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Short communication

Gene for A-type inclusion body protein is useful for a polymerase chain reaction assay to differentiate orthopoxviruses

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Abstract

Orthopoxvirus species were identified and differentiated by polymerase chain reaction amplification of genome DNA using a single primer-pair based on sequences coding for the major protein component of the cowpox virus acidophilic-type inclusion body (ATI). DNA available for 6 of 8 Old World (cowpox, variola, monkeypox, camelpox, ectromelia and vaccinia viruses) and 3 New World (skunkpox, volepox, and raccoonpox) resulted in amplicons that ranged in size from 510 to 1673 base pairs depending on the species, except for raccoonpox virus DNA which did not amplify. *Xba*I digest gel electrophoresis profiles of the amplicons improved resolution of the differences. © 1997 Elsevier Science B.V.

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The genus *Orthopoxvirus* is composed of antigenically closely related viruses of veterinary and zoonotic importance (Fenner et al., 1989). Until the global eradication of smallpox in 1977, the etiologic agent, variola virus, was by far the most important poxvirus of humans; however, viruses belonging to three other species (monkey pox,

cowpox, vaccinia viruses and the vaccinia subspecies buffalopox virus) can also cause human infections.

The laboratory identification of variola virus and differentiation from other orthopoxviruses have conventionally been achieved by biological methods such as determination of pock morphology on the chorioallantoic membrane of chick embryos. Serologic tests are of limited value because genus members are antigenically highly

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cross-reactive (Esposito and Massung, 1995; Esposito and Nakano, 1988). The cross-reactivity is reflected by a high degree of base sequence homology; however, restriction endonuclease cleavage patterns and mapping of genome DNAs have provided distinct profiles that enable species and strain differentiation, including differentiation of variola major from alastrim variola minor strains (Esposito and Knight, 1985). But, generation of DNA restriction profiles and maps is laborious, requiring virus propagation and DNA purification. Variola virus growth is restricted to maximum containment facilities.

To overcome virus propagation and time-consuming biological techniques, rapid polymerase chain reaction (PCR) screening methods are being developed. For example, a primer set specific for detection of variola virus by PCR has been reported (Knight et al., 1995) and a PCR strategy to differentiate orthopoxvirus species based on the hemagglutinin gene has been described (Ropp et al., 1995). Moreover, a PCR assay has been reported that uses a consensus primer pair that targets DNA coding for the acidophilic-type inclusion body (ATI) protein of cowpox virus (Meyer et al., 1994).

In the latter method, primer-annealing positions flanked a section of the cowpox virus ATI open reading frame, which was hypervariable compared with corresponding sequences of camelpox, ectromelia, and vaccinia virus ATI DNA regions; the variations included truncations, deletions, insertions, and base changes. PCR of the four viral DNAs showed amplicons of slightly different size, which were able to be further resolved into different profiles by *Bgl*II digest electrophoresis. However, further studies of ATI sequences of five other species indicated that *Bgl*II digestion would not be optimal for differentiation. We have extended the ATI PCR method and show that *Xba*I digestion of the amplicon readily identifies and differentiates 9 of the 11 known orthopoxviruses (DNAs of two species were unavailable).

The origins of viruses used in the present study have been described elsewhere (Esposito and Knight, 1985; Meyer et al., 1994; Esposito et al., 1995). To develop the current method, viruses

were grown in chick embryo fibroblast, Rat-2 or MA-104 cells and DNAs were extracted from the cytoplasm of infected cells as described elsewhere (Meyer et al., 1997; Esposito and Massung, 1995). DNA amplifications were done in a total volume of 50 μ l reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3 and 2.5 mM MgCl₂) that contained 1 μ l DNA (~250 ng), 200 μ M of each dNTP, 100 ng of forward primer ATI-up-1 (5'-AATACAAGGAGGATCT-3'), 100 ng of reverse primer ATI-low-1 (5'-CTTAACCTTTTCTTTCTC-3') and 2.5 Units of Taq polymerase (Boehringer-GmbH, Germany). Reactions were thermocycled 25 times using a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT). Each cycle included denaturation at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2.5 min. Reaction mixes were then held at 72°C for 10 min followed by storage at 4°C until 5 μ l were analyzed by electrophoresis in 1.0% agarose gels (Meyer et al., 1997; Ropp et al., 1995).

