Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay

Hi-Gung Bae a,1, Andreas Nitsche a,b,*,1, Anette Teichmann a, Stefan S. Bie1 a,2, Matthias Niedrig a

a Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany
b Medizinische Klinik II m.S. Onkologie und Hämatologie, Charité, Humboldt Universität, Berlin, Germany

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Abstract

Yellow fever virus quantitation is performed routinely by cultivation of virus containing samples using susceptible cells. Counting of the resulting plaques provides a marker for the number of infectious particles present in the sample. This assay usually takes up to 5 days before results are obtained and must be carried out under L2 or L3 laboratory conditions, depending on the yellow fever virus strain used. For clinical diagnosis of yellow fever virus infections the cell culture-based approach takes too long and is of limited practical relevance. Recently, due to its considerable sensitivity, PCR has become a promising method for virus detection. However, whilst PCR can detect virus-specific nucleic acids, it does not allow conclusions to be drawn regarding the infectious potential of the virus detected. Nonetheless, for diagnostic purposes, a rapid, specific and sensitive virus PCR is preferable. Therefore, two independent yellow fever virus-specific real-time PCR assays were established and compared the viral RNA loads to the results of a traditional plaque assay. The estimated ratio of yellow fever virus genomes to infectious particles was between 1000:1 and 5000:1; both approaches displayed a comparable precision of B45%. A significant correlation between genome number as determined by real-time PCR and the corresponding number of plaques in paired samples was found with a Pearson coefficient of correlation of r = 0.88 (P < 0.0001).

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1. Introduction

Yellow fever virus is the prototype of the family Flaviviridae (Westaway et al., 1985) which comprises approximately 70 strains (Calisher et al., 1989). The viruses of this family contain non-segmented, positive-stranded RNA genome of 10,862 nucleotides with a 5'cap structure and a non-polyadenylated 3'end. The yellow fever virus genome encodes a polyprotein of 3411 amino acids which is cleaved by proteolytic processing into 11 viral polypeptides (Chambers et al., 1990).

Yellow fever infection in man can result in inapparent infection through to fulminant disease which is invariably fatal, provoking great public health concern for centuries (Fields et al., 2001). However, yellow fever epidemics are still a major problem in endemic areas where suitable vaccination regimes are lacking.

Sporadically, cases of yellow fever virus infection are imported to countries where yellow fever virus is not endemic, and therefore, rapid diagnosis of these infections is an important task, especially to rule out other agents of hemorrhagic fever requiring patient management. The last two yellow fever virus cases imported to Europe clearly proved that a rapid diagnosis is a prerequisite for efficient medical treatment (Teichmann et al., 1999; Colebunders et al., 2002). In these two cases, yellow fever virus detection by conventional RT-PCR and real time RT-PCR was the method of choice for rapid diagnosis, permitting rapid decision regarding the
patient’s condition as well as the management and control of viral hemorrhagic fever (Drosten et al., 2002). Usually, the diagnosis of yellow fever virus is carried out by virus cultivation on susceptible cells followed by immune-fluorescence detection, ELISA, immunohistochemical examination or PCR (Deubel et al., 1997; De Brito et al., 1992; Monath et al., 1989; Monath and Porterfield, 1969). For quantifying the load of infectious particles either in cell culture or serum samples, the plaque assay is still a method used commonly. However, it is a very time-consuming technique that takes at least 5 days (Porterfield, 1959), and moreover, it has to be undertaken under L2 conditions for yellow fever virus strain 17D or L3 conditions for yellow fever virus wild type isolates (WHO, 1971).

With the recent development of quantitative real-time PCR, a more feasible approach to quantifying viral load is available (Mackay et al., 2002). However, from the biological point of view, the exclusive detection of viral nucleic acids provides no information regarding the infectious capability of the virion. A correlation between plaque assay results and quantitation of yellow fever virus genomic RNA could simplify and accelerate the diagnosis of yellow fever virus. Therefore, two novel quantitative real-time PCR assays for the quantitation of yellow fever virus RNA based on the 5’-nuclease chemistry (TaqMan; Holland et al., 1991; Livak et al., 1995) were developed and evaluated. The benefits and pitfalls of a real-time time PCR approach to viral diagnostics have been discussed previously (Mackay et al., 2002). The overall precision of the real-time PCR and plaque assay were determined. Finally, the results obtained by the real-time PCR were compared with the results obtained by the plaque assay carried out routinely, with the aim of assessing a correlation between viral RNA load and plaque assay.

