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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 Orthopoxivirus (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 OPV 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 OPV 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 OPV DNA samples. Our analysis shows that these two polymorphisms were conserved in variola virus genomes and provide a reliable signature of Orthopoxivirus species identification.

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Keywords: ARMS; Variola virus; Orthopoxivirus; 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 Orthopoxivirus (OPV) species including vaccinia, cowpox,
camelpox, monkeypox, mouspox 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 viruses 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 restriction 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 (Grammegna 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 Orthopoxviruses 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 x g and the supernatant was layered onto 10 ml of 36% (w/v) sucrose and centrifuged at 30,000 x g. The pellet was resuspended in 100 µl 10 mM Tris pH 8.0, 1 mM EDTA containing 2.5 µg proteinase K (Boehringer Mannheim), 140 mM NaCl, 1% SDS and 1% 2-mercaptoethanol and incubated at 55 ºC 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 ºC.
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   | 1993              | Dubai, UAE   |                        |
|                       | CP-14  | 1994              |              |                        |
|                       | CP-Saudi | 1995     | Saudi Arabia |                        |
|                       | CP-202/95 | 1995 | Somalia      | CDC                    |
| Cowpox virus          | Brighton | 1939   | UK           | ATCC                   |
|                       | EP-1   | 1971              | Augsburg, GE | H. Meyer               |
|                       | EP-2   | 1973              | Ansbach, GE  |                        |
|                       | Moscow Rat | 1977 | Russia       |                        |
|                       | Capex 5 | 1982              | UK           |                        |
|                       | Capex 3 | 1983              | Somerset, UK |                        |
|                       | OPV 85 | 1985              | Hamburg, GE  |                        |
|                       | OPV 89/1 | 1989        | Mannheim, GE |                        |
|                       | OPV 91/1 | 1991       | Landsberg, GE|                        |
|                       | OPV 91/2 | 1991       | Munich, GE   |                        |
|                       | OPV 91/3 | 1991       | Norway       |                        |
|                       | Beaver | 1997              | Berlin, GE   |                        |
|                       | OPV 98/1 | 1998        | Landsbut, GE |                        |
|                       | OPV 98/4 | 1998       | Göttingen, GE|                        |
|                       | OPV 98/5 | 1998       | Munich, GE   |                        |
| Ectromelia virus      | MP-1   | 1983              | Munich, GE   | H. Meyer               |
|                       | MP-2   | 1983              | Munich, GE   | H. Meyer               |
|                       | MP-4   | 1983              | Munich, GE   | H. Meyer               |
|                       | Moscow | 1992              | Russia       |                        |
|                       | Silverfox | 1992   | Czech Republic|                        |
|                       | US 432221 | 1992 | Bethesda, USA |                        |
| Monkeypox virus       | 79-L005 | 1979              | Democratic Republic of Congo | CDC |
|                       | Z1     | 1997              |              | R. Gopal               |
|                       | AP-1   | 2001              |              | H. Meyer               |
|                       | MSF #6 | 2001              |              |                        |
|                       | MSF #10 | 2001         |              |                        |
|                       | INRB 41 | 2001       |              |                        |
|                       | INRB 45 | 2001       |              |                        |
| Raccoonpox virus      | VR838  | 1983              | USA          | H. Meyer               |
| Vaccinia virus        | Copenhagen | 1958  | Denmark      | H. Meyer               |
|                       | BP-1   | 1971              | India        |                        |
|                       | MVA    | 1962              | Ankara, Turkey |                        |
|                       | Elbree | 1982              | London, UK   |                        |
|                       | RPV    | 1982              | Utrecht, NL  |                        |
|                       | WR     | 1982              | USA          |                        |
| Variola virus         | Minnesota 124 | 1939 | USA          | CDC                    |
|                       | Yamala | 1946              | Japan        |                        |
|                       | Hinden | 1946              | UK           |                        |
|                       | Harvey | 1946              | UK, imported |                        |
|                       | Lee    | 1947              | Korea        |                        |
|                       | Juba   | 1947              | Sudan        |                        |
|                       | Rumbec | 1948              | UK           |                        |
|                       | Higgins | 1948       | unknown      | China                   |
|                       | Horn   | Unknown           |              |                        |
|                       | Harper | Unknown           |              | Japan                   |
|                       | Stillwell | 1952 | UK          |                        |
### Table 1 (Continued)

