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Short Communication

A ligase chain reaction targeting two adjacent nucleotides allows the differentiation of cowpox virus from other *Orthopoxvirus* species

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Abstract

A ligase chain reaction (LCR) assay was developed to distinguish cowpox virus from other *Orthopoxvirus* species. The LCR targets two adjacent adenosine residues which are only present in the A-type inclusion protein gene (ATI-gene) of cowpox virus. Two primer pairs were designed with a one base pair overlap at the junction site and one primer of each pair was labeled radioactively. Detection of the ligation product was achieved after denaturing polyacrylamide gel electrophoresis and autoradiography. Prior to LCR, the corresponding region of the ATI-gene was amplified by a consensus primer-directed polymerase chain reaction. All 18 cowpox virus isolates investigated could be clearly discriminated from 10 vaccinia virus strains, 5 camelpox virus isolates, as well as from mousepox and monkeypox virus reference strains. The LCR method allows a fast identification of cowpox virus isolates and is a feasible tool for the analysis of small mutations within viral genes.

Keywords: Ligase chain reaction; LCR; Radioactive detection; *Orthopoxvirus*; ATI-gene

The ligase chain reaction (LCR) has become a powerful tool for the detection of single base pair differences (for review see Wiedmann et al., 1994). Like the polymerase chain reaction (PCR), LCR can be used to detect trace levels of nucleic acids. LCR involves a number of two-step reactions consisting of a denaturing step and an

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annealing/ligation step in which two complementary primer pairs hybridize to the single-stranded target DNA and are subsequently ligated. Analogous to PCR, the ligation products serve as templates for the next cycle, resulting in an exponential amplification. To avoid template-independent ligation, the primer sets have been designed with non-complementary tails and a one base overlap at the ligation site (Barany, 1991a, b). To date, applications of LCR have focused on the discrimination of targets which differ in the loss or exchange of a single nucleotide (reviewed in Wiedmann et al., 1994). For the detection of differences of more than one nucleotide, other ligase-mediated detection systems such as 'gapped LCR (G-LCR™)' (Birkenmeyer and Mushahwar, 1991) and 'repair chain reaction (RCR)' (Segev, 1992) have been applied. These reactions use primers which are separated by a gap covering the discriminatory bases, which is filled in using the missing nucleotides and a thermostable polymerase. This step is followed by ligation of the elongated primer with the second primer using a thermostable ligase.

We intended to use the LCR in a comparative study concerning the A-type inclusion protein (ATI)-genes of different *Orthopoxvirus* (OPV) species. Although the formation of visible ATIs is restricted to cowpox, mousepox, and racoonpox virus-infected cells (Fenner et al., 1989), antigenetically related so-called LS-proteins have been demonstrated in several other OPV species (Patel et al., 1986). The function(s) of these proteins are to be determined while protection against enviromental damage or/and a misguiding of the immune system have been discussed (Fenner et al., 1989; Osterrieder et al., 1994). In the ATI-genes of both vaccinia virus Western Reserve (WR, Amegadzie et al., 1992) and camelpox virus CP-1 (Meyer and Rziha, 1993) a frame-shift is caused by a double A deletion which leads to a premature stop codon and a shortened open reading frame as compared to cowpox virus KR-2 Brighton (BR, Funahashi et al., 1988). Detection of the two A residues should therefore lead to a specific identification of cowpox virus. In order to demonstrate the double A residues, an LCR method was chosen which avoids fill-in reactions required in 'RCR' or 'gapped LCR'. Sequence alignment of the ATI-genes of BR with CP-1 and WR using the GCG software package (Version 7.0, Devereux et al., 1984) demonstrated a mismatch of three nucleotides (nt) for CP-1 and five nt for WR over a stretch of 58 nt adjacent to the double A deletion (Fig. 1). The reference strains of mousepox MP-1 (Mahnel, 1983) and monkeypox virus Copenhagen strain (von Magnus et al., 1959) are far more divergent in this region. These findings were used to design a primer set (consisting of the four primers KPV1Ra, KPV2Ra, KPV3, and KPV4, see Table 1 and Fig. 1) based on the published sequence of BR with the primer junction targeting the double A residue found in the ATI-gene of cowpox virus. These primers have a one-base overhang at the ligation site and two mismatching bases at the 3′ ends of KPV3 and KPV4 to prevent ligation at this end. In case of the double A deletion, juxtaposed primers should not be ligated due to their overlapping ends at the junction site. Primers KPV1Ra and KPV2Ra were labeled with [γ-^{32}P]ATP (Amersham) according to Barany (1991b) using T4 polynucleotide kinase (New England Biolabs, NEB). Labeled primers were purified with Nensorb 20 columns (NEB) according to the manufacturer's recommendation. A 100 fmol aliquot of each primer was incubated with different amounts of target DNA (see below) in 50 μl LCR-buffer (20 mM Tris, pH 7.6; 100 mM KCl; 10 mM $MgCl_2$; 1 mM EDTA; 10 mM

