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Amplification refractory mutation system PCR assays for the detection of variola and *Orthopoxvirus*

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Abstract

PCR assays that can identify the presence of variola virus (VARV) sequences in an unknown DNA sample were developed using principles established for the amplification refractory mutation system (ARMS). The assay's specificity utilised unique single nucleotide polymorphisms (SNP) identified among *Orthopoxvirus* (OPV) orthologs of the vaccinia virus Copenhagen strain A13L and A36R genes. When a variola virus specific primer was used with a consensus primer in an ARMS assay with different O*rthopoxvirus* genomes, a PCR product was only amplified from variola virus DNA. Incorporating a second consensus primer into the assay produced a multiplex PCR that provided *Orthopoxvirus* generic and variola-specific products with variola virus DNA. We tested two single nucleotide polymorphisms with a panel of 43 variola virus strains, collected over 40 years from countries across the world, and have shown that they provide reliable markers for variola virus identification. The variola virus specific primers did not produce amplicons with either assay format when tested with 50 other *Orthopoxvirus* DNA samples. Our analysis shows that these two polymorphisms were conserved in variola virus genomes and provide a reliable signature of *Orthopoxvirus* species identification.
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Keywords: ARMS; Variola virus; *Orthopoxvirus*; Detection

1. Introduction

The World Health Organisation (WHO) announced the eradication of variola virus (VARV), the causative agent of smallpox in 1980 and subsequently recommended that global vaccination should cease (Fenner et al., 1988). Reference stocks of variola virus are currently maintained in WHO licensed repositories at the Centers for Disease Control & Prevention (CDC), Atlanta and Vector Laboratories, Novosibirsk. There is no information on possible unlicensed stocks that may be held elsewhere in the world, however the deliberate releases of a virulent anthrax strain in the USA in 2001 has highlighted the hazards that unlicensed stocks may represent. Today the majority of children and adults are not vaccinated against smallpox, and the consequences of a re-emergence of variola virus, by whatever means, would be far reaching without effective public health interventions (Gani and Leach, 2001; Meltzer et al., 2001).

The clinical presentation of ordinary, haemorrhagic and flat forms of smallpox have historically been confused with monkeypox, chickenpox, meningococcal and other diseases that produce generalized skin lesions (Henderson et al., 1999; Breman and Henderson, 2002). Laboratory diagnosis of smallpox can take several days if virus culturing is performed requiring biosafety level IV laboratories. A simple, reliable and sensitive diagnostic assay would offer improvement for effective medical and public health countermeasures in the event of a release. The first genetic techniques employed for specific identification of variola virus utilised restriction fragment length polymorphisms (RFLP) of viral genomic DNA (Mackett and Archard, 1979; Esposito and Knight, 1985; Dumbell et al., 1999). This approach has been used extensively to differentiate between other *Orthopoxvirus* (OPV) species including vaccinia, cowpox,

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camelpox, monkeypox, mousepox or ectromelia viruses and for other members of the genus. RFLP of genomic DNA requires lengthy virus culture to generate suitable quantities of high quality DNA. Today, PCR based methods offer considerable improvement in sensitivity and specificity for diagnosis and the subsequent sequencing of a large number of *Orthopoxvirus* genomes (Goebel et al., 1990; Smith et al., 1991; Massung et al., 1994; Shchelkunov, 1995; Antoine et al., 1998; Shchelkunov et al., 1998, 2000, 2002; Gubser and Smith, 2002; Afonso et al., 2002), has significantly increased the potential for developing new genetic based assays.

RFLP analysis of PCR products has been widely adopted for *Orthopoxvirus* species identification. RFLP of amplified A-type inclusion body protein gene sequences have been employed for differentiation of *Orthopoxvirus* species (Meyer et al., 1994, 1997; Neubauer et al., 1997, 1998). A collection of assays have been developed around genetic studies of the haemagglutinin (HA) protein, another *Orthopoxvirus* non-essential gene (Ropp et al., 1995). RFLP examination of the cytokine response modifier B gene has also demonstrated that polymorphisms can be exploited by restriction endonuclease digestion (Loparev et al., 2001). An extensive RFLP survey of 45 strains has also been performed on a set of 10 kb pair amplicons that represent the entire virus genome (LeDuc et al., 2002).

Recently, Lightcycler PCR of the HA gene was employed to amplify products from members of the genus, and could differentiate from other *Orthopoxvirus* species on the basis of melt curve analysis (Espy et al., 2002). However, only a very limited number of strains were investigated in this study. Rapid assays based on fluorogenic Taqman PCR of the HA gene have demonstrated the utility of single nucleotide polymorphisms (SNP) for *Orthopoxvirus* identification (Ibrahim et al., 1997, 1998; Sofi Ibrahim et al., 2003). Rapid fluorogenic assays offer benefits for rapid diagnosis but require expensive equipment and reagents.