2. Material and methods

2.1. Virus cultivation

Various cell lines from vertebrates (Vero B4, STC-1, U-937) and insects (C6/36) were infected with yellow fever virus 17D using a multiplicity of infection (MOI) of 0.5 and cultivated in RPMI 1640 medium (Gibco, BRL) for a period of 8 days. Lyophilised yellow fever virus 17D was obtained from yellow fever virus-vaccine Batch No. 189/00/1, 190/00/1 and 191/00/1 (Robert Koch Institute Berlin, Germany). Assays were carried out either with freshly reconstituted yellow fever virus YFV vaccine ampoules, with frozen yellow fever virus-laden cell culture supernatants or with human sera.

Cell culture supernatant samples were taken daily from infected cell cultures, and the concentration of yellow fever virus was determined using both the plaque assay and the quantitative real-time PCR assay.

Serum from yellow fever virus infected patients was obtained by gravity sedimentation of peripheral blood as described previously (Nitsche et al., 2000). Viral RNA was prepared from serum as described below for cell culture supernatants.

2.2. Plaque assay

The plaque assay was carried out as a modified version of the assay described by De Madrid and Porterfield (De Madrid and Porterfield, 1969). Briefly, $6 \times 10^5$ porcine kidney cells in 200 μl RPMI 1640 were seeded in each well of a 24-well plate. Serial dilutions (1:20 000, 1:40 000 and 1:80 000) of the different viral suspensions were added to the wells (200 μl each). After an incubation period of 4 h, overlay medium (1.6% carboxymethyl-cellulose, 3% fetal calf serum in RPMI) was added, and the plates were incubated for 5 days at 37°C.

After a 15 min fixation step with 4% formalin in PBS, the cells were stained with Naphtalin Black for 20 min. The plaques caused by lysis of infected cells were counted and the calculation of plaque forming units (pfu) was carried out according to Reed and Münch (1938).

2.3. Viral RNA isolation and reverse transcription of yellow fever virus genome

Yellow fever virus 17D RNA was isolated from 140 μl cell culture supernatants and serum using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden). RNA was eluted with 60 μl of sterile water. Total cDNA was produced by reverse transcription (RT) of 2 μl of purified RNA, 2 μl of 10 × buffer, 2 μl of 10 mM DTT, 5 μl of 2.5 mM dNTP (each), 0.5 μl of random hexamers and 6 μl of sterile water. Samples were heated to 72°C for 3 min and subsequently chilled on ice for 3 min to allow primer hybridisation. After the addition of 1 μl of 20 U RNAse inhibitor and 0.5 μl 200 U/μl reverse transcriptase, samples were incubated for 40 min at 42°C for RT. Subsequently, the RT reaction was terminated by enzyme inactivation at 83°C for 5 min.

2.4. Primers and TaqMan probes

The oligonucleotide primers and probes were designed according to the general guidelines for real-time PCR primer and probe design and synthesised by TIB MOLBIOL, Berlin, Germany. To increase the reliability of the real-time PCR diagnosis, two independent PCR assays were established, targeting highly conserved regions within the NS3 region and the 3’UTR region of the yellow fever virus genome, respectively. Both
assays were optimised to be carried out simultaneously under identical conditions. Primer sequences, location and annealing temperatures are shown in Table 1.