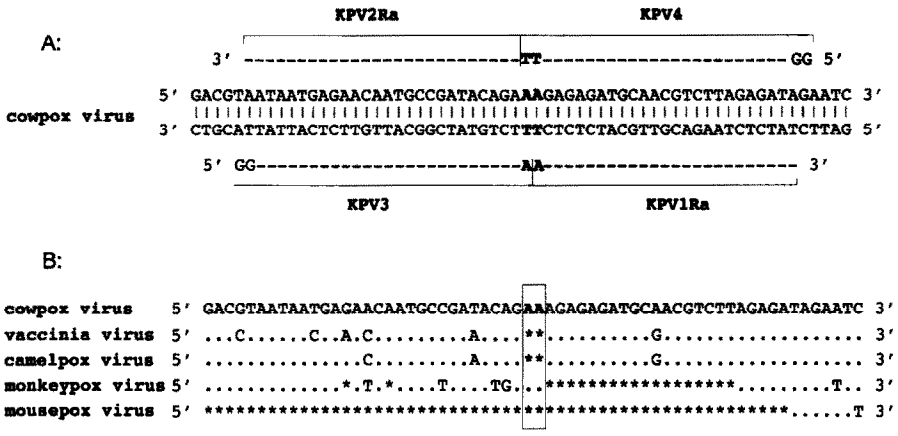

Fig. 1. Target sequences for the LCR primers in the ATI gene of different species of the genus *Orthopoxvirus*. Panel A: The sequence of cowpox virus BR strain and the binding sites for LCR primers KPV1Ra, KPV2Ra, KPV3, and KPV4 are shown with the two adenosine nucleotides at the ligation site depicted in bold. Sequences of the primers which are complementary to the target sequences are shown as bars. Panel B: Sequence comparison of cowpox virus BR with other *Orthopoxvirus* species. Deleted nucleotides are indicated by asterisks, only nonhomologous nucleotides are indicated by letters. The box shows the ligation site used for the design of LCR primers.

dithiothreitol; 1 mM NAD^+; 10 μg of herring sperm DNA; 0.01% Triton X-100) with 50 units Ampligase (Biozym, Hameln, Germany). The reaction mix was overlaid with the same volume of mineral oil and heated at 94°C for 5 min prior to 25 cycles consisting of a denaturing step at 94°C for 1 min and an annealing/ligation step at 65°C for 5 min. A 10 μl aliquot of the LCR samples was mixed with 10 μl deionized formamide, heated at 94°C for 5 min and loaded on a 15% polyacrylamide gel containing 5.8 M urea. Xylene cyanol and bromphenol blue (0.05% each in EDTA 20 mM, 95% formamide) were used as molecular weight markers and run in separate lanes. Electrophoresis was carried out in TBE-buffer (100 mM Tris, pH 8.0; 89 mM borate; 1 mM EDTA) at 150 V for 1.5 h. Gels were autoradiographed using Hyperfilm-βmax (Amersham) for 12 to 24 h. Templates for LCR were prepared by PCR-directed

Table 1
Sequences of the primers used in PCR and LCR

Primer	Sequence (5'-3')	Remarks	T_m (°C) [a]
PCR:			
ATI-up-1	AATACAAGGAGGATCT	Meyer et al., 1994	44
ATI-low-2	CTTAACTTTTTCTTTCTC	Meyer et al., 1994	46
LCR:			
KPV1Ra	AGAGAGATGCAACGTCTTAGAGATA	^{32}P-labeled	70
KPV2Ra	TCTGTATCGGCATTGTTCTCATTATT	^{32}P-labeled	70
KPV3	GGATAATGAGAACAATGCCGATACAGAA		70
KPV4	GGATCTCTAAGACGTTGCATCTCTCTT		70

[a] Calculation of T_m according to Suggs et al. (1981).

