External quality control assessment in PCR diagnostics of dengue virus infections

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Abstract

Background: Increased travelling to countries endemic for dengue fever (DF) demands efficient laboratory diagnostics. Nucleic acid amplification techniques (NAT) are now frequently used for rapid diagnosis of imported viral diseases. Different PCR systems are available.

Objectives: In order to assess the quality of molecular diagnostics of dengue virus infections, an external quality assurance (EQA) in PCR diagnostics was conducted.

Study design: A panel of 10 human plasma samples was prepared and spiked with dengue virus types DEN-1 to DEN-4. In addition, a 10-fold dilution series (1:10–1:10 4 ) of DEN-3 virus was included. The panel was pre-tested by nested RT-PCR, in-house real-time PCR, and a commercial real-time PCR kit. The samples were inactivated by gamma irradiation and shipped in freeze-dried state. Thirteen laboratories, within the European network for the diagnostics of imported viral diseases (ENIVD) took part using either single-round, nested, or real-time RT-PCR methods. Two laboratories used two methods in parallel, summarising up to 15 comparable results.

Results: 33–100% correct results were achieved. All laboratories detected DEN-2 correctly, followed by DEN-1 (14 positive results of 15), DEN-3 (12/15) and DEN-4 (11/15). Testing of the serial dilution revealed low sensitivity in many labs, with results ranging from 33 to 80% of correctly tested samples.

Conclusion: The EQA gives a feedback of the quality of the RT-PCR system used by each respective laboratory. The different test systems and amplification conditions demonstrate the importance of external quality control measures.

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1. Introduction

Dengue virus, which belong as a (+)-stranded encapsulated RNA virus to the family Flaviviridae, genus Flavivirus, is divided in the four serotypes, DEN-1 to DEN-4. The most prominent vector Aedes aegypti is widespread in tropical and subtropical countries especially in urban areas. Dengue infections are mostly asymptomatic in a mild form as dengue fever (DF), but can manifest life-threatening severe form of dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (Guzman and Kouri, 2003). The growing urban populations and the increasing mobility contribute the growing density and geographic expansion of the vectors accompanied by a dramatic increase of dengue infections in the last decade. The estimated number of 50–100 million infections per year results into 250,000–500,000 cases of DHF and 25,000–50,000 deaths each year (Gibbons and Vaughn, 2002). The increase of the severity of the disease is shown by the change of the ratio of DHF to DF shifted from 0.9 in 1980 to 20.4 in 2000 (Guzman and Kouri, 2003). Secondary infections caused by another dengue type as the first dengue infection are significant reasons for the DHF dependence on the age of the patient and his respective health status. In the Americas predominantly DEN-2 and, to a lesser extent, DEN-3 have been found as reinfectious agents causing DHF (Guzman and Kouri, 2003).
Good and reliable diagnostics are important tasks, which rapidly focus on the medical treatment dengue infection. Although there is no specific therapy, quick hospitalisation and adequate therapy can decrease the risk of severe infection.

Especially in the acute phase of the dengue infection before any specific antibody response directed against the dengue virus is detectable, only virus detection, either by RT-PCR and/or virus isolation, will be the matter of choice for the diagnostic. Because virus cultivation is a very time consuming procedure, only RT-PCR can give quick and reliable results within a few hours. Traditional amplification methods like nested or single-tube multiplex RT-PCR published some years ago (Harris et al., 1998; Lanciotti et al., 1992) get more and more replaced by real time, automated RT-PCR assays like the TaqMan or Light Cycler technique (Callahan et al., 2001; Drosten et al., 2002a; Laue et al., 1999; Arthus, Hamburg, Germany).

In order to evaluate the quality of the different PCR methods used for detection of an acute dengue infection, external quality assurance (EQA) by the European network for the diagnostics of imported viral diseases (ENIVD) for the diagnostic laboratories was conducted. Quality assessments are highly effective in mirroring the specificity and sensitivity of the test systems of individual external laboratories. Reflecting EQ controls in detection of HIV or hepatitis B virus (Schweiger et al., 1997; Valentine-Thon et al., 2001), EQA results of the molecular diagnosis, serological diagnosis of dengue infections (Donoso-Mantke et al., 2003) were presented in this publication.

