variola major virus. The amplified fragments from the DNA of three alastrim variola minor virus strains (Brazil-Garcia-1966, Sierra Leone-1968, and Butler-1952) are not cleaved and thus show the 957-bp fragment. In separate experiments (41), *Taq*I and *Hha*I digestions after amplification with EACP1 and EACP2 have been used successfully to verify the present method by confirming the identity of variola major virus and alastrim virus strains from DNA extracted from 20 separate variola virus clinical scab specimens

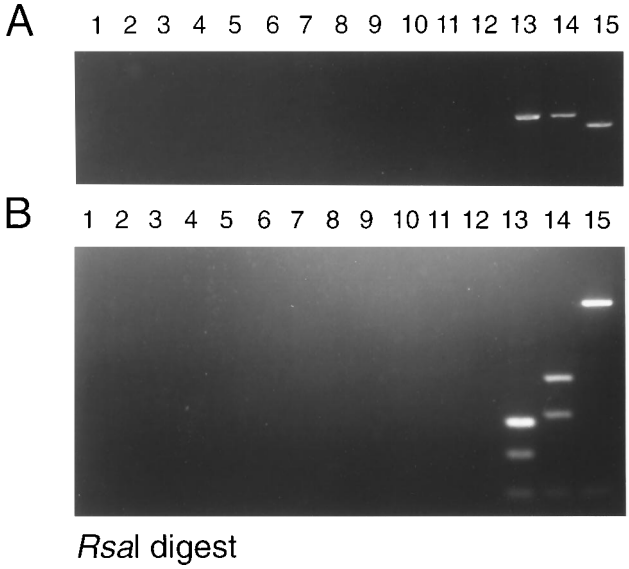

FIG. 1. Identification and differentiation of North American orthopoxviruses from intact genome DNA by *Rsa*I digest electrophoresis of an amplified HA DNA fragment (no product obtained with DNAs 1 to 12). (A) PCR HA DNA products made with consensus sequence primers NACP1 and NACP2 (Table 1). PCR templates were DNAs from the following virus strains (by lane): 1, variola major virus Bangladesh-1975 (VAR-BSH); 2, variola minor alastrim virus Brazil-Garcia-1966 (VAR-GAR); 3, buffalopox virus India-81-1985 (BFL); 4, rabbitpox virus Utrecht (RPV-UTR); 5, vaccinia virus Copenhagen (VAC-CPN); 6, camelpox virus Somalia-1978 (CML); 7, human monkeypox virus Congo-8 (MPV-CNG); 8, monkey monkeypox virus Copenhagen (MPV-CPN); 9, ectromelia virus Moscow (ECT-MOS); 10, cowpox virus 58 (CPV-58); 11, cowpox virus Brighton (CPV-BRT); 12, Tatera gerbilpox virus Dahomey-1971 (GBL); 13, raccoonpox virus Aberdeen-1964 (RCN [652-bp product]); 14, skunkpox virus Colfax-1978 (SKP [658-bp product]); and 15, volepox virus Jasper Ridge (VPX [580-bp product]). (B) *Rsa*I digest fragments of the PCR products in panel A, lanes 13 to 15. The sizes of the digest fragments were as follows: lane 13, RCN, 194, 192, 153, and 113 bp; lane 14, SKP, 264, 202, 113, 71, and 8 bp; and lane 15, VPX, 467 and 113 bp. PCR, restriction digests, gel electrophoresis, and ethidium bromide staining were done as described in the text. The fragment sizes above are from determined HA sequences (4, 26, 41); the determined sequences agreed with sizes of fragments estimated by parallel electrophoresis (not shown) of *Msp*I-digested pBR322 DNA and *Hae*III-digested ϕX174 DNA.