A variola-specific PCR assay was described that could differentiate between variola major and alastrim minor isolates based on the size of the amplified product (Knight et al., 1995). However, a recent evaluation of this PCR revealed that a subset of cowpox viruses also produced amplicons of the same size described for variola minor strains (Meyer et al., 2002). Consequently, more sequence information on variola, vaccinia, cowpox, camelpox and monkeypox virus isolates is required to demonstrate the existence of species-specific sequences. Studies of *Orthopoxvirus* species to date have shown that unique genes are very rare, (Shchelkunov et al., 1998, 2000, 2002; Gubser and Smith, 2002) but species-specific SNPs are frequently observed in most genes. We have sequenced several genes from a large collection of Orthopoxviruses to identify virus specific polymorphisms and have observed that SNPs are usually the only reliable genetic elements for virus identification (Pulford et al., 2002). Frequently no restricticion endonucleases exist that can exploit these unique markers by RFLP. To test this hypothesis, we constructed PCR assays based upon the amplification refractory mutation system (ARMS) (Newton et al., 1989) which is a sensitive technique for interrogating single base pair differences between DNA templates. ARMS has been used to detect a number of different variants of the hepatitis B virus (Gramegna et al., 1993; Liang et al., 1994) as well as identify genetic profiles of virulent, attenuated or vaccine strains of transmissible gastroenteritis virus and porcine respiratory coronavirus (Lai et al., 1995). Using a modification of ARMS we have developed novel gel based single-tube assays which not only detect Old World Orthopoxviruses, but are also specific for variola virus. These multiplex assays employ three primers; two consensus primers generate an amplicon diagnostic of an Old World *Orthopoxvirus* and the third primer simultaneously binds to a variola-specific polymorphism and initiates extension of a shorter PCR product to detect the presence of variola virus. Examination of amplified PCR products by agarose gel electrophoresis allows the distinction of one (*Orthopoxvirus* present) or two (variola virus detected) PCR products. We show here the examination of these assays with DNA samples from an extensive panel including 43 variola and 50 other *Orthopoxvirus* isolates.

2. Methods

2.1. Viruses and cell lines

All variola virus DNA was prepared from strains kept at the WHO repository at the CDC, Atlanta, USA. The panel included DNA from 43 isolates obtained from five continents from between 1939 and 1977 (Table 1). Variola virus isolates were grown on BSC40 cells whereas other Orthopoxviruses (Table 1) were grown on MA104 cells (Pulford et al., 2002). Archived clinical tissue samples were recovered from liquid nitrogen.

2.2. Purification of sample DNA

All chemicals were purchased from Sigma Aldrich unless otherwise stated. Assays were initially developed using *Orthopoxvirus* DNA prepared from partially purified virus. MA104 cells infected with Orthopoxviruses were Dounce homogenised, centrifuged for 15 min at $1000 \times g$ and the supernatant was layered onto 10 ml of 36% (w/v) sucrose and centrifuged at $30{,}000 \times g$. The pellet was resuspended in 100 μl 10 mM Tris pH 8.0, 1 mM EDTA containing 2.5 μg proteinase K (Boehringer Mannhiem), 140 mM NaCl, 1% SDS and 1% 2-mercaptoethanol and incubated at 55 °C for 30 min. The sample was then phenol:chloroform extracted and the aqueous phase collected, 10 μl 3 M NaCl was added and the genomic DNA was precipitated by addition of 2.5 vol. 100% ethanol and centrifugation for 10 min. The DNA pellets were washed with 70% ethanol, air dried, resuspended in 50 μl distilled water and stored at +4 °C.