PCR experiments with primer pair ATI-up-1/ATI-low-1 and template DNA extracted from cowpox, variola, vaccinia, ectromelia and camelpox virus produced individual virus amplicons of variable sizes as indicated in Table 1. The size differences corresponded with differences in base sequences of the ATI coding region in the GenBank database. The 880 bp amplicon observed after amplification of camelpox virus DNA has

Table 1
Comparison of PCR-derived DNA fragments of nine species of the genus Orthopoxvirus amplified with consensus primer pair ATI-up-1/ATI-low-1

Species	Strain	Amplicon size (base pairs)
Variola virus	Bangladesh-1975	1575
Monkeypox virus	Copenhagen	1500
Cowpox virus	Brighton	1673
Vaccinia virus	Western Reserve	1603
Ectromelia virus	Munich-1	1219
Camelpox virus	CP-1	880
Volepox virus	Jasper Ridge	510
Raccoonpox virus	Aberdeen-1964	No amplification
Skunkpox virus	Colfax-1978	530

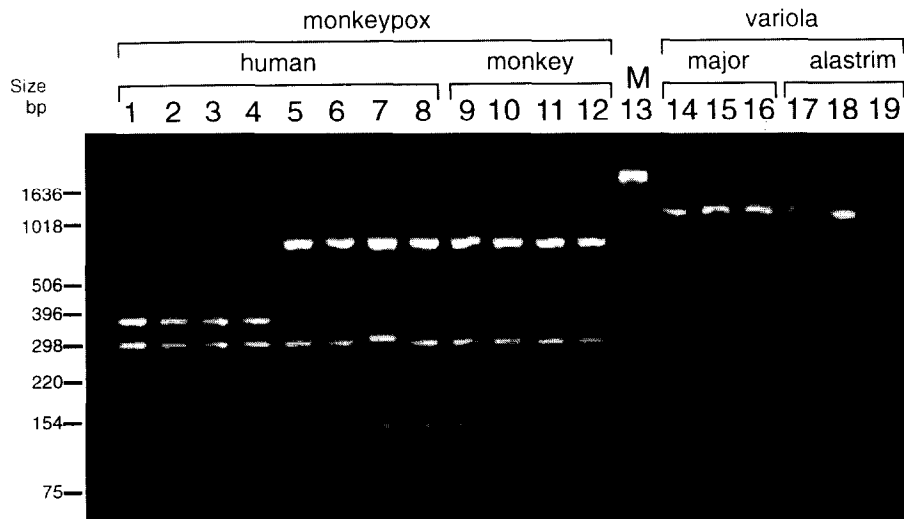


Fig. 1. PCR Identification and differentiation of monkeypox and variola virus strains by electrophoresis of *Xba*I digests of DNA products amplified using primers ATI-up-1 and ATI-low-1. Lanes 1–8, human monkeypox virus isolates Congo-8, 74-0226, 77-0666, 79-0005, 70-0187, 70-0266, 71-0082 and 78-3945; lanes 9–12, monkey monkeypox virus isolates Copenhagen-PVM-1958, Copenhagen-COPE-1958, Paris-PCH-1968 and Utrecht-UTC-6532; lanes 14–16, variola major virus isolates Bangladesh-1975, Congo-1970 and Harvey-1944; lanes 17–19, variola minor alastrim virus isolates Sierra Leone-1968, Brazil-Garcia-1966 and Butler-1952; lane 13, 1 kb DNA-ladder size marker (GIBCO-BRL, Gaithersburg, MD).

previously been shown to be useful for identifying this virus in lesion scabs from infected camels (Pfeffer et al., 1996). The broader utility of the primers for differentiating variola virus, monkeypox, volepox and skunkpox virus DNAs is demonstrated here by the appearance of amplicons of 1575, 1500, 530 and 510 bp, respectively. The only orthopoxvirus yielding no fragment was raccoonpox virus which is known to make a 155 kDa ATI protein (Esposito et al., 1995). The absence of a fragment may be of diagnostic value in some situations; however, the result suggested to us that further confirmatory PCR testing, e.g. HA PCR which gives a definitive-size raccoonpox virus DNA fragment (Ropp et al., 1995), should also be used.

