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Minor groove binder modification of widely used TaqMan hydrolysis probe for detection of dengue virus reduces risk of false-negative real-time PCR results for serotype 4

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ABSTRACT

Dengue is a vector-transmitted viral infection that is a significant cause of morbidity and mortality in humans worldwide, with over 50 million apparent cases and a fatality rate of 2.5 % of 0.5 million severe cases per annum in recent years. Four serotypes are currently co-circulating. Diagnosis of infection may be by polymerase chain reaction, serology or rapid antigen test for NS1. Both pan-serotype and serotype-specific genome detection assays have been described, however, achieving adequate sensitivity with pan-serotype assays has been challenging. Indeed, as we show here, inspection of components and cycling parameters of a pan-serotype RT-qPCR assay in use in laboratories worldwide revealed insufficient probe stability to accommodate potential nucleotide mismatches, resulting in false-negatives. A minor-groove binder (MGB)-modified version of the probe was designed and its performance compared with that of the original probe in 32 samples. Eight of the samples were undetected by the original probe but detected by the MGB modified probe and six out of seven of these that could be serotyped belonged to serotype 4. Sequencing of the region targeted by the probe in these samples revealed two mismatches which were also universally present in all other serotype 4 sequences in a public database. We therefore recommend adoption of this MGB modification in order to reduce the risk of false-negative results, especially with dengue serotype 4 infections.

1. Introduction

Dengue, caused by a member of the family *Flaviviridae,* is the most common arboviral infection worldwide. Globally, case incidence is thought to be 50–100 million per annum, though the true number may be higher as not all infections are apparent and misclassification may occur in regions with a high burden of febrile illness (Bhatt et al., 2013; Stanaway et al., 2016). Clinical infections range widely in severity from asymptomatic to dengue haemorrhagic fever and death. Other than supportive therapy no specific treatment is available and as yet there is no effective vaccine universally recommended for those at risk (Aguiar et al., 2016; Halstead, 2017; World Health Organization, 2017). A vaccine would need to prevent infection in the infection-naïve, such as travellers to dengue-endemic regions, and neonates in endemic countries, as well as those who have experienced primary infection. Recommendations for the only licensed vaccine currently available (the Pasteur CYD-TDV) are limited to those aged 9–45, who are likely to have previously been exposed to infection (Sridhar et al., 2018; Wilder-Smith et al., 2018).

Four serotypes co-circulate in most endemic countries, and these are genetically variable (Costa et al., 2012). Initial infection with any serotype gives type-specific lifelong immunity, however, prior infection

Abbreviations: MGB, minor groove binder; qPCR, quantitative polymerase chain reaction; RT-qPCR, reverse transcription quantitative polymerase chain reaction; C_q, quantification cycle (sometimes referred to as C_t or threshold cycle)

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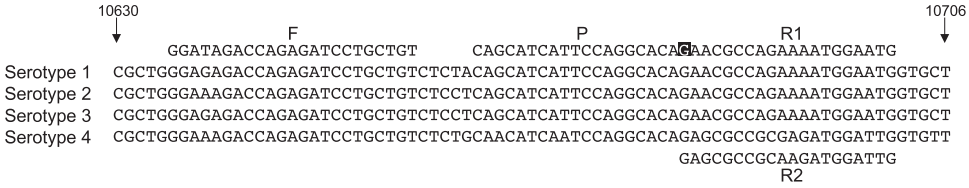

Fig. 1. Representatives of the four dengue serotypes showing primer/probe binding sites. The sequences are: serotype 1 Hawaii (EU848545); serotype 2 New Guinea C (AF038403); serotype 3 Philippines H87 (M93130); serotype 4 Philippines H241 (AY947539). F = forward primer; P = probe; R1 = reverse primer 1; R2 = reverse primer 2. The genomic numbering refers to AF038403 (serotype 2). The black shading represents the single nucleotide overlap between the probe and the reverse primer.

with one serotype can predispose to a more serious episode on subsequent infection with a different serotype (Halstead et al., 1970; Katzelnick et al., 2017).