Table 1
Orthopoxvirus strains used for ARMS and multiplex PCR analysis

Orthopoxvirus species	Strain	Year of isolation	Origin	Collection provided by
Camelpox virus	CP-1	1972	Iran	H. Meyer
	CP-5		Dubai, UAE	
	CP-14	1993		
	CP-17	1994		
	CP-Saudi		Saudi Arabia	
	CP-202/95	1995		
	Somalia		Somalia	CDC
Cowpox virus	Brighton	1939	UK	ATCC
	EP-1	1971	Augsburg, GE	H. Meyer
	EP-2	1973	Ansbach, GE	
	Moscow Rat	1977	Russia	
	Catpox 5	1982	UK	
	Catpox 3	1983	Somerset, UK	
	OPV 85	1985	Hamburg, GE	
	OPV 89/1	1989	Mannhiem, GE	
	OPV 89/2		Bad Kissingen, GE	
	OPV 89/5		Ulm, GE	
	OPV 90/1	1990	Deisenhofen, GE	
	OPV 90/2		Bonn, GE	
	OPV 90/4		Grömitz, GE	
	OPV 91/1	1991	Landsberg, GE	
	OPV 91/2		Munich, GE	
	OPV 91/3			
	Norway	1995	Norway	
	Beaver	1997	Berlin, GE	
	OPV 98/1	1998	Landshut, GE	
	OPV 98/4		Göttingen, GE	
	OPV 98/5		Mülsen, GE	
Ectromelia virus	MP-1	1983	Munich, GE	H. Meyer
	MP-2			
	MP-4			
	Moscow		Russia	
	Silverfox	1992	Czech Republic	
	US #33221		Bethesda, USA	
Monkeypox virus	79-I-005	1979	Democratic Republic of Congo	CDC
	Z1	1997		R. Gopal
	AP-1			H. Meyer
	MSF #6	2001		
	MSF #10			
	INRB 41			
	INRB 45			
Racoonpox virus	VR838		USA	H. Meyer
Vaccinia virus	Copenhagen	1958	Denmark	H. Meyer
	BP-1	1971	India	
	MVA		Ankara, Turkey	
	Elstree		London, UK	
	RPV		Utrecht, NL	
	WR		USA	
Variola virus	Minnesota 124	1939	USA	CDC
	Yamada	1946	Japan	
	Hinden		UK	
	Harvey		UK, imported	
	Lee	1947	Korea	
	Juba	1947	Sudan	
	Rumbec			
	Higgins	1948	UK	
	Horn	Unknown	China	
	Harper	Unknown	Japan	
	Stillwell			
	Butler	1952	UK	

Table 1 (*Continued*)

Orthopoxvirus species	Strain	Year of isolation	Origin	Collection provided by
	Kali Mathu	1953	Madras, India	
	New Delhi		New Delhi, India	
	Herrlich[a]	1958	Bombay, India	
	Kudano[a]	1961	Nigeria	
	7124	1964	Vellore, India	
	7125			
	SAF65-102	1965	Natal, RSA	
	SAF65-103		Transvaal, RSA	
	Hembula[a]		Tanzania	
	Garcia		Brazil	
	V66-39	1966	Sao Paulo, Brazil	
	K1629		Kuwait	
	V68-59	1968	Benin	
	Lahore	1969	Pakistan	
	V68-258		Sierra Leone	
	Congo	1970	Kinshasa, Congo	
	Variolator 4[a]		Afghanistan	
	V70-222		Sumatra	
	V70-228			
	V72-119	1972	Syria	
	V72-143		Botswana	
	ETH72-16		Addis, Ethiopia	
	ETH72-17			
	V73-225	1973	Botswana	
	Nepal 73		Nepal	
	Nur Islam	1974	Bangladesh	
	Shahzaman			
	Solaiman[a]			
	Parvin[a]			
	Mannan[a]			
	V77-1252	1977	Somalia	
	Heidelberg	Unknown	Germany	
	Iran 2602		Tabriz, Iran	
Varicella Zoster	V01-I-01			CDC

[a] DNA samples derived from scab material. All other DNA samples were from cell culture grown virus. *Abbreviation*: Germany, GE; The Netherlands, NL; United Arab Emirates, UAE; Republic of South Africa, RSA; Center for Disease Control, CDC; and American Tissue Culture Collection, ATCC.

All variola virus DNA samples prepared by the CDC were obtained from 0.1 ml of infected cell suspension. Samples were heat inactivated at 55 °C overnight and the samples were checked for sterility in tissue culture before processing with the Aquapure genomic lysis kit (Biorad) according to manufacturer's instructions. DNA prepared from clinical specimens was performed using the Aquapure genomic lysis kit for tissues according to manufacturer's instructions. Final DNA pellets were resuspended in up to 100 μl of DNA hydration buffer and incubated overnight at room temperature before storage at 4 °C.

2.3. ARMS assay design

The variola virus specific primers were designed after compiling sequences for orthologs of the vaccinia virus Copenhagen A13L and A36R genes (Pulford et al., 2002). Primers were designed to interrogate a specific polymorphism and specificity was increased by incorporating an adjacent mis-match base (Fig. 1, Table 2) using principles established for ARMS (Newton et al., 1989; Ferrie

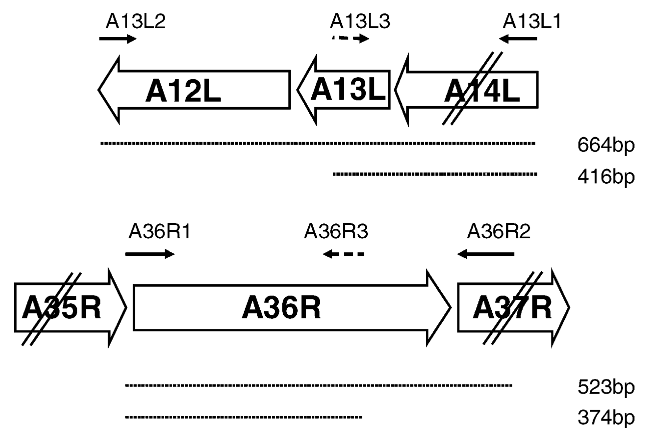

Fig. 1. Diagram (not to scale) illustrating PCR primers, *Orthopoxvirus* genes and their predicted PCR products. Open reading frames are shown as open arrows, correctly orientated in relation to the vaccinia virus Copenhagen genome and incomplete adjacent ORFs are shown with slashed lines. PCR primers are shown as black filled arrows (generic pair) or dashed arrows (variola virus specific). The predicted amplicon size for each primer pair is shown with dotted lines underneath each ORF.

