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

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Introduction

The genus yatapoxvirus belongs to the family *poxviridae*, subfamily *chordopoxvirinae* and currently consists of three members, called tanapoxvirus (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.

Yatapoxviruses are morphologically similar to Orthopoxviruses but antigenically distinct from all other poxviruses.

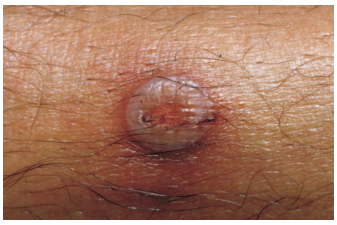

Fig. 1 Umbilicated, painless skin lesion on the right shank (about 14mm in diameter) found in a 49-year old German traveler 1999 returning von Eastern Tanzania (Stich et al. 2002)

Given the fact that early stages of TPV infection can be mistaken with more serious conditions such as monkeypox, smallpox, tularemia or anthrax, a rapid, unambiguous identification is extremely important to exclude the deliberate use of agents of biological warfare and bioterrorism.

Therefore, our aim was to develop an ultra-fast real-time PCR assay for rapid diagnosis. Primers and probe from a recently published TaqMan® assay (Zimmermann et al., 2005) were combined with the RealMasterMix® Probe (Eppendorf, Germany) to reduce initialisation and amplification times. Comparative studies were performed on two different real-time PCR platforms: Mastercycler ep realplex⁴ (Eppendorf, Germany) and Mx3000p (Stratagene, Germany).

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 pCR 2.1 vector. Purified plasmid pCR2.1 was photometically quantified and serially diluted in a 10-fold manner using Tris-EDTA buffer (10 mM/1 mM). The initial concentration of the plasmid was 2.1 x 10^{10} copies/µl.

To investigate potential influence of various PCR profiles on the amplification and detection of native TPV and YLDV DNA, DNA of YLDV strain Davis grown on MA 104 at 34 °C was used. DNA was purified from 100 µl aliquots by magnetic beads separation using MagNA Pure Compact Nucleic Acid Isolation Kit I on the Magna Pure Compact system (Roche, Germany).

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 were mixed with 10 µl reagent consisting IT/ITa primers (400 nM), yielding an 101 bp amplicon, ITs probe (240 nM) (Tib-Molbiol, 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 realplex⁴ S, Eppendorf, Germany and Mx3000P®, Stratagene, Germany). Real-time PCR involved 2 minutes initial DNA denaturation at 95 °C followed by denaturation and annealing/elongation for 40 cycles (95 °C for 15 s and 55 °C for 1 min). During following experiments denaturation and amplification time were progressively reduced. Ct-values (cycle threshold), R^2-values (correlation coefficient), slopes and efficiencies of amplification (Efficiency = $10^{(-1/slope)}$-1) were determined. Threshold and baseline for each platform were individually optimized to achieve best R^2-value and to detect unambiguous positive samples.

thermoprofile	D15s/ A60s	D15s/ A35s	D15s/ A30s	D15s/ A25s	D15s/ A20s	D15s/ A15s	D15s/ A10s	D15s/ A8s	D10s/ A8s	D8s/ A8s	D6s/ A8s	D4s/ A8s	D2s/ A8s	D1s/ A8s
Mastercycler®		43	39	35	33	30	27	26	23	22	21	18	15	14,5
Mx3000p®	134	71	67	64	60	57	54	52	49	48	47	46	44,5	44

Table 1: In order to determine the optimal combination of runtime and sensitivity, the thermoprofile was gradually modified. First of all the amplification time (A in sec) and then the denaturation time (D in sec). For both platforms total times are shown in minutes.

Dilution	Realplex® mean value	Mx3000p® mean value	Realplex® standard deviation	Mx3000p® standard deviation
10^{-5}	19,94 (n=36)	17,40 (n=39)	2,3	0,8
10^{-6}	23,31 (n=36)	20,50 (n=39)	2,2	1,1
10^{-7}	27,07 (n=36)	23,74 (n=36)	2,8	0,7
10^{-8}	30,39 (n=36)	26,94 (n=36)	2,8	0,6
10^{-9}	33,53 (n=18)	31,15 (n=32)	3,1	1,4
10^{-10}	n.d.	n.d.	-	-

Table 2: Results: Mean value and standard deviation of the crossing points of all thermoprofiles for each dilution; n= number of detected replications, n.d. = not detected;

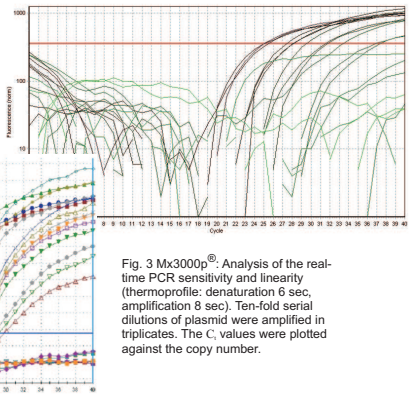

Fig. 2 Mastercycler®: Analysis of the real-time PCR sensitivity and linearity (thermoprofile: denaturation 6 sec, amplification 8 sec). Ten-fold serial dilutions of plasmid were amplified in triplicates. The C_t values were plotted against the copy number.

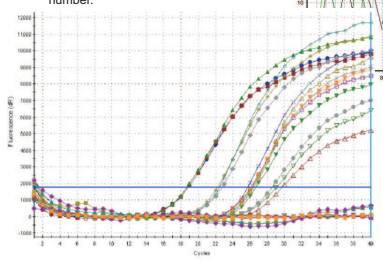

Fig. 3 Mx3000p®: Analysis of the real-time PCR sensitivity and linearity (thermoprofile: denaturation 6 sec, amplification 8 sec). Ten-fold serial dilutions of plasmid were amplified in triplicates. The C_t values were plotted against the copy number.

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 cycler, 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 economic 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 realplex⁴ 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 shortened 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 necessary 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 cyclers 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

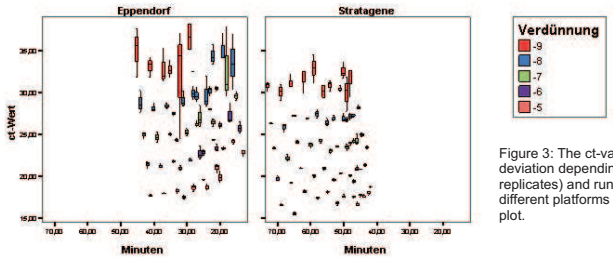

Figure 3: The ct-values with standard deviation depending on the dilution (3 replicates) and runtime for the two different platforms displayed using box plot.

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 realplex⁴ 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 realplex⁴ 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.