Diagnosis of dengue is on clinical suspicion based on symptoms and travel history; in addition a wide variety of laboratory methods are in current use. Blood samples may be analysed for RNA, IgM and IgG or NS1 antigen; the presence of RNA and/or IgM suggesting acute infection. Point of care diagnostic tests are also available, although the sensitivity of these tests when performed in the field has been variable (Pang et al., 2017; Sa-ngamuang et al., 2018). Molecular techniques are widely used, though the resource requirements for these tests mean that their implementation may not be feasible in resource-limited settings. Molecular tests to detect viraemic infections include qualitative/nested PCR, reverse-transcription quantitative PCR (RT-qPCR) and isothermal amplification methods (Domingo et al., 2010; Poersch et al., 2005). Some tests seek to differentiate serotypes for epidemiological purposes, while others are intended to be pan-serotype. Serotyping of dengue infection has limited applicability in immediate clinical management. However, epidemiological surveys are important to assess whether a predominant serotype in a locale is supplanted, bringing with it the risk of secondary infections and more severe consequences.

A widely cited pan-serotype RT-qPCR assay published in 2002 (Drosten et al., 2002) that is apparently used by laboratories in many countries (Domingo et al., 2010) was identified in a literature search and evaluated using archived samples. An initial failure to detect one of four samples tested led to a closer inspection of this assay and, as a result, to its improvement through minor groove binder (MGB) modification of its TaqMan probe (i.e. 5' hydrolysis probe). The MGB-modified and un-modified probe (herein designated the 'original' probe) assays were tested on a larger group of samples and two sequence mismatches between the 'original' probe and dengue serotype 4 sequences were identified. The MGB modification was thus shown to prevent false-negative results being generated with serotype 4 samples.

2. Materials and methods

2.1. Samples

Samples #1-21: Archived EDTA-plasma samples from University College London Hospital that had been submitted for arbovirus testing between January and August 2016 were reviewed. 21 samples that had been reported elsewhere as positive for dengue virus RNA were identified and retrieved from storage at -20 °C The total storage time at −20 °C varied between 5 and 14 months prior to nucleic acid extraction (mean 10 months). Samples #22-32: Archived samples were stored at −80 °C since 2012 (Samples #22, 26–30), 2016 (Samples #23-25) or 2018 (Samples #31-32). All samples from Brazil were processed at the Flavivirus Laboratory, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

2.2. Ethical approval

The study was approved by the local University College London Hospital Clinical Governance committee following regulatory requirements for diagnostic assay development for Samples #1-21 and the Ethical Screening Committee of Fiocruz (CAAE): 90249218.6.1001.5248 (2.998.362) for Samples #22-32.

2.3. Nucleic acid extraction

RNA was extracted from plasma samples manually using the Qiagen viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (except volumes as follows) or the Qiagen EZ1 BioRobot. Sample input and elution volumes for manual extraction were matched to the BioRobot for the samples from UCLH: 200 µl sample input and 90 µl elution. For samples from Brazil, only 100 µl was available, which was supplemented with 100 µl negative normal human serum, and extracted as described. Extracted RNA was stored at -80°C.

2.4. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Real-time RT-qPCR was performed in accordance with the MIQE guidelines (Bustin et al., 2009) using TaqMan Fast Virus 1-Step Master Mix (cat. no. 4444432, Life Technologies, Paisley, UK), primers at 600 nM and probe at 100 nM (sequences as in (Drosten et al., 2002) and Fig. 1, synthesized by Sigma, Haverhill, UK); either a 6-carboxy-fluorescein (FAM)/ tetramethylrhodamine (TAMRA) probe for the 'original' assay (Drosten et al., 2002) or a FAM/ minor groove binder (MGB)-non-fluorescent quencher probe (synthesized by Life Technologies) for the MGB-modified assay (Fig. 1). Each well contained 15 µl total reagents with 1 µl RNA extract, and each condition was run in triplicate wells. Cycling conditions (ABI 7500 thermal cycler, Applied Biosystems) were: 50 °C 5 min, 95 °C 20 s, then 45 cycles of 95 °C 3 s, 60 °C 1 min. Negative controls (H_2O) were run on every plate. Data were analysed using the ABI 7500 software v2.0.6.

2.5. Synthesis of RNA by in vitro transcription

Transcripts used to generate the dengue RNA dilution series were synthesised using the T7 MegaScript kit (Life Technologies) and PCR amplicons as template, amplified from Sample #14 (serotype 2). RNA from this clinical sample was initially reverse transcribed using Superscript IV and random hexamers (both Life Technologies). Amplified DNA template was produced by nested PCR using primers EG273/EG250 then EG295/EG296 (Table 1) and Platinum SuperFi polymerase (cat. no. 12351010, Life Technologies). Transcripts were cleaned up using the MegaClear kit (Life Technologies) and the yield assessed using the Nanodrop One^C (Thermo Scientific, Paisley, UK).