Table 2
Orthopoxvirus strains and isolates investigated

Species	Strain/Isolate	Origin/Reference
Cowpox virus ($n = 18$)	KR-2 Brigthon	WHO-Reference strain; Downie, 1939
	OPV 88	Cat; Mahnel, 1991
	OPV 89/1 to OPV 89/5	Cats; Mahnel, 1991
	OPV 90/1 to OPV 90/5	3 Cats, 1 man, 1 dog; Meyer et al., 1993; Eis-Hübinger et al., 1990; von Bomhard et al., 1991
	OPV 91/1 to OPV 91/4	Cats; Meyer et al., 1994
	WV1	cheetah; Baxby et al., 1982
	L97	Cat; Bennett et al., 1989
Vaccinia virus ($n = 10$)	Elstree	WHO-Reference strain [a]
	Western Reserve (WR)	WHO-Reference strain [a]
	CVA	Mayr et al., 1975
	IHD-J	Wokatsch, 1973
	Hagen	Wokatsch, 1973
	Levaditi	Wokatsch, 1973
	Bern	Wokatsch, 1973
	M1	Wokatsch, 1973
	Rabbitpox Uetrecht strain	Fenner, 1958
	Buffalopox BP-1	Baxby and Hill, 1971
Camelpox virus ($n = 5$)	CP-1	Ramyar and Hessami, 1972
	CP-2 to CP-5	Meyer et al., 1993
Mousepox virus ($n = 1$)	MP-1	Mahnel, 1983
Monkeypox virus ($n = 1$)	AP-1	von Magnus et al., 1959

[a] Used as vaccine strains during smallpox eradication (see Fenner et al., 1989).

amplification of ATI-specific sequences of all strains and isolates investigated (Meyer et al., 1994; Table 2). DNA concentrations were estimated by comparison with linear plasmid pTZ18R DNA (Pharmacia) run in the same gel.

The results of an LCR using PCR-amplified DNA of the five OPV reference strains are shown in Fig. 2. Only in the reaction with cowpox virus BR an LCR product, whose size corresponds to the molecular weight of two ligated primers, is present indicating a successful ligation. Five attomoles (i.e. 5 pg) were easily detected after 12 h exposure. In contrast, ligation products were not observed when PCR-amplified DNA from vaccinia, camelpox, mousepox, or monkeypox virus reference strains was used as templates (shown for 100 pg in Fig. 2). This indicated that the LCR chosen indeed allows differentiation of cowpox virus from other OPV species. In order to check the reliability of the assay, 33 OPV isolates of different origin (Table 2) were examined. Seventeen OPV isolates from cats, one human, one dog, and one cheetah isolate, phenotypically classified as cowpox viruses (Table 2), displayed a ligation product using 50 pg of the respective PCR fragment as a template; results for eight representative OPV isolates are shown in Fig. 3. These results suggested that the target sequence, i.e. the primer sequences including the two adenosine residues, is highly conserved among the ATI genes of cowpox viruses. This is in agreement with previous reports showing high sequence conservation within the ATI genes of cowpox (Meyer et al., 1993, 1994),