### 2.5. Real-time PCR

Quantitative real-time PCR was performed in a Perkin–Elmer 7700 Sequence Detection System (Applied Biosystems, Foster City). Amplifications were carried out in 50 µl reaction mixtures containing 2 µl of template cDNA, 0.5 µM of each primer, 0.1 µM of TaqMan probe, 0.2 mM of each dNTP and 4 mM MgCl₂. As a passive fluorescent reference dye 1 µM ROX (6-carboxy-X-rhodamine) was added to the mixture. Cycling conditions were as follows: initial denaturation at 94 °C for 3 min, 45 cycles with 94 °C for 30 s and 60 °C for 1 min.

For calibration of the real-time PCR, two independent plasmids pYFV-NS3amp and pYFV-3’UTRamp were constructed by cloning the respective amplicons of 104 and 228 bp into a TOPO TA Cloning vector, according to the manufacturer’s instructions (Invitrogen, Leek, The Netherlands). Serial dilutions of 10⁸–10¹ plasmids/ml were used to generate calibration curves as described previously (Nitsche et al., 1999).

### 2.6. Test evaluation

Several independent steps can affect the reproducibility and sensitivity of an RT-PCR reaction. Therefore, the variability of the complete RT-PCR procedure was determined by undertaking 10 RT reactions in parallel on aliquots of one identical RNA sample. This was repeated four times. Subsequently, the cDNA was quantified in duplicate by real-time PCR. To compare real-time PCR and plaque assay results, three yellow fever virus samples were examined in parallel with plaque assay and the real-time PCR using three different sample dilutions (non-diluted, 1:100, 1:1000). The three dilutions were split up in six aliquots to determine the intra-assay variability and experiments were repeated on 4 consecutive days to determine the inter-assay variability. The correlation between viral genome concentra-

### 3. Results

#### 3.1. Reproducibility of the real-time PCR assay

Two quantitative real-time PCR assays for the detection of yellow fever virus were designed and evaluated. First, to allow quantitation of yellow fever virus genomic RNA, calibration curves were generated by amplification of serial dilutions of the plasmids pYFV-NS3amp and pYFV-3’UTRamp. C_T values were determined in quadruplicate and plotted against the plasmid copy number. Fig. 1 presents the resulting calibration curves from both assays, indicating a PCR efficiency of 98.7%, a linear detection range between 10⁸ and 10¹ plasmids and a high correlation of R² > 0.999 between cycle number and plasmid number for both independent PCR assays.

Real-time PCR reproducibility was determined by repeated amplification of two 10-fold dilutions of the

### Table 1

<table>
<thead>
<tr>
<th>Sequence of primer/TaqMan probe</th>
<th>K02749</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS3 sense</td>
<td>4857-1875</td>
<td>62.1</td>
</tr>
<tr>
<td>NS3 antisense</td>
<td>4961-4942</td>
<td>62.2</td>
</tr>
<tr>
<td>NS3 TaqMan</td>
<td>4893-4921</td>
<td>71.7</td>
</tr>
<tr>
<td>3’UTR sense</td>
<td>10109-128</td>
<td>58.8</td>
</tr>
<tr>
<td>3’UTR antisense</td>
<td>10337-318</td>
<td>59.1</td>
</tr>
<tr>
<td>3’UTR TaqMan</td>
<td>10237-266</td>
<td>68.2</td>
</tr>
</tbody>
</table>

Abbreviations: F, 6-carboxyfluorescein attached to 5’-terminus (FAM), T, 5-carboxytetramethylrhodamine (5-TAMRA) attached to 5-ethylamino-dThymidin. Melting temperature was calculated by nearest neighbour method as described elsewhere (Breslauer et al., 1986).

Fig. 1. Calibration curves of the two independent real-time PCR assays NS3 (black circles) and 3’UTR (open triangles). Plasmid copy numbers are plotted against the corresponding C_T value. The slope indicates a PCR efficiency of > 98.7% for both assays.
plasmids pYFV-NS3amp and pYFV-3’UTRamp and by amplification of two 10-fold dilutions of three different yellow fever virus 17D vaccine batches. The intra-assay precision could be determined as <20% for the vaccine samples and <13% for the plasmid dilutions for both assays. Only ten plasmid copies, the lowest number detectable, showed a reduced reproducibility of <34% for both assays. The inter-assay precision could be determined as <30% for both real-time PCR assays. Only low copy numbers showed a lower reproducibility of <45%. However, although a reduced reproducibility in the template range close to the detection limit was observed, 100% of the samples containing ten plasmid copies could be detected, indicating a high reliability even in the low range of low template concentrations (Peccoud and Jacob, 1996).