2. Material and methods

2.1. Nested RT-PCR

RT and first PCR were performed in a one-step assay. Portions of 5 µl RNA were given to the RT-PCR mix of 10 mM Tris–HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.5% Tween 20, 0.5% Nonidet P 40, 200 µg/ml BSA, 50% glycerol, 200 µM dNTPs, 2 U AMV Reverse Transcriptase (Promega, Mannheim, Germany), 1 U Dynazyme™ DNA polymerase (Biometra, Göttingen, Germany) and 0.25 µM of each primer (sense: 5'-aaacaggctttataa3', antisense 5'-ctcctcagggagcaagttc-3') in a 20 µl reaction volume. After RT at 42°C for 50 min followed by 30 s at 94°C cDNA was amplified for 15 s at 94°C, 30 s at 53°C and 45 s at 72°C by 38 cycles and a final step at 72°C for 5 min. The primers anneal to YF and Japanese encephalitis virus cDNA as well and produce in case of dengue viruses amplicons of approximately 2050 bp in length. For nested amplification 1 µl amplification was transferred into a mix containing 300 µM dNTPs, 2 U Bio Therm™ DNA polymerase (Rapidzym, Luckenwalde, Germany), 16 mM (NH₄)₂SO₄, 67 mM Tris–HCl (pH 8.8), 1.5 mM MgCl₂, 0.01% Tween 20, the sense primer and the type specific antisense primers (Lanciotti et al., 1992). The primers were used in concentrations of 0.15 µM each in a 20 µl reaction volume. Cyclic amplification was done as mentioned above, except the annealing time was reduced to 15 s, and resulted in PCR products of 482, 119, 290 and 392 bp in size, which were compared to a 100 bp DNA ladder (Sigma-Aldrich, Taufkirchen, Germany).

2.1.2. TaqMan PCR

The viral RNA was reverse transcribed and amplified in a two step modus. RT was done in a 20 µl reaction mixture containing 100 U SuperScript II RNase H Reverse Transcriptase (Invitrogen, Karlsruhe, Germany), 10 µM dithiotreitol, 0.85 mM dNTP, 1.5 µl random primer (Gibco), 40 U RNAsin and 7 µl viral RNA. RT was done at 42°C for 50 min. TaqMan PCR was performed as described by Laue et al. (1999) with some modifications. The 25 µl PCR mix contained 2 µl cDNA, 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 500 µM dNTPs, 2.5 mM MgCl₂, 1.25 U Platinum Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany), 0.25 µl ROX, 30 pmol of each sense, 10 pmol of each antisense primer and 5 pmol of each probe (TIB Molbiol, Berlin, Germany). Cycling was done in thin-walled 96 well plates in an ABI Prism 7700 sequence detector (PE Applied Biosystems, Foster City, California). DNA fragments between 162 and 229 bp in size were amplified. The PE 7700 Sequence Detection System Software was used for analysis.
2.1.3. Light cycle RT-PCR

The assay was done with the Dengue LC RealArt™ RT-PCR Kit (Arthus, Hamburg, Germany) in a single-step procedure according to the manufacturer’s instruction on the Light Cycler (Roche Diagnostics, Mannheim, Germany). For analysis 5 μl RNA were added. A dengue virus DNA fragment of 146 bp in size was amplified and measured by fluorescence intensity (fluorometric channel F3/F1) using a 5′-nuclease detection probe. Analysis was done with the Light Cycler software (Version 3.5). The kit detects dengue virus without information of the type.

2.2. External quality assurance

2.2.1. Participating laboratories

The following 13 external laboratories took part in the QA: Antonio Tenorio (Centro Nacional de Microbiología, Majadahonda, Spain); Detlev Schultz (IKMI, St. Gallen), Switzerland; Vance Vorndam (Dengue Branch CDC, San Juan), Puerto Rico; Thomas Laue, (Arthus GmbH, Hamburg), Germany; Tatjana Avsic-Zupanc (University of Ljubljana), Slovenia; Anna Papa, Antonios Antoniadis (Aristotle University of Thessaloniki), Greece; Jan Groen (Erasmus MC Rotterdam), The Netherlands; Felicity Burt (NIH, Bethesda, Maryland), Republic of South Africa; Ake Lundkvist (Karolinska Institute, Stockholm), Sweden; Anders Fomsgaard (SSI, Copenhagen, Denmark); Howard Tolley (CAMR, Porton Down), UK; Jean Paul Durand (IMTSSA, Marseille) France; Christian Drosten, Herbert Schmitz (BNI, Hamburg), Germany.