As Table 1 shows, certain fragments are rather similar in size, therefore to differentiate more clearly viruses, endonuclease assays were done by adding 5 Units of *Xba*I (Boehringer GmbH) to 30 μ l of the thermocycled mixtures. Digests were incubated at 37°C for 2 h and cleavage products were separated by electrophoresis in 3% NuSieve-GTG agarose that contained 1% SeaKem-GTG agarose (FMC, Germany).

Fig. 1 shows an agarose gel electropherogram developed for a variety of orthopoxviruses of known origin and taxonomic status. Patterns are shown for DNAs of 4 monkeypox viruses isolated from primates, 8 monkeypox viruses from human cases, and 6 human variola virus isolates from different parts of the world isolated between 1944 and 1977. Lanes 14–19 show digest products using the 1570-bp amplicons generated from 6 different variola virus DNAs with ATI-up-1/ATI-low-1 primers. The digest pattern of the monkeypox virus DNAs (lanes 1–12) is clearly different from that of the variola viruses (lanes 14–19). Moreover, amplicon restriction patterns for monkeypox viruses from Zaire (lanes 1–4) show that the largest fragment is about 450 bp smaller than the corresponding fragment from non-Zairian monkeypox virus isolates (lanes 5–12) that originate from countries of coastal West Africa or from a primate imported into Europe from Africa. The results correlate with differentiation of Zairian and non-Zairian monkeypox viruses by DNA restriction maps (Esposito and Knight, 1985). The estimated sizes of the *Xba*I fragments determined

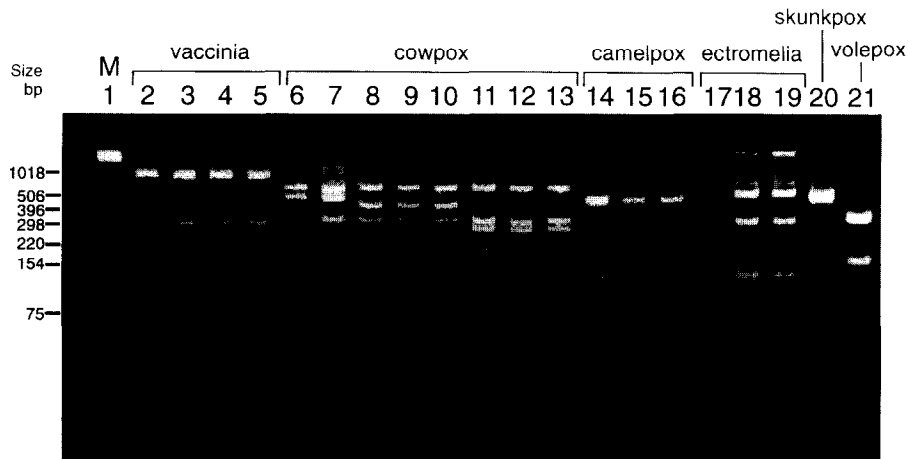


Fig. 2. Identification and differentiation of vaccinia virus, cowpox, camelpox, ectromelia, skunkpox virus and volepox virus by electrophoresis of *Xba*I digests of DNA products amplified using primers ATI-up-1 and ATI-low-1. Lane 1, 1 kb DNA-ladder size marker (GIBCO-BRL); lanes 2–5, vaccinia virus strains WR, IHD, CVA, and M1; lanes 6–13, cowpox virus isolates 88Lu, 91/3, EP-1, EP-3, EP-4, 89/3, 89/4, and 90/2; lane 14–16, camelpox virus isolates CP-1, Saudi and Niger; lane 17–19, ectromelia virus isolates M-1, M-2, and M-3; lane 20, skunkpox virus Colfax-1978; lane 21, volepox virus Jasper Ridge.

by gel electrophoresis of the variola virus ATI amplicon agreed precisely with reported sequence data (Massung et al., 1994).

In Fig. 2, *Xba*I digest patterns are shown for ATI-PCR-amplified genome DNAs of vaccinia, cowpox, camelpox, ectromelia, skunkpox, and volepox viruses; a distinguishing profile is apparent for each species. In separate experiments to examine the reproducibility of the present method, we were able to amplify and restrict amplicons derived from 20 different isolates of camelpox virus, which showed profiles identical to those in lanes 14–16 of Fig. 2. Nine isolates of ectromelia virus from mice also showed profiles identical to those in lanes 17–19.