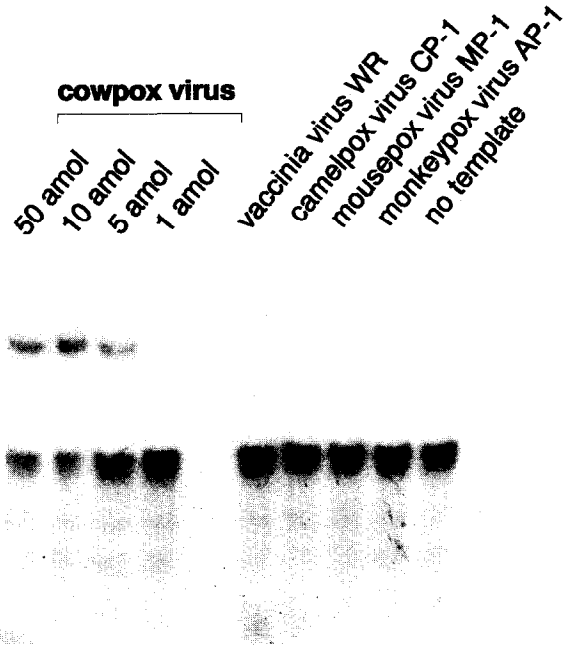

Fig. 2. Ligase chain reaction using reference strains of five *Orthopoxvirus* species. Cowpox virus BR showed a ligation product even with 5 attomoles of template. The reference strains for the other four OPV species (100 pg PCR-directed DNA) as well as the negative control (no OPV-DNA) did not react.

camelpox (Meyer and Rziha, 1993), and mousepox virus (Osterrieder et al., 1994). Based on the published ATI gene sequences of OPV reference strains, a common mechanism for ATI gene truncation in vaccinia and camelpox virus has been assumed (Meyer and Rziha, 1993). Therefore, a panel of eight vaccinia virus strains (Table 2) was tested by LCR in order to determine the consistency of the double A deletion among this OPV species. No ligation could be observed in any of the strains at the conditions described above. According to the sequence data of strain WR (Amegadzie et al., 1992), a mismatch of five nt between target DNA and primers exists (Fig. 1). Even at a reduced annealing/ligation temperature (58°C) no ligation with 100 pg of the PCR-amplified DNA was observed (Fig. 4), suggesting indeed considerable mismatch between primer and target DNA. Furthermore, amplicons (100 pg) from four isolates of camelpox virus (Table 2) failed to yield a ligation product (data not shown).

In order to determine the sensitivity of a direct LCR without primary PCR amplification, purified viral DNA (Meyer et al., 1993) from the five OPV reference strains was used directly in LCR. After 25 cycles an LCR product was only observed with cowpox viral DNA. These results confirmed the specifity of this LCR assay. Since about 250 pg of the viral DNA (equal to 2 attomoles) could be detected after an exposure of 12 h (data not shown), the sensitivity of the LCR was approximately the same regardless of whether PCR products or total viral DNA served as a template. Nevertheless, the

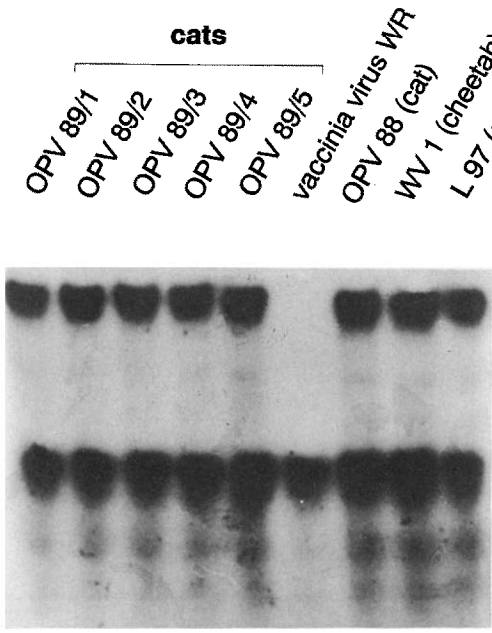

Fig. 3. Results of the LCR with seven cowpox virus isolates from cats, one isolate from a cheetah, and the vaccinia virus reference strain W R. A ligation product is seen in all eight cowpox virus isolates.

sensitivity of the direct LCR was approximately 100-fold lower as compared to direct PCR (H. Meyer, unpublished data).