2.6. Sequencing

RNA extracts were reverse transcribed using Superscript IV with reverse primer EG244 (Table 1) (Samples #2, #3, #6-17 and #19-21) according to the manufacturer's instructions. Where amplification by nested PCR was initially unsuccessful, random hexamers were used for the reverse transcription step (Samples #4 and #18). Nested PCR

Table 1
Primer usage for nested PCR amplification of nucleic acid prior to sequencing and for generation of transcript. Both pan-serotype and serotype-specific primers were used. The serotype-specific PCRs were employed for Samples #14 (serotype 2), #6 and #19 (serotype 4) to amplify the assay primer-binding region (Drosten et al., 2002), and for generation of transcript from Sample #14 (serotype 2). The published sources of these primer sequences is also given, where not originating from this study. All primer sequences are presented 5′ to 3′. * Numbers refer to the final amplicon (i.e. the second round of amplification).

Genomic region (numbers refer to GenBank:AF038403)	1st round		2nd round	
	Forward	Reverse	Forward	Reverse
10,467-10,660* (pan-serotype, all samples, sequencing)	EG272 GTGCTGCCTGTAGGTCC This study	EG244 TTCTGTGCCTGGAATGATGCTG (Alm et al., 2014)	EG273 CCATGGAAGGTGTACGC This study	EG248 GAGACAGCAGGATCTCTGGTC (Huhtamo et al., 2010)
10,479-10,701* (Serotype 2 specific, for Sample #14, sequencing)	EG273 CCATGGAAGCTGTACGC This study	EG250 TCAACAGCACCATTCCAT This study	EG282 TACGCATGGCGTAGTGGA This study	EG223 CATTCCATTTTCTGGCGTTC (Drosten et al., 2002)
10,479-10,701* (Serotype 4 specific, for Samples #6 & #19, sequencing)	EG273 CCATGGAAGCTGTACGC This study	EG251 TCAACAACACCAATCCAT This study	EG283 TACGCGTGGCATATTGGA This study	EG224 CAATCCATCTTGCGGCGCTC (Drosten et al., 2002)
10492-10704* (Serotype 2 specific, for Sample #14, transcription)	EG273 CCATGGAAGCTGTACGC This study	EG250 TCAACAGCACCATTCCAT This study	EG295 TAATACGACTCACTATAGGGTGGACTAGCGGTTAGAGGAG This study	EG296 CACCATTCCATTTTCTGGCGTTC This study

reactions were carried out on all samples using the primers in Table 1 and Platinum SuperFi Taq polymerase according to the manufacturer's instructions in 25 µl (first round) and 50 µl (second round) reactions. After the second round, 5 µl PCR reaction product was analysed by agarose gel electrophoresis to ensure unique bands of the correct size had been obtained, and DNA from the remaining 45 µl reaction product was purified by the QIAquick PCR purification kit (Qiagen). Bi-directional Sanger sequencing of the purified amplicon was carried out by Genewiz Ltd. (Essex, UK).

2.7. Serotyping

To assign serotype to Samples #1-21, the segment that was sequenced (corresponding to 10467–10660, numbering according to GenBank AF038403) was used as input for a nucleotide BLAST search (www.ncbi.nlm.nih.gov/BLAST) and the serotype of the closest matched dengue sequences from the database noted. None of the sequences from this study matched more than one serotype. Samples #22-32 were assigned a serotype using the method described in (Santiago et al., 2013).

2.8. Sequence analyses to explore probe/target mismatches in serotype 4 samples

Analysis of. ab1 files, alignment to template and inspection of sequences was carried out using DNADynamo (Blue Tractor Software Ltd).

To create a multiple sequence alignment, all whole virus genome sequences from samples taken from a human host, classified as dengue serotype 4 that included data between nucleotides 10,635 and 10,701 (the qPCR amplicon from (Drosten et al., 2002), numbering according to AF038403) were downloaded from the Virus Pathogen Database and Analysis Resource (www.viprbrc.org), aligned using MUSCLE via the online interface, then opened in Seqotron (Fourment and Holmes, 2016) for further analysis. This dataset comprised 192 genomes. The section encompassing the 'original' assay primers and probe was selected and extraneous sequence data removed, as well as any genomes with missing data for this section. The resulting FASTA file contained 147 sequences from geographically diverse locations. To create Supplementary Table 1, the percent identity of each nucleotide at each position was calculated using consensus mode in DNADynamo.

