Development of Quantitative PCR Tests for the Detection of the Orthopox Virus Adsorption Protein Gene (ORF D8L) on the LightCycler

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Introduction

The genus *Orthopoxvirus* (OPV) includes several species of well-known pathogens [13, 21]. Although the Variola and Alastrim viruses were eradicated by a worldwide compulsory vaccination with vaccinia virus, monkeypox and, especially in Europe, cowpox virus strains may cause diseases in humans. Human cowpox is a relatively rare zoonosis and cows are currently not known to be involved. In recent years, virus strains have drawn the attention of the medical profession by causing localized and generalized skin infections in humans [5, 6, 12, 14, 17, 24] as well as in cats [7, 10, 22] or zoo-animals like large felides or elephants [3, 4, 18, 25]. Usually, a low infectivity for healthy persons is observed with benign skin lesions. However, in immunocompromised and non-vaccinated individuals the infection can lead to severe diseases which may end lethally [8, 11, 12].

A permanent natural cowpox virus reservoir has not yet been identified, but based on epidemiological surveys, it is assumed that these virus strains are maintained in wild-life rodents [2, 16, 18]. The virus spreads between the rodents which are considered to be intermediate hosts, whereas, cats, cows, elephants, and human beings are final hosts.

Because of the wide host range but unclear ecology of OPV, it is important to investigate their natural distribution, reservoirs, and infective routes. This makes very sensitive and quick diagnostic tools necessary. Several PCR methods for OPV detection and differentiation have recently been developed [9, 19, 20]. In this connection we evaluate a new quantitative LightCycler PCR for rapid detection of OPV strains, based on the amplification of the vaccinia virus open reading frame D8L-analogues [15], the gene for the 32 kDa adsorption protein.

Materials

Ultracentrifuge Optima L60 (Beckman, Munich)  
ALFexpress Automatic DNA Sequencer (Amersham Pharmacia, Freiburg)

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Senator-Gerauer-Str. 23, 85586 Poing, Germany
LightCycler Instrument (Roche Diagnostics, Mannheim)
DNA Star Lasergene, DNA and Primer analysis software (gatc, Konstanz)

TaqStart Antibody (Clontech, Heidelberg)
Eagle’s MEM containing 5% FCS
Proteinase K, 14 mg/ml (Roche Diagnostics, Mannheim)
Sodium dodecylsulfate (SDS), 20% (Sigma, Munich)
Phenol-Chloroform-Isomylalcohol 25:24:1 (Roth, Karlsruhe)
Amplification Primers (MWG Biotec, Ebersberg)
Hybridization Probes (TIB MOLBIOL, Berlin)
Sequencing Primers (MWG Biotec, Ebersberg)
LightCycler-DNA Master SYBR Green I (Roche Diagnostics, Mannheim)
LightCycler DNA Master Hybridization Probes (Roche Diagnostics, Mannheim)

Procedure

Sample Preparation
To isolate orthopox virus (OPV) DNA from tissue cultures infected with several virus strains, 500 μl aliquots were exposed to three alternating heating (100°C 5 min) and freezing steps (−70°C) before 5 μl proteinase K, 22.5 μl TEN buffer and 22.5 μl SDS were added. After an incubation of 2 h at 56°C, DNA was extracted from proteins and lipids with the same volume of phenol-chloroform, set on ice for 30 min and centrifugated at ca. 9,000×g and room temperature for 15 min. The supernatants were carefully collected and mixed with 1/10 volume of a 5 M potassium-acetate solution and the double volume of absolute ethanol. After precipitation over 30 min at −70°C, DNA was pelleted at 17,600×g and 4°C for 30 min, washed three times with 70% ice-cold ethanol, dried and resuspended in PCR water. This semipurified DNA was stored at −20°C until use.

Additionally, purified DNA was prepared according to the same procedure from cowpox virus KR2 Brighton ultra-centrifuged on sucrose gradients [9].

Primer Design
Primer design was performed under the prerequisite to amplify the whole D8L gene (GeneBank accession number: M35027) in various orthopox virus species. From published sequence data [15, 23] and the data of our group it was known that N- and C-termini of D8L-analogues of several OPV strains were conserved. Primers were designed by the DNA Star computer program. Also, 3’-Pentamer Stability ΔG was set to −13.5 kCal/mol. Six bp of dimer or hairpin duplexing was accepted. The average cut off for mispriming (−ΔG/primer length) was set to 0.76. Hybridization probes were designed by TIB MOLBIOL (Berlin) with a moderate stringency according to published guidelines [1]. For sequencing, the PCR primers were conjugated to Cy5 at the 5’-terminus. Oligonucleotides and characteristics are shown in Table 1.