Representative amplification plots of the NS3 assay carried out on yellow fever virus 17D in two serial 10-fold dilutions on the first day tested are shown in Fig. 2. All samples were measured in quadruplicate. Based on the calibration curves shown in Fig. 1 results obtained from both real-time PCR assays were averaged. Yellow fever viral could be quantified in the three vaccine batches as follows: batch 189/1: 2.2 × 10^6, batch 190/1: 3.0 × 10^6 and batch 191/1: 3.9 × 10^6 genome equivalents (GE)/ml. Serial serum samples of a patient suffering from fulminant yellow fever virus infection showed a decrease in yellow fever virus genome load from 1.6 × 10^6 to 9.5 × 10^5 GE/ml serum on the last 3 days before his death (data not shown).

All yellow fever virus control samples were detected as positive by both real-time PCR assays from cell culture as well as from diagnostic human serum samples. Yellow fever virus free cell culture supernatants as well as plasma samples of healthy blood donors remained negative in all PCR reactions carried out. In addition, there were no cross reactions with other flaviviruses including Japanese encephalitis virus, tick-borne encephalitis virus, West Nile virus and the dengue viruses 1–4.

3.2. Reproducibility of the RT reaction

In order to measure the extent of variation in the RT reaction, the cDNA concentration of ten independent RT reactions were determined in duplicate by employing real-time PCR. Applying the calibration curves as described above, quantitation by real-time PCR resulted in genome loads of 1.4 × 10^6 GE/assay (S.D. = 16%) for the NS3 assay and 1.8 × 10^6 GE/assay (S.D. = 12%) for the 3’UTR assay, respectively. When reducing RNA concentration 20-fold prior to cDNA synthesis, variations of 9.1 × 10^4 GE/assay (S.D. = 16%) for the NS3 assay and 1.0 × 10^5 GE/assay (S.D. = 10%) for the 3’UTR assay, respectively, were obtained. Variation fell within the range of the overall assay variation observed with both real-time PCR assays.

3.3. Reproducibility of the plaque assay

The plaque assay results of the three yellow fever virus 17D vaccine batches (189/1, 190/1, 191/1) were 8.2 × 10^5, 7.2 × 10^5 and 9.2 × 10^5 pfu/ml. Determining the intra-assay variability, <33% variation for the three independent yellow fever virus 17D vaccine batches was observed. Maximum inter-assay variation was <44%.

Fig. 2. Representative amplification plots of the NS3 real-time PCR assay. Two serial 10-fold dilutions of yellow fever virus 17D vaccine batch 189 were measured in quadruplicate. The high reproducibility can be seen by the small variation in C_T values.
3.4. Correlation between plaque assay and real-time PCR assay

To elucidate if there was a correlation between the quantitative results of the real-time PCR assays and the plaque assay, an NS3-specific real-time PCR assay and a plaque assay were carried out on the same samples of different yellow fever virus concentrations. In general, viral genome numbers determined by real-time PCR were 1000–5000-fold higher than the infectious particles that were determined by the plaque assay. A significant correlation of $r = 0.88$ with $P < 0.0001$ was found plotting the viral genome concentration as determined by real-time PCR versus plaque number (Fig. 3).