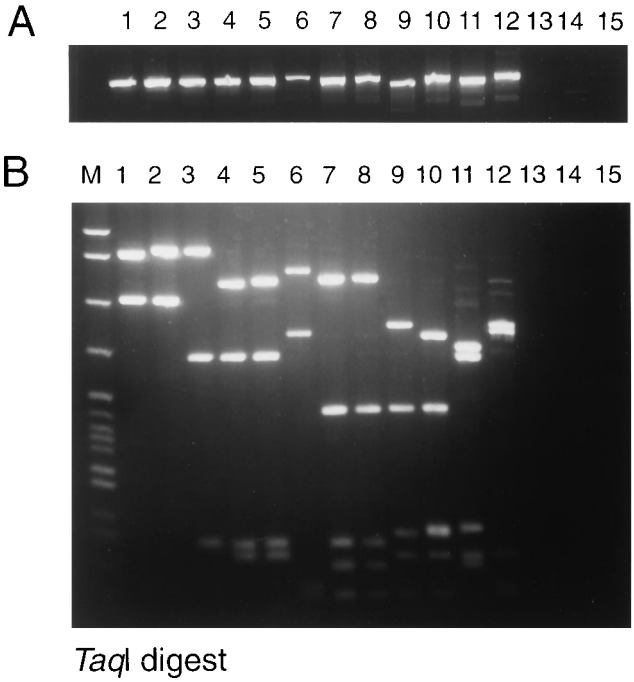

FIG. 2. Identification of Eurasian-African orthopoxviruses from intact genome DNA by *Taq*I digest electrophoresis of an amplified HA DNA fragment. (A) Electrophoresis of amplified HA fragments produced by using the consensus sequence primers EACP1 and EACP2 (Table 1). Template DNAs and sizes of PCR products from the viruses were (by lane) as follows: 1, VAR-BSH, 942 bp; 2, VAR-GAR, 957 bp; 3, BFL, 945 bp; 4, RPV-UTR, 938 bp; 5, VAC-CPN, 948 bp; 6, CML, 960 bp; 7, MPV-CNG, 942 bp; 8, MPV-CPN, 942 bp; 9, ECT-MOS, 846 bp; 10, CPV-58, 942 bp; 11, CPV-BRT, 894 bp; 12, GBL, 960 bp; 13, RCN, no products; 14, SKP, no products; and 15, VPX, no products. (B) Electrophoresis of the *Taq*I digest of the fragments in panel A. The sizes of the digest fragments were (by lane) as follows: 1, VAR-BSH, 536 and 406 bp; 2, VAR-GAR, 551 and 406 bp; 3, BFL, 545, 295, and 105 bp; 4, RPV-UTR, 442, 295, 104, and 97 bp; 5, VAC, 451, 295, 105, and 97 bp; 6, CML, 474, 331, 80, and 75 bp; 7, MPV-CNG, 451, 220, 105, 91, and 75 bp; 8, MPV-CPN, 451, 220, 105, 91, and 75 bp; 9, ECT-MOS, 343, 220, 111, 97, and 75 bp; 10, CPV-58, 324, 220, 115, 111, 97, and 75 bp; 11, CPV-BRT, 303, 289, 115, 96, and 91 bp; and 12, GBL, 342, 331, 97, 80, 75, and 35 bp. PCR, restriction digests, gel electrophoresis, and ethidium bromide staining were done as described in the text; virus abbreviations are defined in the legend to Fig. 1. The fragment sizes presented above are from the HA sequences determined (4, 26, 41), which agreed with the sizes of the fragments observed in comparison with the *Msp*I-digested pBR322 DNA size marker (M).

