Fast real-time PCR for the detection of tanapox virus and yaba-like disease virus

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Introduction

The genus yatapoxivirus belongs to the family poxviridae, subfamily chordopoxvirinae and currently consists of three members, called tanapoxavirus (TPV), yaba-like disease virus (YLDV) and yaba monkey tumor virus (YMTV). TPV and YLDV may infect monkeys and humans, whereas YMTV so far has infected only non-human primates naturally. It has been assumed that TPV and YLDV may be different strains of the same virus. TPV infection is a rare zoonosis and believed to be endemic throughout tropical Africa. TPV infection in humans results in an acute febrile illness with one or more localized small, pock-like lesion. During the third week of infection, the skin lesion ulcerates and finally heals leaving a scar. Increased international travel has led to appearance of TPV-infections outside Africa. Several authors pointed out that TPV-induced lesions can easily be confused with smallpox or monkeypox.

Materials and Methods

Virus and Plasmid

TPV and YLDV DNA was purified as recently reported (Zimmermann et al., 2005). In brief, a 465 bp PCR amplicon of the PstI L fragment of TPV (Accession number AF153912) was cloned into pGEM-T Easy (Promega, Madison, Wisconsin). Recombinant plasmid DNA was used as template for PCR amplification using primers TPV-F (5’-CTGGTACTTCTGTTTGGAAAG-3’), and TPV-R (5’-CCAGGATCTGTTATGTTGG-3’), yielding an 826 bp fragment. The linearized plasmid TPV was used as standard. YLDV DNA strain Davis grown on MA 104 at 34 °C was used.

Real-Time PCR

Based on IT-TaqMan® assay (Zimmermann et al., 2005) the PCR profile was successively modified in order to shorten experimental procedure. Different from IT-TaqMan® assay, using 2x PCR buffer (Applied Biosystems, Germany) 10 µl purified template DNA was mixed with 10 µl a reagent consisting IT/ITa primers (400 nM), yielding an 101 bp amplicon, ITs probe (240 nM) (TibMolbiol, Germany), RealMasterMix Probe (Eppendorf, Germany) and PCR-graded water (Eppendorf, Germany) to a final volume of 20 µl. Comparative studies were performed on two different real-time PCR platforms: Mastercycler ep realplex® 134 71 67 64 60 57 54 52 49 48 47 46 44,5 44 (Eppendorf, Germany) and Mx3000p® 134 71 67 64 60 57 54 52 49 48 47 46 44,5 44 (Stratagene, Germany). It has been assumed that TPV and YLDV may be different strains of the same virus. TPV infection is a rare zoonosis and believed to be endemic throughout tropical Africa. TPV infection in humans results in an acute febrile illness with one or more localized small, pock-like lesion. During the third week of infection, the skin lesion ulcerates and finally heals leaving a scar. Increased international travel has led to appearance of TPV-infections outside Africa. Several authors pointed out that TPV-induced lesions can easily be confused with smallpox or monkeypox.

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Results and Discussion

Among other effects, like product length of the PCR amplicon, total running time of PCR experiments is limited by the heat transfer from the catalyst, through the consumable into the reaction volume. Thus, thin-walled and high quality consumables are prerequisites. It is well known that the transition time between different temperatures should be as short as possible to avoid formation of non-specific coproducts and to reduce progressively decreasing enzyme activity. Due to the implication of the results for some assays as for smallpox or yaba-like disease virus calculations reduced runtimes are desirable. Therefore real-time PCR instruments with enhanced ramping rates are an attractive tool to accelerate PCR experiments. Thus, we compared the performances concerning speed of the TaqMan® assay on two commercial real-time PCR platforms, Mastercycler ep realplex® S and Mx3000P®. First, we substituted the formerly used TaqMan® Universal PCR Master Mix (Applied Biosystems, Germany) with RealMasterMix Probe (Eppendorf, Germany) to reduce the runtimes. Further, starting from the original PCR profile with 40 cycles amplification (95 °C for 15 s and 55 °C for 60 s) we shorted the cyclic denaturation and annealing/elongation phases stepwise. At the end of the optimization procedure we decided to use following protocol for further investigations: 2 minutes initial denaturation and 40 cycles of amplification/detection (95 °C for 6 s and 55 °C for 8 s), due to its short running time in combination with adequate R2-values and satisfying sensitivity. Shorter times were not possible, since 5 seconds or less probably were not sufficient enough for effective denaturation of PCR products and resulted in a clear loss of sensitivity. Amplification times were not possible to reduce more than to a final time of 8 seconds due to technical necessity for fluorescence measurement. Using the RealMasterMix Probe and this thermoprofile a 10-fold loss of sensitivity for the Mx3000P® and a 100-fold loss for the Mastercycler® was seen. Due to the high viral load of clinical samples this was approved to be acceptable. To judge the performance of the cycles while reducing denaturation and amplification time, we estimated the standard deviation of the three replicates of the dilution series. As anticipated the standard deviations increased while reducing runtime as for higher dilutions (see figure 3). Also the standard deviations were higher for the Mastercycler® than for the Mx3000P® probably because of the faster increase and decrease of the silver bloc and therefore the runtime is explicit shorter. Considering these results the developed assay is not suitable for quantification but for a qualitative conclusion. Under this new PCR conditions we finally determined the optimal concentration and proportion of forward and reverse primer as well as probe by primer matrix analysis. The optimal annealing/elongation temperature of the PCR was ascertained by using the thermal gradient function of the cycler in the range between 55 °C to 60 °C. No significant difference was observed for 55 °C to 60 °C.

Conclusion

- We developed an ultra-fast probe based real-time PCR assay to detect tanapox virus and yaba-like disease virus.
- The runtime could be reduced from 134 minutes to 48 minutes (Mx3000P®) and 22 minutes (Mastercycler ep realplex® S) respectively.
- The change of reagents and the reduction resulted in a 10-fold loss of sensitivity for the Mx3000P® and a 100-fold loss for the Mastercycler ep realplex® S.
- Due to relative high standard deviations the assay is suitable for qualification, but not for quantification.
- Further experiments shall show the sensitivity of the assay using probit analysis.