Table 2
Primers used for PCR and sequencing

Name	Type	Primer sequence			
Multiplex primers					
A13L1	C	5′-GACTTTAGTAAGTCTACCAGTCCCACTC-3′			Sense
A13L2	C	5′-AAGATTATTGTTGCCTCCTTTGAC-3′			Antisense
A13L3	S	5′-TGTTTCTGGAGGAGGC*A*g*G*G-3′			Antisense
A36R1	C	5′-TCTTATCACAGTGACCGTAGTTGC-3′			Sense
A36R2	C	5′-GTAATGAACGGATTTGACTTGCTAC-3′			Antisense
A36R3	S	5′-TTTGTTCATTACAATCATTATTTATTAGg*C*-3′			Antisense
Control Templates (CT)					
A13L3CT		5′-TGTTTCTGGAGGAGGC*A*A*G*TTTAAATTCGGACT-3′			
A36R3CT		5′-TTTGTTCATTACAATCATTATTTATTAGC*C*CGCGTGCTTCCAG-3′			
Design at Primer 3′-end					
Position at 3′	−4	−3	−2	−1	
A13L ortholog					
Orthopoxvirus	C	G	A	A	
Variola virus	C	*A*	A	*G*	
A13L3 primer	C	*A*	g	*G*	
A36R ortholog					
Orthopoxvirus	A	G	C	A	
Variola virus	A	G	C	*C*	
A36R3 primer	A	G	g	*C*	

Primer types include *Orthopoxvirus* consensus (C) and variola virus specific (S) oligonucleotides. Variola virus specific polymorphisms are shown underlined and mismatch bases introduced into each primer sequence are shown in lower case.

et al., 1992). A third *Orthopoxvirus* primer upstream of the variola-specific primer was included to generate a multiplex assay (Fig. 1). The *Orthopoxvirus* generic product in both multiplex assays is larger than the variola virus specific amplicon.

2.4. Primers

High quality HPLC purified primer sets were used for multiplex and ARMS assays (Cruachem). Primers were designed with Oligo 6.0 software to have matching melting temperatures with minimum secondary structure and primer-dimer character. Ten micromoles working stocks of each primer were prepared and were added in varying ratios for optimal multiplex assay performance.

Positive control templates (CT) were generated from vaccinia virus IHD-J DNA by PCR using synthetic oligonucleotide primers that contained variola virus specific polymorphisms (Table 2, underlined). The CT primers were combined with the consensus primer in a PCR and the products were purified using a Qiaquick column and diluted 1/10,000 before they were used as control samples in PCR (see Fig. 2).

2.5. PCR components

All PCR reagents were purchased from Roche. Master mixes of reagents were prepared and 25 μl volumes were dispensed with 1 μl of sample DNA for PCR. Each A13L PCR assay included 200 nM of generic primers and 400 nM of variola-specific primer, whereas A36R PCR assays used 400 nM of generic primer and 200 nM of variola-specific primer. All PCR assays used 320 nmol of dNTPs (Roche), 3 mM $MgCl_2$ and 0.05 U/μl Taq DNA polymerase (Roche). Variola virus PCR assays contained between 0.3 and 2.8 ng of infected cell culture DNA per reaction.