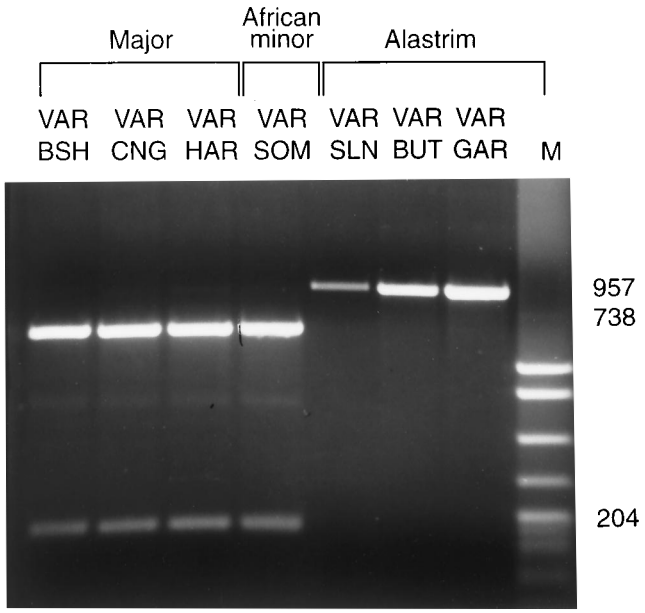

FIG. 3. Differentiation of alastrim variola minor virus strains from other variola virus strains. Electrophoresis of *Hha*I digests of DNA products amplified with primers EACP1 and EACP2 (Table 1) and genome DNA of different variola virus strains was done after species identification by *Taq*I digestion as illustrated in Fig. 2. Except as noted, virus abbreviations are defined in the legend to Fig. 1. VAR-CNG, variola major virus Congo-1970; VAR-HAR variola major virus Harvey-1944; VAR-SOM, African variola minor virus Somalia-1977; VAR-SLN, alastrim variola minor virus Sierra Leone-1968; VAR-BUT, alastrim variola minor virus Butler-1952. The fragment sizes shown are from the HA sequences determined (4, 26, 41), which agreed with the sizes of the fragments observed in comparison with the *Msp*I-digested pBR322 DNA size marker (M).

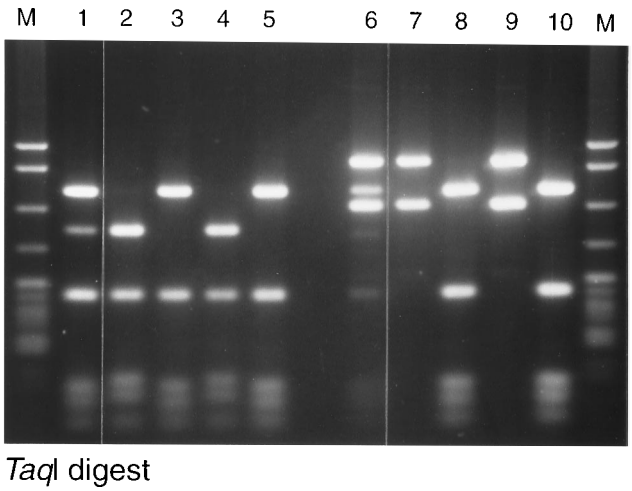

FIG. 4. Examples of differentiation of individual Eurasian-African orthopoxvirus DNAs in deliberately mixed genome DNA samples. First, *Taq*I digest electrophoresis was performed after amplification of HA DNA with the consensus primers (Table 1). When the cleavage fragment patterns were compared with those depicted in Fig. 2, a prediction of the virus DNAs in the mixture was made. Second, *Taq*I digest electrophoresis was done separately with products of individual amplifications performed with appropriate primers selected from Table 2 and paired as described in Table 3. For illustrative purposes, the results of the first (lanes 1 and 6) and second (lanes 2 to 5 and 7 to 10) electrophoresis steps are combined in this figure; virus abbreviations are defined in the legend to Fig. 1. Lanes 1 to 5 show DNA fragment patterns after *Taq*I cleavage of HA DNA amplified either from a 1:1 mixture of the MPV-CNG and ECT-MOS genome DNAs (lanes 1 to 3) or from the individual (not cross-contaminated) DNAs (lane 4, MPV-CNG; lane 5, ECT-MOS). PCRs were done with the primers EACP1 and EACP2 (lane 1), G-ECG and G-EMBRV (lanes 2 and 4), and G-MPV and G-EMBRV (lanes 3 and 5). Lanes 6 to 10 show DNA fragment patterns after *Taq*I cleavage of HA DNA amplified either from a 1:1 mixture of the VAR-GAR and MPV-CNG genome DNAs (lanes 6 to 8) or from the individual (not cross-contaminated) DNAs (lane 9, VAR-GAR; lane 10, MPV-CNG). PCR was done with the primers EACP1 and EACP2 (lane 6), G-VAR and G-CGV (lanes 7 and 9), and G-MPV and G-EMBRV (lanes 8 and 10). Lane M, *Msp*I-digested pBR322 DNA size marker.

and 6 separate specimens from virus-infected chicken embryo chorioallantoic membranes.