3. Results

3.1. A widely used pan-serotype RT-qPCR assay can yield false-negative results

In 2010, an international external quality assessment exercise for molecular diagnostics of dengue was reported, in which participating laboratories were able to compare the performance of their assays (Domingo et al., 2010). The most widely used published assay in that international assessment was the RT-qPCR pan-serotype assay described by Drosten et al. in 2002 (Drosten et al., 2002). This assay was therefore selected for further evaluation. The primer and probe binding sites for this assay are shown in Fig. 1, and are located in the 3′ terminal non-coding region of the genome, where there is a high degree of sequence homogeneity between serotypes. In order to encompass the genetic diversity of serotype 4 there are two reverse primers (R1 and R2) in the mix at a 1:1 ratio.

Preliminary tests on four known RNA-positive samples (sample numbers #18 - #21) unexpectedly gave one false-negative result. The properties of the primers and probe were therefore investigated further. The melting temperatures (T_m) of the oligonucleotides were calculated by several alternative methods as shown in Table 2. These calculations revealed that the T_m of the probe was approximately the same as that of the primers. This was a surprising finding given that TaqMan probe

Table 2
Melting temperature (T_m) calculations for oligonucleotide primers and probe described in (Drosten et al., 2002). The MGB-modified probe (EG242) has an identical sequence to the 'original' probe in the table, but carries 5'FAM and 3'MGB-NFQ modifications.

Oligonucleotide name	Oligonucleotide sequence (5' to 3')	Sigma Aldrich[a] T_m (°C)	OligoCalc[b] T_m (°C)	OligoAnalyzer[c] T_m (°C)	PrimerAnalyser[d] T_m (°C)
Forward	GGATAGACCAGAGATCCTGCTGT	64.6	64.6	66.2	69.4
Reverse R1	CATTCCATTTTCTGGCGTTC	64.2	56.4	61.4	64.0
Reverse R2	CAATCCATCTTGCGGCGCTC	72.1	62.5	67.3	70.8
Probe	FAM-CAGCATCATTCCAGGCACAG-TAMRA	66.9	60.5	62.2	65.2

Abbreviations: FAM - 6-carboxy fluorescein; TAMRA – tetramethylrhodamine; MGB – minor groove binder; NFQ – non-fluorescent quencher.
[a] SigmaAldrich OligoEvaluator online calculator; nearest neighbour method (Borer et al., 1974); www.sigmaaldrich.com.
[b] OligoCalc online calculator; salt adjusted (Kibbe, 2007); http://www.basic.northwestern.edu/biotools/oligocalc.html.
[c] OligoAnalyzer online calculator 'qPCR' setting; Allawi's thermodynamic parameters (Allawi and SantaLucia, 1997); https://eu.idtdna.com/calc/analyzer.
[d] PrimerAnalyser online calculator; Allawi's thermodynamic parameters; http://primerdigital.com/tools/PrimerAnalyser.html.

design guidelines state that the probe T_m should be about 10 °C higher than that of the primers (Bustin, 2000). If the probe T_m is too low then stable binding may not occur before displacement and hydrolytic cleavage by the DNA polymerase during amplification. An inadequate T_m difference between that of the probe and that of the primers would be likely to make probe hybridization unstable in the face of even minor sequence mismatch.

3.2. MGB-modified probe has optimised characteristics and reduces risk of false-negatives

Heterogeneity in the bases upstream of the probe sequence, and the proximity of the reverse primer downstream of the probe, meant that extending the probe to increase its T_m was not an option. To optimise the assay we therefore elected to synthesise an alternative probe of identical sequence and length but with a 3' minor groove binder (MGB) moiety. This stabilises the duplex, increasing the T_m of the probe by between 10 and 20°C, without lengthening it (Garson et al., 2012). This alternative probe was synthesised (EG242) and the assay was designated 'MGB-modified'.