4. Discussion

The detection of viruses by rapid and reliable techniques is still one of the most demanding tasks in virus diagnosis. Cell culture-based methods which determine the number of infectious virus particles, are time consuming and complex. The need for alternative fast and sensitive diagnostic tools has provided PCR (Niesters, 2001). Beside many qualitative approaches, quantitative PCR assays have evolved rapidly during recent years. In addition to the option of reliable, accurate quantification, attributes including speed, sensitivity and a reduced risk of amplicon carry-over contamination are restricted to real-time PCR-based methods. Therefore, two independent real-time PCR-based assays have been developed to be carried out under identical conditions. These assays demonstrated all benefits of real-time PCR: speed ($< 3$ h including sample preparation), a very low detection limit of ten GE per reaction and a linear quantification range of seven orders of magnitude which should encompass the general clinical requirements. Despite the different amplicon lengths produced by the two assays (104 vs. 228 bp), detection limits and precision were nearly identical under optimised conditions. Both assays produced a high overall precision of $< 30\%$ and still $< 45\%$ in the lowest detection range of ten GE per assay. This precision is comparably high, considering that the RT reaction can vary from 5 to 95% efficiency (Ferré et al., 1994). An increased variation in low template concentration ranges close to the detection limit can be caused solely by statistical distribution (De Vries et al., 1999). However, it has been suggested, that the problem of inter-assay variation is mainly attributed to RNA preparation and sample processing, not to the PCR reaction itself (Keilholz et al., 1998). Considering all the different steps included in the real-time PCR detection, an overall precision of $< 45\%$ is high.

Although each assay was constructed to amplify several different yellow fever virus strains, the use of two independent PCR assays, spanning approximately 5000 nt, reduced the risk of false positive results due to sequence heterogeneity in the primer or probe binding regions. However, results obtained from both assays were comparable consistently and no assay failures with the different templates tested were found. Considering that absolute quantification of RT-PCR reactions is still demanding, the use of an external calibration curve in separate reactions was preferred. Although external calibration curves are not perfectly quantitative because of uncontrolled and unmonitored inter-tube variations (Mackay et al., 2002), the use of an internal standard within the PCR reactions can influence the detection limit, and therefore, reduce the diagnostic potential of the real-time PCR assays. The use of the internal reference dye ROX controls at least for tube-to-tube variations.

However, one of the main drawbacks of (real-time) PCR in viral diagnosis is the lack of a croculation between viral nucleic acid detection and the presence of infectious particles. Unfortunately, infectious particles can only be demonstrated by virus amplification using laborious cell culture systems. To determine whether there is a correlation between the concentrations of genomes and the number of infectious particles, results from quantitative real-time PCR were compared with the results obtained from the plaque assay carried out routinely on identical samples. As expected, values for the genome detecting real-time PCR were approximately 1000–5000-fold higher compared with the infectious particle detecting plaque assay. This difference may result from the production of defective non-infectious particles, as previously shown for HTLV-1 (Morozov and Weiss, 1999). The extent of this production varies from virus to virus and even within virions of

Fig. 3. Correlation between concentration of yellow fever virus nucleic acids and plaque number of yellow fever virus containing cell culture supernatants ($n = 36$). Results obtained with the real-time PCR assay are plotted against the pfu values, as determined by the plaque assay. Pearson analysis revealed a significant of $P < 0.001$ and $R = 0.88$. 

the same stock the ratio between infectious particles and viral genome copies can vary significantly. The overall precision of the plaque assay was comparable to the precision of the real-time PCR assays. Despite a distinct correlation between genomic load and plaque number being unlikely, the amount of yellow fever virus genome on the one hand was compared with the number of infectious virus particles on the other. Pearson analysis revealed a significant correlation between genomic load and plaque number.

Because real-time PCR can only detect a biological molecule, i.e. the genome, but not a biological feature, i.e. the infectious potential of a cell, PCR can never replace the plaque assay. However, due to comparable precision, quantitative PCR has the additional benefits of flexibility, feasibility and speed. The last yellow fever infection cases have shown that a fast and sensitive yellow fever virus detection assay that includes quantification can be an important factor for adequate treatment of the patient. Hence, real-time PCR has proven to be the method of choice for monitoring of antiviral treatment (Held et al., 2000). Whenever the identification of infectious virus particles is required, for example during vaccine production and control, the plaque assay, although very time consuming and laborious, is appropriate. In this regard, the real-time PCR assay described now is a very useful addition to diagnostic procedures but cannot replace the plaque assay.

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