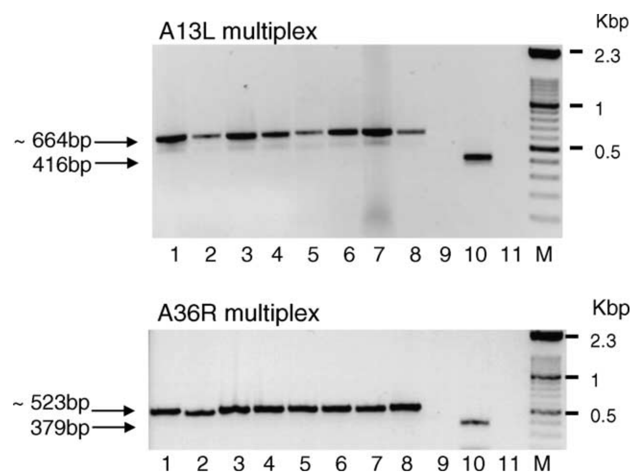

Fig. 2. The specificity of multiplex PCR assays with a panel of DNA from different *Orthopoxvirus* species. Multiplex assays were performed on DNA prepared from purified viruses as described in Section 2. DNA samples included; (1) vaccinia virus Copenhagen, (2) vaccinia virus MVA, (3) camelpox virus CP1, (4) cowpox virus Brighton, (5) cowpox virus EP1, (6) cowpox virus Norway, (7) ectromelia virus MP1, (8) monkeypox virus Z1, (9) racoonpox virus VR838, (10) variola virus synthetic control template, (11) water and (M) 100 bp ladder (Roche). Ten microliters of PCR product was loaded on a 1% agarose gel.

2.6. PCR cycling

All PCR assays were performed with an MJ Research PTC 200. Assays were optimised for specificity using the MJ 96V thermal gradient block and with a panel of *Orthopoxvirus* DNA or synthetic control templates. Multiplex and ARMS assays with variola virus genomic DNA were performed at the CDC, Atlanta using thermal cycling programs with predicted temperature algorithms. The optimised protocol for both assays was 94 °C 5 min; (94 °C 30 s, 60 °C 45 s, 72 °C 30 s) × 20 cycles; (94 °C 30 s, 60 °C 45 s, 72 °C 30 s + 10 s/cycle) × 10 cycles; 72 °C 10 min; +4 °C.

3. Results

3.1. Specificity of assay primers

To evaluate the specificity of the multiplex PCR we tested the A13L and A36R multiplex assays with a small panel of *Orthopoxvirus* DNA samples (Fig. 2). Synthetic control templates were used to confirm the functioning of the variola virus specific primer in each multiplex assay (lane 10). The shorter variola-specific amplicon was absent from all the *Orthopoxvirus* genome samples (lanes 1–9) but was produced by the control template (lane 10) confirm-

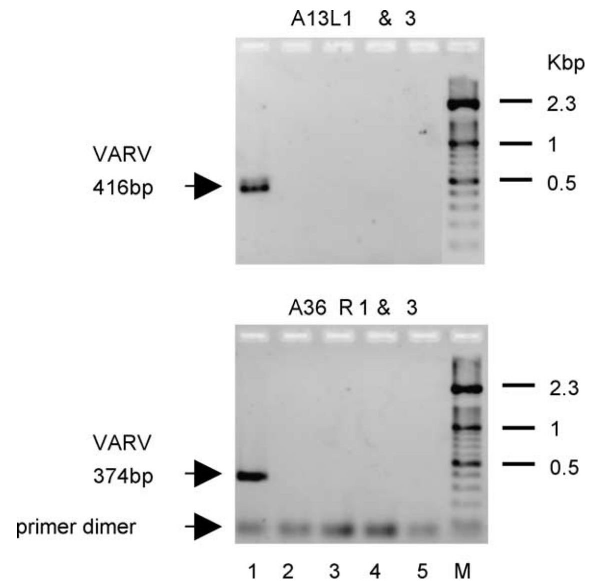

Fig. 3. Testing the variola virus (VARV) primer specificity using an ARMS PCR. Reaction mixes included either variola virus specific primer A13L-3 or A36R-3, matched with a single *Orthopoxvirus* generic forward primer (i.e., A13L-1 or A36R-1, respectively). One nanogram of viral DNA from (1) variola virus Congo, (2) camelpox virus Somalia, (3) cowpox virus Brighton, (4) monkeypox virus 79-I-005, (5) vaccinia virus Copenhagen or (6) mock infected BSC40 cell DNA was added to 25 μl of PCR mix. DNA markers (100 bp ladder, Roche) are shown in the last lane on each gel (M). PCR primers were used in equal concentrations. Primer-dimers are present at the base of the A36R1 and three gel.

Table 3
Performance of A13L and A36R PCR assays with an extensive panel of *Orthopoxvirus* DNA excluding variola virus