To investigate whether adding the MGB modification to the probe made a difference to assay characteristics (PCR efficiency and detection limit) a dilution series of a sample with a high viral load (Sample #14) was prepared. Ten-fold serial dilutions were made and analysed by both the MGB-modified and the 'original' assays in triplicate (Fig. 2a). The fitted regression line for the MGB-modified probe showed detection at approximately 1 C_q lower than the 'original' probe across most of the dilution range (Fig. 2a). The slopes, -3.33 and -3.17, representing a nominal PCR efficiency of ˜99.7% and ˜106.6%, and the R squared values (0.998 and 0.994) for the MGB-modified and 'original' assays respectively, were not significantly different. The dilution series was repeated a second time with RNA transcript generated from Sample #14 to assess the minimum copy number detectable by the two assays (Fig. 2b). Again, fitted regression lines for the MGB-modified probe showed detection at approximately 1 C_q lower than the 'original' probe across most of the dilution range, equivalent to approximately a 2-fold increase in sensitivity (Fig. 2b). The limit of qualitative detection for both assays was approximately 100 RNA copies per reaction, though the limit of quantitation was ˜1000 copies. Similar slopes, nominal PCR efficiencies and R squared values were seen (-3.26 and -3.24, ˜102.5% and ˜103.4%, 1.000 and 0.999 for the MGB-modified and 'original' assays respectively) (Fig. 2b).

To compare the performance of the MGB-modified and 'original' assays on a wide variety of samples, 32 archived samples reported elsewhere as dengue RNA-positive were tested. Overall, 29 of 32 samples were found to be positive with the MGB-modified assay but only 21 of 32 with the 'original' assay. Sample #5 yielded one positive well out of three repeats and Sample #25 yielded two positive wells out of three repeats with the MGB-modification but zero of three were positive with the 'original' probe for both these samples. For every positive sample, the C_q with the MGB-modified assay was approximately one cycle lower than generated by the 'original' assay (Fig. 3 and Table 3).

3.3. False-negative results with 'original' probe were from serotype 4 samples

To determine whether dengue serotype may be associated with the inability of the 'original' assay to detect certain samples, a region between nucleotides 10467–10660 (numbering based on serotype 2, GenBank AF038403) was amplified by nested PCR and sequenced for 19 of Samples #1-21, yielding the serotype data shown in Table 3. Two samples, samples #1 and #5, did not yield amplicons for sequencing. Samples #22-32 were serotyped by the molecular assay described in (Santiago et al., 2013) (Table 3). The samples that were undetectable with the 'original 'assay but were detectable in triplicate with the MGB-modified assay (i.e. Samples #6, #19, #27, #28, #29 and #31) were all serotype 4, suggesting that this serotype (an early evolutionary breakaway from the lineage that led to serotypes 1–3, (Costa et al., 2012)) may be particularly poorly detected by the 'original' version of this assay. To understand why the 'original' probe without the MGB modification may not have detected these samples, the region covering the qPCR amplicon was sequenced, corresponding to 10479–10701 (numbering according to GenBank AF038403) from Samples #6 and #19. These data were appended to the sequencing data generated for serotyping (10467–10660, Table 3) and are shown in Fig. 4. Sample #14, a serotype 2 sample which was detected by both the MGB-modified and 'original' probes was also sequenced in this region to provide a control for comparison. The sequencing clearly demonstrated two mismatches in the probe binding region, for serotype 4 samples only. The destabilizing effect of these mismatches would have been negated by the MGB moiety in the modified probe.

3.4. Two mismatches in probe binding site are present in all available serotype 4 sequences

We investigated whether the 'original' probe mismatches with serotype 4 samples found in this study are also found in previously sequenced samples from around the globe. A multiple sequence alignment was prepared from all available dengue serotype 4 genomes from the Virus Pathogen Database and Analysis Resource (www.viprbrc.org) with sequence data covering the entire forward primer and reverse primer binding region (n = 147 sequences). This demonstrated that the two mismatches found in the probe-binding region of Samples #6 and #19 in this study are in fact universally present in all available dengue serotype 4 genomes (Supplementary Table 1).