2.2.2. RT-PCR techniques used by the participants

The RT-PCR techniques used by the 13 participating laboratories included nested (Lanciotti et al., 1992; ter Meulen et al., 2000) and one step RT-PCR (n = 6), single-tube multiplex (Harris et al., 1998) (n = 1), real time RT-PCR by TaqMan (Laue et al., 1999) (n = 1) or Light Cycler instruments (Arthus, Hamburg, Germany) (n = 2). Five laboratories used their individual (in house) RT-PCR assays including two individual TaqMan systems. Two of the laboratories examined the samples by two different methods, single-tube multiplex and Light Cycler RT-PCR, or one-step nested RT-PCR, respectively. The results of each PCR method were scored separately. Thus, altogether 15 different results obtained of the 10 samples were evaluated (Table 1). Predominantly the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) was used for RNA extraction.

2.2.3. Evaluation of the results

The results were scored in reflection of sensitivity and specificity. Correct negative and positive results up to determination of the correct dengue type were assessed by two criteria. One point was given for correct dengue positive results without type differentiation or incorrect type determination. False positive or false negative results were scored with no points. The test results were sent out in an anonymous form giving an individual identification code for each laboratory.

3. Results

3.1. Pre-evaluation of the EQA panel

Investigation of the QA samples by nested RT-PCR showed different results before and after gamma irradiation (Table 1). Without irradiation the results, which have been expected, were detectable in all samples with exception of DEN-3 in dilution 1:104. After gamma irradiation only positive results were obtained into the 1:10 diluted samples. In the 1:102 and 1:103 diluted sample the expected 290 bp band was no more detectable. After TaqMan and Light Cycler RT-PCR, in which short DNA fragments were amplified, all dengue virus samples showed positive results, including DEN-3 in the highest dilution, 1:105.

3.2. Comparison of the EQA results

The 13 participating laboratories are ranged according to the quality of their results (Table 1). With exception of laboratory K and O, which used the Light Cycler system, all other participants used dengue virus type specific amplification systems. Beside the Light Cycler system, which permits only qualitative results per se, laboratory K used also a type specific RT-PCR. The laboratories A and B, who accomplished the pre-check, could not detect DEN-3 cDNA in this low concentration. Since 10 out of 13 laboratories had received negative results this sample was not included in the scoring (Table 1). Only four laboratories (P in both assays, T, D and K with the Light Cycler system) detected DEN-3 cDNA in this low concentration. Since 10 out of 13 laboratories had received negative results this sample was not included in the scoring (Table 1). Only two laboratories (A, B) reached the maximum score (18 points for 100%) with nested and TaqMan RT-PCR, respectively. Laboratory P, used two different assays and could detect DEN-4 only by nested RT-PCR. However, with the nested RT-PCR they received positive results also with the YF and TBE virus sample. Laboratory E, could not differentiate in their in house TaqMan assay between DEN-1, DEN-2 or DEN-4 in the respective samples.

4. Discussion

The presented EQA study evaluates the results of 13 international laboratories, regarding sensitivity and specificity of their RT-PCR in detection of dengue virus. As shown in previous EQA for serology of Hanta and dengue freeze drying and gamma irradiation is a very suitable method for samples preparation (Biel et al., 2003; Donoso-Mantke et al., 2003). Also in an EQA for PCR diagnosis of Filo-, Lassa-
and Orthopox-viruses the gamma irradiation turned out to be highly efficient for inactivation of infectious agents (Niedrig et al., 2003).

As found in the pre-evaluation study, the gamma irradiation could have a significant effect on the sensitivity of the PCR. The damage of the viral genome by nicking is an expected result caused by the irradiation process and correlates with the doses. Therefore, the diagnostic procedures based on long amplification products will be affected compared to methods relying on short amplicons. This can be seen in Table 1 for our nested RT-PCR (with 2050 bp for RNA target sequence) compared to the real-time methods (<600 bp). This clearly demonstrates that for PCR diagnostic short fragment of viral RNA should be selected as target sequence. As seen in Table 1 the nested PCR seems not affected by gamma irradiation procedure for the samples with high dengue concentration.