Virus species	Strains	A13L multiplex		A13L ARMS	A36R Multiplex		A36R ARMS
		Consensus product	Variola product		Consensus product	Variola product	
Camelpox virus 7 strains	CP-1,CP-5, CP-14, CP-17, CP-Saudi, CP-202/89, Somalia	7+	7∅	7∅	7+	7∅	7∅
Cowpox virus 21 strains	Brighton, Beaver[c], Catpox 3[c], Catpox 5[a], EP-1[c], EP-2, Moscow Rat[c], Norway, OPV 85[c], OPV 89/1, OPV 89/2[b], OPV 89/5, OPV 90/1, OPV 90/2[a,c], OPV 90/4[a,c], OPV 91/1,OPV 91/2[b], OPV 91/3, OPV 98/1[a,c], OPV 98/4[c], OPV 98/5[a,c]	13+, 2∅, 1 ± weak, 5 n.d.	∅,5 n.d.	16∅, 5 n.d.	19+, 2 n.d.	19∅, 2 n.d.	8∅, 13 n.d.
Ectromelia virus 6 strains	MP-1, MP-2, MP-4, Moscow, Silverfox, US #33221	5 + 1∅	6∅	6∅	6+	6∅	6∅
Monkeypox virus 8 strains	Copenhagen, AP-1, MSF #6, MSF #10, INRB 45, INRB 41, Z1, 79-I-005	6 + 1∅ 1 ± weak	8∅	8∅	8+	8∅	8∅
Racoonpox virus	VR838	1∅	1∅	1∅	1∅	1∅	1∅
Vaccinia virus 7 strains	BP-1, Copenhagen, Elstree, IHDJ, MVA, RPV, WR	7+	7∅	7∅	7+	7∅	7∅

Assays were performed with A13L3 or A36R3 variola virus specific primers as either two primer ARMS or three primer multiplex assays. Symbols used show the presence (+) or the absence (∅) of a PCR product in these assays, (n.d.) were assays not done. Symbols are prefixed with the number of strains with that result. Cowpox virus strain OPV 91/1 and monkeypox virus strain IRNB41 did not produce significant quantities of A13L consensus amplicon (±weak).

[a] Strains not tested with any A13L assay.
[b] Strains not tested with any A36R assay.
[c] Strains not tested in an A36R ARMS two primer assay.

ing assay specificity. The consensus primers for the A13L and A36R genes produced PCR amplicons for all the Old World (lanes 1–8), but not for the New World (lane 9) *Orthopoxviruses*.

A more extensive survey was then performed to evaluate the reliability of variola virus specific amplification using a larger panel of *Orthopoxvirus* DNA samples. These cross-reactivity assays are summarised in Table 3. Multiplex and ARMS assay formats were employed with 7 camelpox, 21 cowpox, 6 ectromelia, 8 monkeypox and 7 vaccinia virus isolates. In all assays performed no variola virus product was generated in the A13L or A36R ARMS or multiplex assay formats. However, the A13L consensus primers proved unreliable at amplifying a generic *Orthopoxvirus* product with some cowpox virus strains (Table 3).

3.2. Performance of ARMS with variola DNA

To establish the specificity of the variola-specific primer we then performed some ARMS assays using a small panel of *Orthopoxvirus* genomes including variola genomic DNA (Fig. 3). The combination of the A13L1 and 3 primers produced a 416 bp product with variola DNA (lane 1), but no product with any other Orthopoxviruses in this experiment (lanes 2–5). A similar result was obtained with the A36R1 and 3 primers which produced a 374 bp variola-specific product (lane 1), but no product with the other *Orthopoxviruses* (lanes 2–5).

3.3. Confirmation of SNP targets in variola virus

The ARMS is an imperfect method for identifying SNPs because the absence of an amplified product reveals lit-