Interestingly, after examining the DNA of 22 cowpox virus isolates from 7 different animal species (isolates described in Meyer et al., 1994), ATI-PCR and *Xba*I amplicon digestion, showed differences in the electrophoresis profiles that indicated the current method would be useful for strain (subspecies) differentiation. To provide an example of this, lanes 6–13 show profile differences for 8 of the 22 cowpox virus DNAs. The results with cowpox virus DNAs were in contrast

to those described above using monkeypox viruses of different geographic origin. There was no apparent correlation between restriction pattern and either the temporal or geographic origin of the cowpox virus strain and the different profiles did not correlate with the different host animal from which the virus was isolated.

It is concluded from the present study that the ATI-PCR amplicon restriction fragment length polymorphism assay provides an additional method to identify and differentiate orthopoxvirus isolates. The present method combined with procedures described previously (Ropp et al., 1995; Meyer et al., 1994; Knight et al., 1995) provides a series of PCR protocols targeting different genes to differentiate more accurately orthopoxvirus infections in the diagnostic laboratory.

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References

- Esposito, J.J. et al. (1995) Poxviridae. In: F.A. Murphy, C.M. Fauquet, D.H.L. Bishop, S.A. Ghabrial, A.W. Jarvis, G.P. Martelli, M.A. Mayo, M.D. Summers (Eds.), *Virus Taxonomy, Sixth Report of the International Committee on Taxonomy of Viruses*. Springer-Verlag, Berlin, pp. 79–91.
- Esposito, J.J. and Knight, J.C. (1985) Orthopoxvirus DNA: Comparison of restriction profiles and maps. *Virology* 143, 230–251.
- Esposito, J.J. and Massung, R.F. (1995) Poxviruses infecting humans. In: P.R. Murray, F. Tenover, E.J. Baron, M.A. Pfaller, and R.H. Tenover (Eds.), *Manual of Clinical Microbiology*, 6th edn., American Society for Microbiology, Washington, D.C., pp. 1131–1138.
- Esposito, J.J. and Nakano, J.H. (1988) Poxviridae: the poxviruses. In: E.H. Lennette, P. Halonen, and F.A. Murphy, (Eds.), *Laboratory Diagnosis of Infectious Diseases, Vol. 2. Viral, Rickettsial and Chlamydial Diseases*, Springer-Verlag, Berlin, pp. 177–207.
- Fenner, F., Wittek, R. and Dumbell, K.R. (1989) *The Orthopoxviruses*. Academic Press, New York.
- Knight, J.C., Massung, R.F. and Esposito, J.J. (1995) Polymerase chain reaction identification of smallpox virus. In: Y. Becker and G. Darai (Eds.), *Diagnosis of Human Viruses by Polymerase Chain Reaction Technology*, 2nd edn., Springer-Verlag, Berlin, pp. 297–302.
- Massung, R.F., Liu, L., Jin, Q., Knight, J.C., Yuran, T.E., Kerlavage, A.R., Parson, J.M., Venter, J.C. and Esposito, J.J. (1994) Analysis of the complete genome of smallpox variola major strain Bangladesh-1975. *Virology* 201, 215–240.
- Meyer, H., Pfeffer, M. and Rziha, H.-J. (1994) Sequence alterations within and downstream of the A-type inclusion protein genes allow differentiation of Orthopoxvirus species by polymerase chain reaction. *J. Gen. Virol.* 75, 1975–1981.
- Meyer, H., Ropp, S.L. and Esposito, J.J. (1997). Poxvirus diagnostic protocols. In: A. Warnes and J. Stephenson (Eds), *Methods in Molecular Biology: Diagnostic Virology Protocols*, Humana Press, Totowa, NJ, in press.
- Pfeffer, M., Meyer, H., Wernery, U. and Kaaden, O.-R. (1996) Comparison of camelpox viruses isolated in Dubai. *Vet. Microbiol.* 49, 135–146.
- Ropp, S.L., Jin, Q., Knight, J.C., Massung, R.F. and Esposito, J.J. (1995) Polymerase chain reaction strategy for identification and differentiation of smallpox and other orthopoxviruses. *J. Clin. Microbiol.* 33, 2069–2076.