Results reported by the 13 laboratories show a great variation between 6 and 18 scoring points corresponding to 33–100% of the correct results. Laboratories A, B show the best results considering the sensitivity and specificity. Laboratory E recognises all dengue positive samples but was not able to type DEN-1, DEN-2 and DEN-4 correctly. The scores obtained by the laboratories B, E, and T performing TaqMan RT-PCR ranged from 15 to 18 scoring points, demonstrating a high quality of the respective method. Both laboratories using Light Cycler PCR received a score of 11, which reflects not reaching the reasonable sensitivity and virus specificity of their achievable results. Generally, this method is described as highly sensitive in detection of viral DNA or RNA, also in case of dengue virus (Drosten et al., 2002a; König et al., 2001; Schröter et al., 2001). Interestingly both laboratories (O, K) obtained different results using the same commercial assay which is also in

### Table 1
Comparison of the pre-evaluation results obtained with different RT-PCR techniques

<table>
<thead>
<tr>
<th>Lab RT-PCR technique</th>
<th>Sample no.</th>
<th>Score*</th>
<th>% Correct results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-evaluation</td>
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<tr>
<td>Nested a (not irrad.)</td>
<td>++ ++ ++ ++ ++ ++ (-) ++ ++ - - - - 18 100</td>
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<tr>
<td>Nested b (irrad.)</td>
<td>++ ++ ++ ++ ++ ++ ++ (-) ++ ++ - - - - 16 89</td>
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<tr>
<td>TaqMan c</td>
<td>++ ++ ++ ++ ++ ++ ++ (-) ++ ++ - - - - 15 83</td>
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<tr>
<td>Light Cycler f (irrad.)</td>
<td>++ ++ ++ ++ ++ ++ ++ (+) (+) - - - - 14 78</td>
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<tr>
<td>EQA results</td>
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<tr>
<td>A Nested d</td>
<td>++ ++ ++ ++ ++ ++ (-) ++ ++ - - - - 12 67</td>
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<td>B TaqMan e</td>
<td>++ ++ ++ ++ ++ ++ (+) ++ ++ - - - - 12 67</td>
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<td>C Nested f</td>
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<td>D Nested g</td>
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<td>E In-house h</td>
<td>++ ++ ++ ++ (+) ++ ++ - - - - 6 33</td>
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</table>

Correct positive/negative results (%)

93 100 80 67 67 33 74 74 87 80

The samples were tested before and after gamma irradiation (irrad.) by nested RT-PCR, by TaqMan and Light Cycler RT-PCR after irradiation, only.

EQA results of the respective 13 laboratories. *The score reflects the quality of the diagnostic results: ++, positive result and correct typing (score 2); +, positive, no information on type or incorrect type (score 1); (+), false positive (score 0); −, negative result (score 2); (−), false negative (score 0); sample #18 is not part of the score.

* Lanciotti et al. (1992).
* Lane et al. (1999).
* In house TaqMan RT-PCR.
* In house TaqMan RT-PCR systems.
* Harris et al. (1998).
* Commercial test system: artus, Germany (not type specific).
* ter Meulen et al. (2000).
The laboratories, which failed to detect DEN-4 or DEN-3 RNA should improve the primer sequences in conformity to both types. Although laboratory U, N and Q used the nested RT-PCR failed to detect DEN-3 even the amount of RNA was approximately $10^7$ geq/ml in the 1:10 dilution. The inhibition of RT-PCR by other factors as described earlier can be excluded since all samples originate from one plasma pool (Drosten et al., 2002b). The sensitivity and specificity should be influenced predominantly by the selection of primers and the annealing conditions. Other factors can interfere amplification, too, e.g. the thermal cycler, the detection system (Raggi et al., 2003), and the length of RNA target sequences. It is important to address the issue that current primers and probes used by many laboratories will not detect all dengue virus strains. Thus, a good laboratory should always have at least two primers and probes systems in order to detect different strains and mutated variants.

In conclusion, the present results demonstrate the importance of external quality control measures in PCR diagnostics of dengue virus infections as done in the present EQA study. The evaluation show the variability of the RT-PCR techniques and the variability in sensitivity and specificity. Overall, RT-PCR is an efficient method in detection of dengue virus infections, and it will be a helpful tool for clinicians for detection of an acute dengue infection if sampling and diagnostic PCR is performed seriously.

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