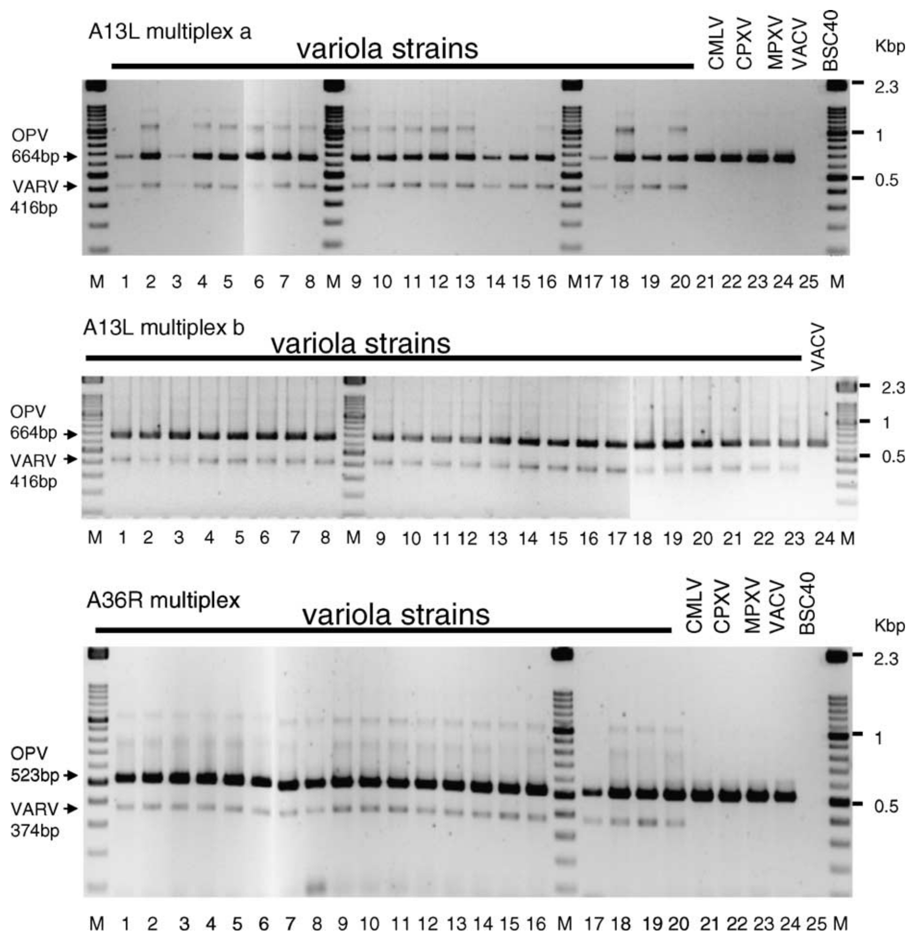

Fig. 4. Multiplex PCR performed on panels of variola virus genomic DNA samples extracted from virus infected BSC40 cells. DNA samples used for A13L multiplex a and the A36R multiplex included variola strains; (1) Congo, (2) Heidelberg, (3) Eth72-16, (4) v70-228, (5) v68-59, (6) Minnisota 124, (7) Juba, (8) Nepal 73, (9) K1629, (10) Harper, (11) Butler, (12) Horn, (13) Hinden, (14) 7125, (15) Kembula, (16) SAF65-103, (17) Higgins, (18) Iran 2602, (19) v77-1252, (20) Solaiman, and control strains included (21) camelpox virus (CMLV) Somalia, (22) cowpox virus (CPXV) Brighton, (23) monkeypox virus (MPXV) 79-I-005, (24) vaccinia virus (VACV) Copenhagen and (25) BSC40 mock infected cells. DNA samples used with the A13L multiplex b PCR included variola strains; (1) Garcia, (2) Yamada, (3) Lahore, (4) Lee, (5) V70-222, (6) Shah, (7) Kali Mathu, (8) Rumbec, (9) v77-1605, (10) 102, (11) v72-119, (12) v68-258, (13) 7124, (14) v73-225, (15) Ethiopia 17, (16) New Delhi, (17) Stillwell, (18) Harvey, (19) Nur Islam, (20) v66-39, (21) 72-143, (22) Variolator 4, (23) Somalia strains and control DNA from (24) vaccinia virus Copenhagen. DNA markers (100 bp ladder, Roche) are shown in lanes marked (M). Ten microliters of each PCR was run on a 1.5% agarose gel and stained with ethidium bromide to visualize DNA bands by UV illumination.

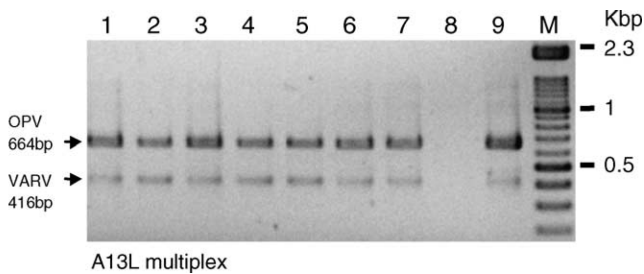

Fig. 5. Performance of a variola virus specific multiplex PCR assays with DNA extracted from clinical samples including variola viruses; (1) Solaiman (1.82 ng), (2) Kudano (1.23 ng), (3) Herrlich (8.59 ng), (4) Hembula (0.60 ng), (5) Variolator 4 (0.11 ng), (6) Parvin (3.83 ng), (7) Mannan (11.03 ng), (8) Varicella zoster V01-I-01 (2.37 ng) and (9) variola virus Congo infected BSC40 cell extract (1.85 ng) in a 25 μl multiplex PCR assay containing A13L1, two three three primers per tube. Amplified products were visualized by agarose gel electrophoresis and are indicated with arrows. A 100 bp ladder (Roche) is shown marked (M).

tle information about the assay performance. Therefore, we tested all variola DNA samples with the multiplex assay format to report the presence of *Orthopoxvirus* DNA and to confirm if the hypothesised variola-specific SNPs were present. As anticipated, all the variola virus genomes, produced two PCR amplicons, the smaller band represented a variola-specific product and the larger band was an *Orthopoxvirus* generic amplicon (Fig. 4). No bands were amplified from mock-infected BSC40 cell DNA in any assay (lane 25). A second panel of 23 more variola isolates was also tested with the A13L3 multiplex assays (Fig. 4, A13L multiplex b). Two PCR products were always amplified from all 43 variola strains tested demonstrating that the polymorphisms exploited by these multiplex assays were conserved.