| Orthopoxvirus species | Strain       | Year of isolation | Origin          | Collection provided by |
|-----------------------|--------------|-------------------|-----------------|------------------------|
| Kali Mathu            | 1953         | Madras, India     | New Delhi, India|                        |
| New Delhi             | 1953         | New Delhi, India  |                 |                        |
| Hector               | 1958         | Bombay, India     |                 |                        |
| Keilana              | 1961         | Nigeria           |                 |                        |
| 7124                  | 1964         | Vellore, India    |                 |                        |
| 7125                  | 1965         | Natal, RSA       |                 |                        |
| SAF65-102             | 1964         | Transvaal, RSA    |                 |                        |
| Tzanzania             | 1965         | Tanzania          |                 |                        |
| V66-39                | 1966         | Sao Paulo, Brazil |                 |                        |
| K1429                 | 1968         | Kuwait            |                 |                        |
| V66-59                | 1968         | Benin             |                 |                        |
| Lahore                | 1969         | Pakistan          |                 |                        |
| V66-58                | 1969         | Sierra Leone      |                 |                        |
| Congo                 | 1970         | Kinshasa, Congo   |                 |                        |
| Variolator 4           | V70-222      | Afghanistan       | Somalia         |                        |
| V70-228               | V72-119      | Syria             | Botswana        |                        |
| V72-143               | ETH72-16     | Addis, Ethiopia   |                 |                        |
| ETH72-17              | V73-225      | Botswana          |                 |                        |
| Nepal 73              | V74-229      | Nepal             | Bangladesh      |                        |
| Nur Islam             | 1974         |                   |                 |                        |
| Shalataman            | 1977         | Somalia           |                 |                        |
| Suleiman              | 1977         |                   |                 |                        |
| Parme               | 1977         |                   |                 |                        |
| Mannara               | 1977         |                   |                 |                        |
| V77-1252              | Heidelberg   | Unknown           | Germany         |                        |
| Iran 2002             | V77-1252     |                   | Tabil, Iran     |                        |
| Variola-Zoster        | V01-I-01     |                   | CDC             |                        |

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...
Table 2

| Name               | Type | Primer sequence                  |
|--------------------|------|----------------------------------|
| Multiplex primers  |      |                                  |
| A13L1              | C    | 5′-GACTTCTAGTCAAGTCTACAGGACGC-3′  |
| A13L3              | S    | 5′-TTTTGTAGTGAGGAGGGGCTAGG-3′    |
| A36R3              | S    | 5′-GACTTGACTTGAGGAGGGGCTAGG-3′   |
| Control Templates (CT) | | 5′-TTTTGTAGTGAGGAGGGGCTAGG-3′    |

Design at Primer 3’-end

Position at 3’ −4 −3 −2 −1

A13L ortholog
Orthopoxvirus C G A A
Variola virus C Δ A G
A13L3 primer C Δ g G

A36R ortholog
Orthopoxvirus C G C A
Variola virus C G C C
A36R3 primer C G g C

Primers 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 Quiaquick 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 3.2 mM of dNTPs (Roche), 3 mM MgCl2 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](image-url)
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. Multi-plex 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 nanograms 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.]

| Virus species  | Strains                                      | A13L multiplex | A13L ARMS | A36R Multiplex | A36R ARMS |
|----------------|-----------------------------------------------|----------------|------------|----------------|------------|
| Camelpox virus | 7 strains                                     | 7+             | 7+         | 7+             | 7+         |
| Cowpox virus   | 21 strains                                    | 13+ 2+ ± weak  | 5 n.d.     | 16+ 5 n.d.     | 19+ 2 n.d. | 19+ 2 n.d. | 8+ 13 n.d.  |
| Ectromelia virus| 6 strains                                    | 5 + 12±       | 6±         | 6±             | 6±         | 6±         |
| Monkeys virus  | 8 strains                                    | 6 + 12± ± weak | 8±         | 8±             | 8±         | 8±         |
| Racoonpox virus| VR838                                         | 1±            | 1±         | 1±             | 1±         | 1±         |
| Vaccinia virus | 7 strains                                    | 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 91/3 and monkeypox virus strain BEN41 did not produce significant quantities of A13L consensus amplicon (twocycle).

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-
With arrows. A 100 bp ladder (Roche) is shown marked (M).

Products were visualized by agarose gel electrophoresis and are indicated.

PCR assay containing A13L1, two three three primers per tube. Amplified

format to report the presence of
tele information about the assay performance. Therefore, we
tested all variola DNA samples with the multiplex assay
to confirm if the hypothesised variola-specific SNPs were present. As anticipated, all the variola virus genomes, pro-
duced two PCR amplicons, the smaller band represented a variola-specific product and the larger band was an Or-
thropoxvirus generic amplicon (Fig. 4). No bands were am-
plicated 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 polymor-
phisms exploited by these multiplex assays were conserved.

3.4. Performance of PCR assay with clinical samples

To evaluate the performance of the multiplex for diagno-
sis 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 var-
iola 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 re-
markable 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 specif-
cally priming and extending from variola virus SNPs’s. The
A13L-3 primer utilised two variola unique polymor-
phisms 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 532 bp am-
plicon 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 polymor-
phism and an adjacent base mismatch C→G to create
further instability at the 3′-end. This primer worked specifi-
cally 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 prod-
uct amplification (Fig. 4). The A13L multiplex also revealed
minor ∼100 bp bands on gels with samples containing var-
iola virus DNA only (Fig. 4). The size of this extra band cor-
responds to the sum of the consensus and specific products
together, and may represent a heteroduplex. The produc-
tion of potential minor heteroduplex complexes was also ob-
served 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). Or-
thropoxvirus genes frequently contain repetitive sequence el-
ements (Massing et al., 1996). Consequently, the structural
characteristics of this sequence might explain the low abun-
dance of variola virus specific products in the A13L-3 mul-
tiplex 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 prod-
uct. 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 corre-
sponding 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, OPV89/2, 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 genotypes.

The clinical presentation of a generalised exanthem 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 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 differentiation of old world orthopoxviruses: restriction fragment length polymorphism of the crmB gene region. J. Clin. Microbiol. 39 (4), 1494–1502.

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