3.5. No false positive signals are seen in dengue-negative samples

To ensure that the MGB-modified probe maintained its specificity and did not yield false positive results with other members of the family

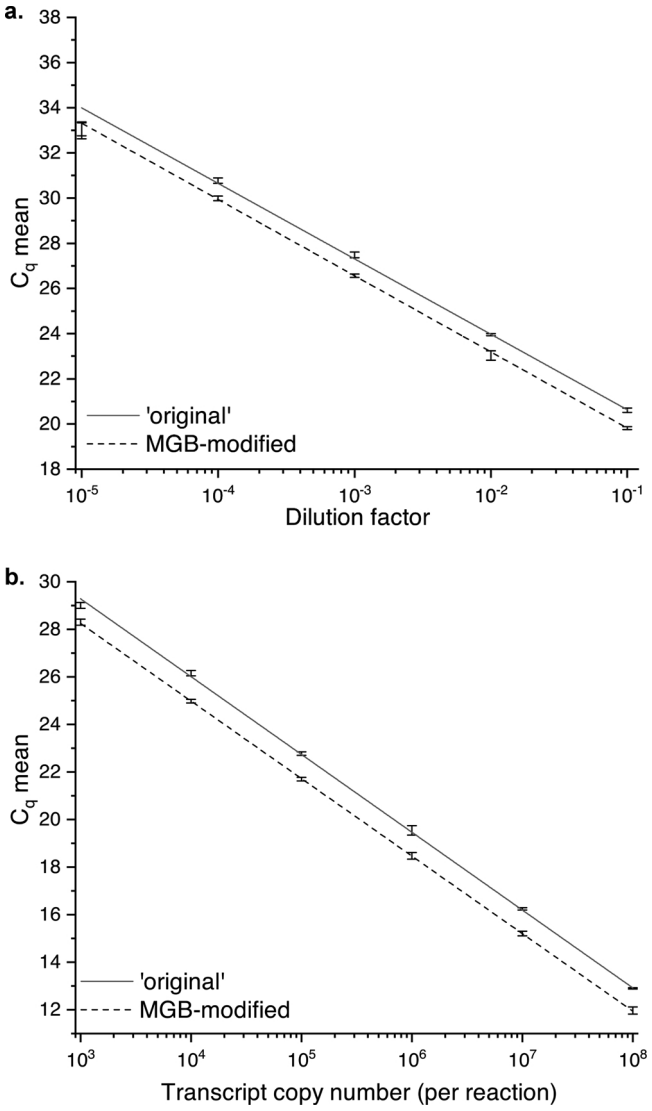

Fig. 2. Amplification of samples generated from ten-fold serial dilutions.
Each dilution was analysed in triplicate using both the MGB-modified probe and the 'original' probe. Error bars represent the standard deviation of the mean of positive wells. a) Ten-fold serial dilutions were made with Sample #14 nucleic acid extract, covering the dilution range from 10^{-1} to 10^{-8}. The nominal PCR efficiency, R^2, and slope for the MGB-modified assay were 99.7%, 0.998 and -3.33, and for the 'original' assay were 106.6%, 0.994 and -3.17. b) Ten-fold serial dilutions were made with RNA transcript generated from Sample #14 template. Between 10^0 and 10^8 copies per sample were tested; for both assays, only samples with 10^2 copies or more yielded a positive result. The nominal PCR efficiency, R^2, and slope for the MGB-modified assay were 102.5%, 1.000 and -3.26, and for the 'original' assay were 103.4%, 0.999 and -3.24.

Flaviviridae, 16 samples from patients with hepatitis C and 22 samples from patients with Zika virus were tested in vitro by qPCR. None of the samples tested by qPCR yielded false-positive results. An *in silico* analysis using NCBI Primer-BLAST also failed to find any cross-reactivity with the human genome, or with the genomes of other members of the *Flaviviridae* including the following viruses: hepatitis C, chikungunya, Zika and yellow fever.

4. Discussion

The incidence of dengue is slowly rising worldwide and vigilance is increasingly required in non-endemic countries where dengue is