3.4. Performance of PCR assay with clinical samples

To evaluate the performance of the multiplex for diagnosis we performed assays using DNA extracted from lesion or scab samples obtained from smallpox and chickenpox skin biopsies. These samples offered a significant test for the variola virus specific multiplex PCR because the DNA was of unknown quality being prepared from archived patient scabs collected during the eradication campaign. Seven separate variola virus isolates (Fig. 5, lanes 1–7) and one chickenpox (lane 8) sample prepared from scabs were compared using the A13L3 multiplex assay only. All seven biopsy samples produced two amplicons. No products were amplified from varicella zoster DNA (lane 8) confirming the suitability of this assay for diagnosis from clinical specimens.

4. Discussion

This paper describes an easy method for performing fast, reproducible and specific PCR assays with the minimum of equipment or reagents. To achieve this goal we compared *Orthopoxvirus* ortholog sequences of the vaccinia virus Copenhagen A13L and the A36R genes using a panel of African and Eurasian viruses. These two genes both code for virus membrane proteins, and although they are present in all Orthopoxviruses so far sequenced, they display remarkable sequence heterogeneity (Pulford et al., 2002). We exploited this diversity to design some new variola virus specific PCR assays.

The principles established for the ARMS PCR were exploited to design an oligonucleotide capable of specifically priming and extending from variola virus SNP's. The A13L-3 primer utilised two variola unique polymorphisms in close proximity and introduced a mismatch base A → G to generate further instability at the primer 3′-end (Table 2). This primer produced a characteristic 416 bp amplicon with variola virus DNA (Fig. 3, lane 1) in the ARMS assay. Priming events from *Orthopoxvirus* genomes other than variola virus were inhibited strongly by this cluster of three non-matched bases at the 3′-end of the A13L3 oligonucleotide primer making it highly specific.

The A36R-3 primer contained a single variola polymorphism and an adjacent base mismatch C → G to create further instability at the 3′-end. This primer worked specifically with variola DNA in an ARMS assay when combined with the A36R-1 primer, and also worked specifically in a multiplex with all variola viruses tested. The consensus primer pair A36R-1 and A36R-2 faithfully produced a larger ∼523 bp amplicon with all Orthopoxviruses tested.

The quantity of shorter variola virus specific amplicons should normally exceed longer PCR products because they are synthesised more efficiently. However, the incorporation of a mis-match base into the variola-specific primers reduces their binding energy and subsequently the efficiency of product amplification (Fig. 4). The A13L multiplex also revealed minor ∼1100 bp bands on gels with samples containing variola virus DNA only (Fig. 4). The size of this extra band corresponds to the sum of the consensus and specific products together, and may represent a heteroduplex. The production of potential minor heteroduplex complexes was also observed for other multiplex assays we performed (not shown) which may be the result of the repetitive sequence elements identified in the A13L orthologs (Pulford et al., 2002). *Orthopoxvirus* genes frequently contain repetitive sequence elements (Massung et al., 1996). Consequently, the structural characteristics of this sequence might explain the low abundance of variola virus specific products in the A13L-3 multiplex assays.

Knight et al. (1995) described a variola virus specific PCR assay that could differentiate between variola alastrim minor and major isolates based on the size of the amplified product. A recent evaluation of this published diagnostic method with a large panel of other Orthopoxviruses revealed that a subset of cowpox viruses also produce amplicons corresponding exactly with the size described for variola minor strains (Meyer et al., 2002). We analysed the performance of the ARMS and multiplex A13L and A36R assays with the same cowpox virus subset (Table 3, cowpox viruses EP-2,

OPV89/1, OPV89/5, OPV90/1, OPV91/1 and OPV91/3) and observed that no variola-specific amplicons were produced from these or any other *Orthopoxvirus* DNA samples.

It is clear that the production of an ever-expanding *Orthopoxvirus* sequence database (http://www.poxvirus.org/index.html) has given researchers the opportunity to produce many new assays (Espy et al., 2002), but the real value of any diagnostic assay can only be provided by the quantity and diversity of the samples tested. The ARMS and multiplex assays described herein were performed and validated with a very large panel of variola virus and *Orthopoxvirus* strains to confirm that the SNPs probed by these assays are preserved and unique to variola virus genomes.

The clinical presentation of a generalised exanthum is not uncommon. Smallpox was often confused with a range of common skin conditions, and frequently chickenpox and monkeypox have been difficult to differentiate from smallpox at early times in clinical presentation (Fenner et al., 1988; Breman and Henderson, 2002). The assays described here, were able to differentiate between DNA from smallpox, monkeypox and chickenpox and have demonstrated their reliability and specificity with a large collection of *Orthopoxvirus* samples, including clinical specimens. The application of these multiplex and ARMS PCR assays offer a simple and inexpensive alternative to the sequencing of amplicons generated from A13L or A36R orthologs for *Orthopoxvirus* identification.

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