Oligonucleotides of higher template-primer sequence specificity. During experimentation with the consensus primers, we examined orthopoxvirus DNAs that had been inadvertently cross-contaminated and noted complex *Taq*I digest patterns with fragment sizes (Fig. 2B) indicative of two viruses. Because such a situation has been encountered in routine diagnostics (for example, see references 12, 15, 20, and 36 to 38 regarding so-called variola virus-like "whitepox" viruses), we designed the primers shown in Table 2 on the basis of the actual sequences around the HA start and stop codons. With no base mismatches, we were able to attain greater sequence specificity during annealing of primer to template. After several tests to examine specificity, we concluded that pairing the oligonucleotides as indicated in Table 3 usually provided for preferential HA amplification of particular Eurasian-African orthopoxvirus species DNA in mixed DNA samples.

Figure 4 presents two examples that show the high level of specificity of DNA synthesis when two separate primer pairs from Table 3 are used for amplification from deliberately mixed virus genome DNA samples. Figure 4, lane 1, shows the results of *Taq*I digest electrophoresis after PCR amplification when the consensus primers EACP1 and EACP2 were used with a sample of monkeypox virus DNA mixed with ectromelia virus DNA. Comparison of the digest fragment sizes with the fragments in the patterns in Fig. 2B was done to identify presumptively the individual virus DNAs in the mixture. Appropriate primer pairs were then selected from Table 3. Figure 4, lanes 2 and 3, shows *Taq*I digest electrophoresis patterns after separate amplifications in which the mixed DNAs were primed with the monkeypox virus-distinguishing oligonucleotides G-MPV and G-EMBRV or the ectromelia virus-distinguishing oligonucleotides G-ECG and G-EMBRV. Controls (lanes 4 and 5) included *Taq*I digests of products made by using the specific primers and the individual, not cross-contaminated virus DNAs. Similarly, lanes 6 through 8 show *Taq*I electropherograms after a mixture of variola virus and monkeypox virus DNAs was amplified by using the consensus primers EACP1 and EACP2 (lane 6), the variola virus-distinguishing primers G-VAR and G-CGV (lane 7), or the monkeypox virus-distinguishing primers G-MPV and G-EMBRV (lane 8). Controls (lanes 9 and 10) included *Taq*I digests of products made with the specific primers and the individual, not cross-contaminated DNAs.

Using the primer pairs shown in Table 3, we have amplified from various mixtures of orthopoxvirus DNA (41) and have observed a general preference of the oligonucleotides (Tables 2 and 3) to prime and produce a product from the genome template that contains an HA sequence with the lowest number of template-to-primer base sequence mismatches. The precise match of the base sequences, not the template concentration, appeared to be the major influence governing which virus species HA was amplified. However, if both genome templates contained an equal number of template-to-primer base sequence mismatches, the genome DNA sample present in the highest concentration served as the preferred template under