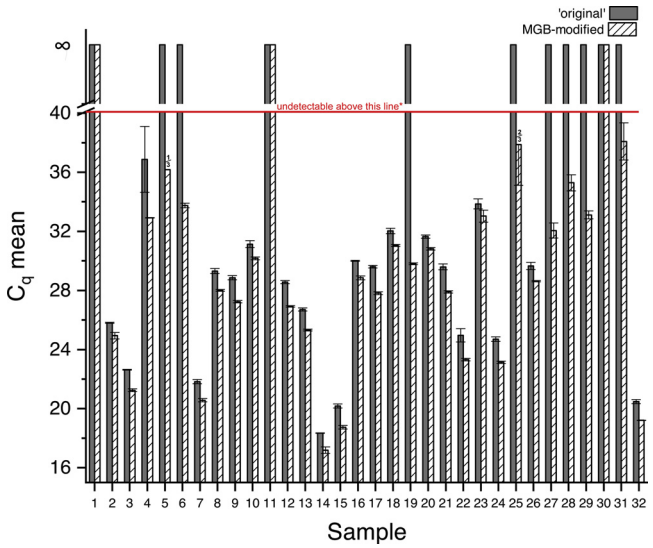

Fig. 3. A comparison of C_q values obtained with 32 archived samples assayed by the MGB-modified and the 'original' assay.
Each sample was analysed in triplicate; error bars represent the standard deviation of the mean of positive samples. If fewer than 3 of 3 wells yielded positive results (Samples #5 and #25), the number of positive wells is given above each bar. Samples for which 3/3 wells were negative are shown with C_q mean above 40 cycles.

Table 3
Dengue serotypes of samples in this study. Serotype for Samples #1-21 was identified through BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the sequenced amplicon corresponding to 10467–10660 (according to GenBank AF038403). Serotype for Samples #22-32 was deduced via the serotyping assay described in (Santiago et al., 2013).

Sample	C_q (MGB-modified probe)	C_q ('original' probe)	Serotype
Sample 1	undetected	undetected	Did not amplify
Sample 2	24.9	25.8	3
Sample 3	21.2	22.6	2
Sample 4	32.9	36.9	2
Sample 5	36.2	undetected	Did not amplify
Sample 6	33.7	undetected	4
Sample 7	20.6	21.8	1
Sample 8	28.0	29.3	3
Sample 9	27.2	28.9	1
Sample 10	30.2	31.1	1
Sample 11	undetected	undetected	2
Sample 12	26.9	28.6	3
Sample 13	25.3	26.7	2
Sample 14	17.2	18.3	2
Sample 15	18.7	20.2	2
Sample 16	28.9	30.0	2
Sample 17	27.8	29.6	2
Sample 18	31.0	32.0	2
Sample 19	29.8	undetected	4
Sample 20	30.8	31.6	3
Sample 21	27.9	29.6	3
Sample 22	23.3	25.0	1
Sample 23	33.0	33.9	1
Sample 24	23.1	24.7	1
Sample 25	37.9	undetected	1
Sample 26	28.6	29.7	1
Sample 27	32.1	undetected	4
Sample 28	35.3	undetected	4
Sample 29	33.1	undetected	4
Sample 30	undetected	undetected	4
Sample 31	38.1	undetected	4
Sample 32	19.2	20.5	2

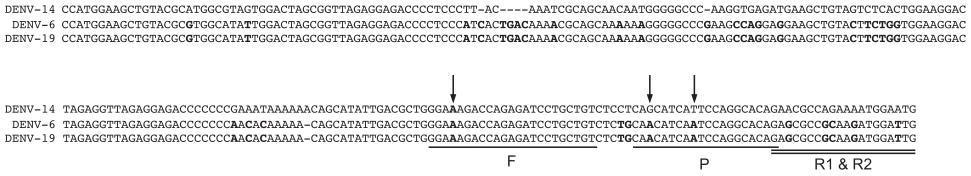

Fig. 4. Sequence alignment of Samples #6, #14 and #19.
The amplicon covering the section 10467–10701 (numbering according to AF038403) including binding sites for the primers and probe is shown for Samples #6, #14 and #19. A section between 10479–10701 was amplified, sequenced and appended to data from 10467 to 10660, used to serotype all samples (Table 3). Samples #6 and #19 are serotype 4; Sample #14 is the high titre sample used to generate Fig. 2 and is serotype 2. Mismatches to the assay primers and probe are shown with arrows and highlighted in bold. Outside of the primer/probe binding regions (delineated by horizontal lines), sequence differences between the samples are also highlighted in bold. There are two different reverse primers (R1 and R2 mixed 1:1) therefore although there are five variant sites between the two serotypes in this region, all have ~100 % homology with one of the reverse primers.