The LCR presented in this communication allows the specific detection of cowpox virus DNA and permits the discrimination of closely related DNA sequences differing in a deletion of two nucleotides. Using 'gapped LCR', Birkenmeyer and Armstrong (1992) achieved specific detection of *Neisseria gonorrhoeae* based on a two base pair difference as compared to other *Neisseria spp.* In this assay the specifity is dependent upon the correct nucleotide triphosphate substitution and a perfect match at the 3′ end of the upstream 'G-LCR' primer which is required for elongation by the thermostable polymerase. As mentioned above, non-specific or template independent ligation was not observed in our LCR assay indicating specifity equal to the 'gapped LCR'. In contrast to the 'gapped LCR' (Birkenmeyer and Mushahwar, 1991), however, no fill-in reaction is required in the LCR, therefore eliminating the need for inclusion of DNA polymerase and the missing nucleotides in the reaction mix. Furthermore, while gapped LCR is limited to the detection of base pair changes from A-T/T-A to G-C/C-G or vice versa, LCR can target all possible deletions.

To our knowledge, this LCR assay is one of the first reports of an LCR that uses viral targets and the first application of an LCR for the discrimination of related DNA sequences based on the presence or absence of two bases. While providing superior specifity this LCR still has some disadvantages for diagnostic purposes, such as low

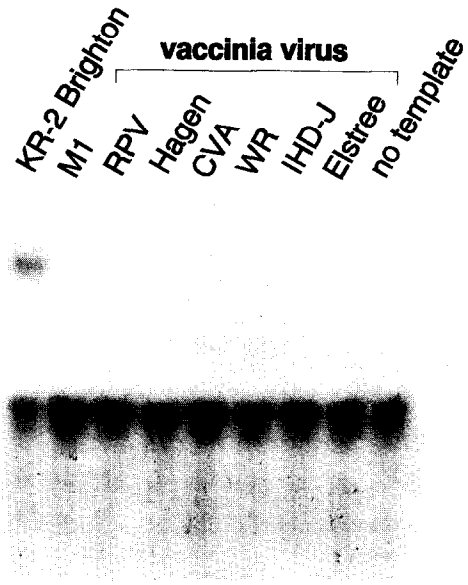

Fig. 4. Results of the LCR with seven vaccinia virus strains. No ligation products can be seen. In cowpox virus BR the expected ligation product was detected. The negative control lacking template DNA is shown in the last row.

sensitivity as compared to PCR, relatively high costs, and the use of radioisotopes. Non-radioactive detection methods of LCR products have already been reported for *Mycobacterium tuberculosis* (Winn-Denn et al., 1993), and *Listeria monocytogenes* (Wiedmann et al., 1993), and are currently under investigation for OPV. A non-isotopic detection method will provide a cheaper, faster, and more convenient way to detect LCR products and therefore will make this assay more suitable for routine applications.

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References

Amegadzie, B.Y., Sisler, J.R. and Moss, B. (1992) Frame-shift mutations within the vaccinia virus A-type inclusion protein gene. Virology 186, 777–782.

Barany, F. (1991a) The ligase chain reaction (LCR) in a PCR world. PCR Meth. Applic. 1, 5–16.

Barany, F. (1991b) Genetic disease detection and DNA amplification using cloned thermostable ligase. Proc. Natl. Acad. Sci. USA 88, 189–193.

Baxby, D. and Hill, B.J. (1971) Characteristics of a new poxvirus isolated from Indian buffaloes. Arch. ges. Virusforsch. 35, 70.

Baxby, D., Ashton, D.G., Jones, D.M. and Thomsett, L.R. (1982) An outbreak of cowpox in captive cheetahs: virological and epidemiological studies. J. Hyg. 89, 365–372.

Bennett, M., Gaskell, R.M., Gaskell, C.J., Baxby, D. and Kelly, D.F. (1989) Studies on poxvirus infections in cats. Arch. Virol. 104, 19–33.

Birkenmeyer, L.G. and Mushahwar, I.K. (1991) DNA-probe amplification methods. J. Virol. Methods 35, 117–126.

Birkenmeyer, L.G. and Armstrong, A.S. (1992) Preliminary evaluation of the ligase chain reaction for specific detection of Neisseria gonorrhoeae. J. Clin. Microbiol. 30, 3089–3094.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12, 387–395.