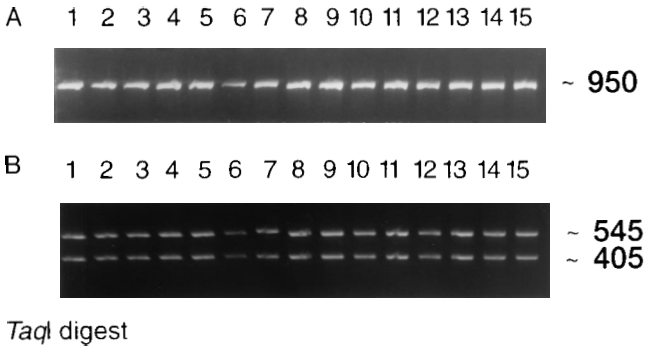

FIG. 5. Identification of variola virus HA DNA in smallpox scab specimens from the Centers for Disease Control and Prevention Smallpox Virus Specimen Repository. (A) Electrophoresis of HA DNA with a size of ~950 bp, depending on strain, was amplified with primers G-VAR and G-CGV from genome DNAs extracted from different crusted-scab specimens. (B) Electrophoresis of *Taq*I digests (~545- and 405-bp fragments) of the PCR products in panel A. The 15 lanes show the following different specimens: 1, Herrlich Bombay-1958; 2, Chandra Bangladesh-1974; 3, Jalaluddin Bangladesh-1974; 4, Hawa Bangladesh-1974; 5, Parvin Bangladesh-1974; 6, Solaiman Bangladesh-1974; 7, Kudano Nigeria-1961; 8, Level Liverpool-1958; 9, Variolator 4 Afganistan-1971; 10, Ethiopia 17; 11, Ethiopia 16; 12, Mannan Bangladesh-1974; 13, Hembula Tanzania-1965; 14, Variolator 2 Afghanistan-1972; and 15, Shahzaman Bangladesh-1974. *Hha*I digest electrophoresis (41) of the PCR products in panel A indicated that these were variola major viruses, except for specimen 7, which was an alastrim virus sample.

the standard PCR conditions described in Materials and Methods.

The primer pairs in Table 3 have enabled specific diagnosis of orthopoxviruses, if present, in several samples examined, including routine diagnostic specimens and coded samples whose identity was unknown to the person performing the assay. The primer pairs in Table 3 have also proven useful for identifying virus in certain samples that we noted gave no products after priming with the consensus primers. Taken together, the results above indicated that both the consensus primers and the primers in Table 3 would be needed to effectively diagnose and differentiate orthopoxviruses by PCR and restriction digestion of the product.

Tests of clinical materials. Heretofore, we have described the results of assays that used orthopoxvirus DNA extracted from infected tissue culture cells. However, we have also successfully identified orthopoxvirus species in crusted-scab clinical samples from human variola virus and human or monkey monkeypox virus infections. Figure 5 provides an example of PCR confirmation of variola virus in human scab samples in the Centers for Disease Control and Prevention Smallpox Specimen Repository. DNA from the specimens was extracted in the Centers for Disease Control and Prevention Maximum Containment Laboratory by using the methods described above. For the PCR, primer G-VAR was paired with G-CGV (Table 3), the fragment amplified (950 bp) from the DNA samples was then treated with *Taq*I, and digest fragments were resolved by electrophoresis. Figure 5 shows the distinguishing 545- and 405-bp *Taq*I digest DNAs observed after several different smallpox lesion samples were tested. Subsequently, identical results were obtained with consensus primers, and *Hha*I digests showed whether the samples were alastrim virus or variola major virus strains (41).

Primers for virus identification without endonuclease digestion. During analyses of the HA sequences of different orthopoxviruses (26), we also noted the possibility of making yet another set of primers based on other sequences that are highly species specific within the HA open reading frame. We reasoned that such primers might be useful for verifying results from the PCR assays described above or for developing a test based on rapid hybridization. Table 4 describes the oligonucleotide pairs, and Fig. 6 is a diagram illustrating the annealing locations of the second set of primer pairs that we derived with a series of different sequence primers through several experiments. Such experiments also established optimal reaction conditions by titration to amplify a single fragment with a size that would indicate the species of orthopoxvirus without restriction digestion. Using the primers in Table 4 and the reaction conditions described in Table 5, we performed separate