imported by returning travellers (Neumayr et al., 2017; Stanaway et al., 2016). As yet, there is no vaccine that can be universally recommended and no cure, and morbidity and mortality remains high in countries that can least afford the dual burdens of healthcare costs and loss of human capital. A clear and accurate understanding of incidence is key to obtaining a full epidemiological picture of the disease, and accurate diagnostic tools are essential. In this study, we have shown how the sensitivity of a pan-serotype RT-qPCR assay, employed to diagnose viraemic dengue infection can be significantly improved by MGB modification of the TaqMan hydrolysis probe. Dengue serotype 4 comprises a significant minority of infections worldwide, and therefore the diagnostic tools chosen by laboratories must reliably detect this serotype. In the quality assessment study of 2010 (Domingo et al., 2010), samples of dengue serotype 4 were included at a level of 10^5 genome equivalents per mL, two orders of magnitude higher than the minimum detection requirement to achieve an 'acceptable' classification in this exercise. Despite this relatively high level, 14/46 laboratories failed to classify the serotype 4 sample as dengue positive. Several pan-serotype dengue assays proposed in the literature include more than one primer (or probe) to ensure detection of dengue serotype 4 (Alm et al., 2014; Dumoulin et al., 2008; Santiago et al., 2013). Interestingly, the modification to the (Drosten et al., 2002) assay proposed by Dumoulin et al. is the addition of a second probe identically located but with two nucleotides changes to map exactly the dengue serotype 4 consensus sequence (Dumoulin et al., 2008). When the (Drosten et al., 2002) assay was originally published in 2002, MGB-probes were only just becoming available; their widespread use not common until a few years later. It is therefore appropriate to update this assay using technological advances that have become available since that time.

The MGB modification acts as a molecular clamp, increasing the affinity of the probe for its complementary sequence and hence increasing the T_m. In this way, the qualities of the probe can be modified without changing sequence or length. This has been previously shown to make assays more tolerant of mismatches between the probe and target sequence and can raise the T_m of the probe to around 10 °C higher than the T_m of the primers (Garson et al., 2012; Nolan et al., 2006). While the effect on detection of the serotype 4 samples was dramatic in terms of preventing false-negatives, the detection of other serotypes was not impaired, and indeed, appeared to be improved by a mean of about one cycle compared to the non-MGB probe. There is therefore a clear case for laboratories that use the 'original' assay to switch to using an MGB-modified probe, which will increase reliability of detection of serotype 4 samples without adversely affecting detection of serotypes 1, 2 and 3 or compromising specificity.

In terms of the travel history of patients from whom Samples #1-21 derived, more than half had been to Thailand or Indonesia (including Bali), in line with previous data from travellers from Europe with dengue (Neumayr et al., 2017) (Supplementary Table 2). Samples #22-32 were from patients resident in Brazil. Out of the 32 total samples, seven were found to be serotype 4 and of the 21 European samples, two were found to be serotype 4. This is also broadly in line with sentinel European laboratory data that found this serotype to be the least commonly isolated (Neumayr et al., 2017). Both of the sequenced viruses isolated from these samples had two mismatches with the probe (out of 20 nucleotides), in common with all serotype 4 sequences available in a public database that covered this section of the genome. Whilst these mismatches might not prevent very highly viraemic samples from being detected, they would be likely to cause a false-negative result to be returned if the level of viraemia was moderate or low.

Although all archived samples from UCLH analysed here had been reported as dengue RNA-positive when tested as fresh samples, they had been stored at -20 °C for up to 14 months (average of 10 months) and due to clinical requirements, had been subjected to freeze thaws before commencing the present study. The Brazilian samples were stored at -80 °C after collection. We speculate that the storage at -20 °C, and/or a reduced input level of sample through both reduced extraction volume and reduced sample input to the RT-qPCR assay, may account for the failure of both the modified and the 'original' assays to detect Samples #1, #11, and #30 and for the difficulty in obtaining amplicons for sequencing from Samples #1 and #5.

5. Conclusion

In conclusion, we have shown that an MGB-modification of a TaqMan probe from a widely used RT-qPCR assay can improve the sensitivity of the assay for dengue serotype 4 samples, which contain two mismatches with respect to the probe sequence. In light of this, we would suggest that laboratories that use the 'original' assay to detect dengue viraemia use the MGB-modified probe to avoid false-negative results from infections with dengue serotype 4.

Author contributions

EG, EN and JG designed the study. EG, JH, RF and PS performed experiments. EN provided samples. EG and JG analysed data and wrote the paper. All authors had the opportunity to review the manuscript before submission.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.03.006.

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