Downie, A.W. (1939) Study of the lesions produced experimentally by cowpox virus. J. Pathol. Bacteriol. 48, 361.

Eis-Hübinger, A.M., Gerritzen, A., Schneweis, K.E., Pfeiff, B., Pullmann, H., Mayr, A. and Czerny, C.P. (1990) Fatal cowpox-like virus infection transmitted by cat. Lancet (Oct. 6), 880.

Fenner, F. (1958) The biological characteristics of several strains of vaccinia, cowpox and rabbitpox viruses. Virology 5, 502.

Fenner, F., Wittek, R. and Dumbell, K.R. (1989) The Orthopoxviruses. Academic press, New York, pp. 100–102.

Funahashi, S., Sato, T. and Shida, H. (1988) Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. J. Gen. Virol. 69, 35–47.

Mahnel, H. (1983) Attenuierung von Mäusepocken (Ektromelie)-Virus. J. Vet. Med. B 30, 701–707.

Mahnel, H. (1991) Katzenpocken in Deutschland. Tierärztl. Praxis 19, 419–422.

Mayr, A., Hochstein-Mintzel, V. and Stickl, H. (1975) Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. Infection 3, 6–14.

Meyer, H. and Rziha, H.-J. (1993) Characterization of the gene encoding the A-type inclusion protein of camelpox virus and sequence comparison with other orthopoxviruses. J. Gen. Virol. 74, 1679–1684.

Meyer, H., Osterrieder, N. and Pfeffer, M. (1993) Differentiation of species of the genus Orthopoxvirus in a dot blot assay using digoxigenin-labeled DNA-probes. Vet. Microbiol. 34, 333–344.

Meyer, H., Pfeffer, M. and Rziha, H.-J. (1994) Alterations within and downstream of the A-type inclusion protein genes allow the differentiation of orthopoxviruses by polymerase chain reaction. J. Gen. Virol. 75, 1975–1981.

Osterrieder, N., Meyer, H. and Pfeffer, M. (1994) Characterization of the gene encoding for the A-type inclusion body protein of mousepox virus. Virus Genes 8, 125–135.

Patel, D.D., Pickup, D.J. and Joklik, W.K. (1986) Isolation cowpox virus A-type inclusions and characterization of their major protein component. Virology 149, 174–189.

Ramyar, H. and Hessami, M. (1972) Isolation, cultivation and characterization of camel pox virus. J. Vet. Med. B 19, 182–189.

Segev, D. (1992) Amplification of nucleic acid sequences by repair chain reaction. In: C. Kessler (Ed.), Nonradioactive labeling and detection of biomolecules. Springer Laboratory, Berlin, pp. 212–218.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K. and Wallace, R.B. (1981) Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences. In: D. Brown and C.F. Fox (Eds.), Developmental Biology Using Purified Genes. Academic press, New York, p. 683.

von Bomhard, D., Pfleghaar, S., Mahnel, H. and Schneekloth-Dücker, I. (1991) Fallbericht: Katzenpockeninfektion als Zoonose für Hund und Mensch. Kleintierpraxis 36, 511–514.

Von Magnus, P., Andersen, E.K., Petersen, B.K. and Birch-Andersen, A. (1959) A pox-like disease in cynomolgus monkeys. Acta Pathol. Microbiol. Scand. 46, 156.

Wiedmann, M., Barany, F. and Batt, C.A. (1993) Detection of *Listeria monocytogenes* with a nonisotopic polymerase chain reaction-coupled ligase chain reaction assay. Appl. Environ. Microbiol. 59, 2743–2745.

Wiedmann, M., Czajka, C., Wilson, W., Luo, L., Barany, F. and Batt, C.A. (1994) LCR-overview and applications. PCR Meth. Applic. 3, S51–S64.

Winn-Deen, E.S., Batt, C.A. and Wiedmann, M. (1993) Non-radioactive detection of *Mycobacterium tuberculosis* LCR products in a microtitre plate format. Mol. Cell. Probes 7, 179–186.

Wokatsch, R. (1972) Vaccinia virus. In: M. Majer and S. Plotkin (Eds.), Strains of Human Viruses. Karger Verlag, Basel, pp. 241–257.