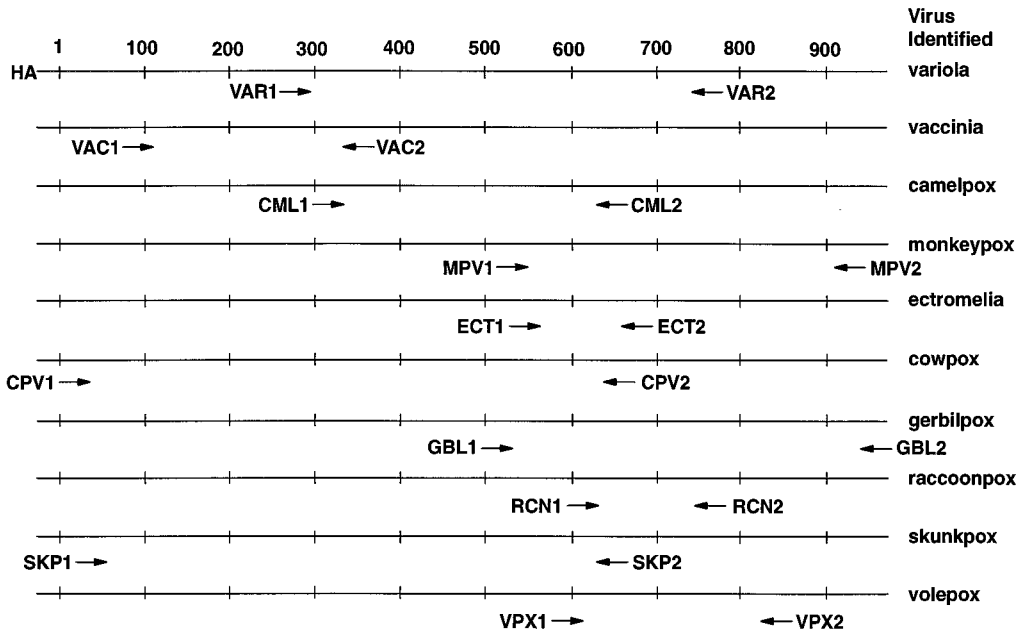

FIG. 6. Virus types identified and illustration of the annealing locations within the HA open reading frame of the primer pairs described in Table 4.

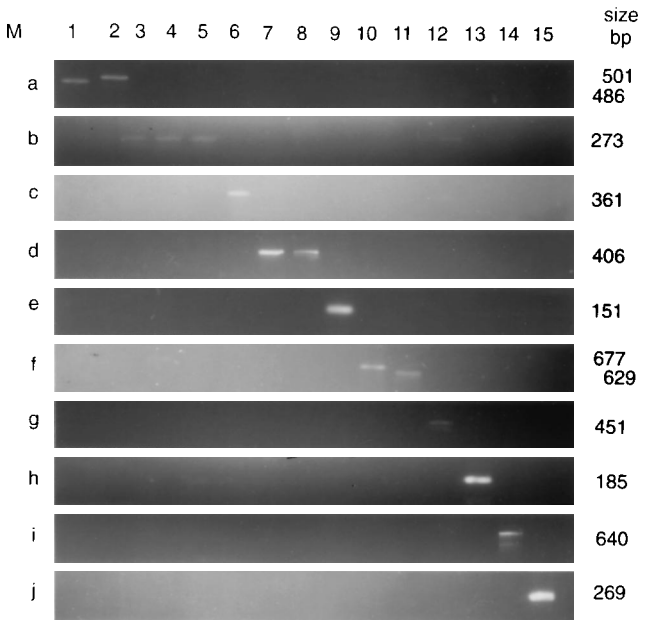

FIG. 7. Amplified HA DNA fragments of different sizes identify different orthopoxvirus strains. Separate electrophoresis separations of HA DNA products were done after amplification of different orthopoxvirus genome DNAs by using the primer pairs described in the legend to Fig. 6 and Table 4 and PCR modifications described in the text and Table 5. Virus abbreviations are defined in the legend to Fig. 1. Amplifications were done with DNA from the following viruses (by lane): 1, VAR-BSH; 2, VAR-GAR; 3, BFL; 4, RPV-UTR; 5, VAC-CPN; 6, CML; 7, MPV-CNG; 8, MPV-CPN; 9, ECT-MOS; 10, CPV-58; 11, CPV-BRT; 12, GBL; 13, RCN; 14, SKP; and 15, VPX. The following primer pairs (Table 4 and Fig. 6) were used: a, VAR1 with VAR2; b, VAC1 with VAC2; c, CML1 with CML2; d, MPV1 with MPV2; e, ECT1 with ECT2; f, CPV1 with CPV2; g, GBL1 with GBL2; h, RCN1 with RCN2; i, SKP1 with SKP2; and j, VPX1 with VPX2. The base pair sizes of the amplified DNAs are shown. (Note band in row b, lane 12: the data here and in other experiments [41] with the earliest-passage material in our collection indicated that our GBL stock likely had been inadvertently cross-contaminated with a vaccinia virus strain during isolation and passage in 1975.)

PCR amplifications from various orthopoxvirus DNA preparations. Figure 7 shows the electrophoresis fragments observed after selected DNA preparations were amplified with the extremely specific HA primers listed in Table 4 and the precise dNTP, $MgCl_2$, and primer concentrations described in Table 5.

Regarding specificity, we note here that among several of the virus species used for Fig. 7, primer and template sequence matches differed by only 1 bp at the 3′ end.

We also note that in separate experiments (41), we gradually decreased or increased the PCR stringency by varying selected components shown in Table 5. Generally, the consequence of decreasing stringency was a loss of specificity, and the result of increasing stringency was a decrease in the amount of PCR product. In fact, with increasing stringency, the PCR products became more and more difficult to visualize in stained gels without resorting to blot hybridization. Our experiences have indicated that the use of such highly specific primers for routine diagnostics is feasible; however, we recommend caution in interpreting the results because the specificity of the primers relies on precise use of PCR conditions and the quality of the DNA preparation. Because the extreme specificity brings the potential for false-positive or false-negative results, direct sequencing of products as we have done should provide definitive virus identification.

DISCUSSION

The present report describes rapid and accurate PCR methods based on the HA gene for identifying orthopoxviruses and differentiating species and sometimes strains. One method uses restriction cleavage of PCR products produced either with a set of HA consensus sequence primers or with a set of more specific sequence primers; both sets are designed to amplify most of the HA open reading frame. Amplifications with the Eurasian-African or North American virus HA consensus primer pairs, depending on the subgroup, provided a PCR product that inherently indicates the presence of an orthopoxvirus, because among poxviruses, only the orthopoxviruses produce an HA. *Taq*I, *Hha*I, and *Rsa*I restriction digests provided sufficient differentiation to readily identify species, and certain subspecies and strains could also be discerned.

In applying these protocols to suspected orthopoxvirus samples, it is suggested that the PCR be done first with the consensus primers, and if no fragment results, the specific primers should be used to attempt amplification of the HA open reading frame (Tables 1 and 2). In testing the consensus sequence primers, we observed no product after PCR from one genome DNA sample each of a parapoxvirus, yatapoxvirus, suipoxvirus, and avipoxvirus. Furthermore, in our searches of the GenBank and EMBL nucleotide sequence databases, queries with the primer sequences herein showed a significant match only with orthopoxvirus HA DNA sequences.

A second method that uses optimized reaction conditions and primers of precise sequence match to amplify a fragment whose presence and size identify the species is described. Because of the high stringency of this method, only certain strains might be able to be discriminated. Experience with more samples will help determine the efficacy of this approach. PCR under such defined conditions might require direct sequencing to confirm virus identity.

The protocols described here were designed for reproducibility in various laboratories, including hospital facilities with PCR and agarose gel electrophoresis capabilities. It may be that certain primers described here could be used in other detection and differentiation methods that are based on nucleotide sequences, for example, in automated DNA or RNA hybridization techniques.

The primers that we developed worked very well with purified virus DNA of known concentration. They have been successfully used to verify virus identity in a limited number of clinical samples. However, until extensive experience and determination of limits of sensitivity with various materials are gained, identification of orthopoxviruses by PCR should serve as an adjunct to other established diagnostic procedures.

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