1 **Minor groove binder modification of widely used TaqMan hydrolysis probe for detection**

2 **of dengue virus reduces risk of false-negative real-time PCR results for serotype 4**

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21

22 **Summary**

23 Dengue is a vector-transmitted viral infection that is a significant cause of morbidity and

24 mortality in humans worldwide, with over 50 million apparent cases and a fatality rate of 2.5% of

25 0.5 million severe cases per annum in recent years. Four serotypes are currently co-circulating.

26 Diagnosis of infection may be by polymerase chain reaction, serology or rapid antigen test for

1

27   NS1. Both pan-serotype and serotype-specific genome detection assays have been described,

28   however, achieving adequate sensitivity with pan-serotype assays has been challenging.

29   Indeed, as we show here, inspection of components and cycling parameters of a pan-serotype

30   RT-qPCR assay in use in laboratories worldwide revealed insufficient probe stability to

31   accommodate potential nucleotide mismatches, resulting in false-negatives. A minor-groove

32   binder (MGB)-modified version of the probe was designed and its performance compared with

33   that of the original probe in 32 samples. Eight of the samples were undetected by the original

34   probe but detected by the MGB modified probe and six out of seven of these that could be

35   serotyped belonged to serotype 4. Sequencing of the region targeted by the probe in these

36   samples revealed two mismatches which were also universally present in all other serotype 4

37   sequences in a public database. We therefore recommend adoption of this MGB modification in

38   order to reduce the risk of false-negative results, especially with dengue serotype 4 infections.

39

40   **Keywords**: dengue, TaqMan, false-negative, sensitivity, serotype 4, minor groove binder

41

42   **Abbreviations:**

43   MGB = minor groove binder

44   qPCR = quantitative polymerase chain reaction

45   RT-qPCR = reverse transcription quantitative polymerase chain reaction

46   C_q = quantification cycle (sometimes referred to as C_t or threshold cycle)

47

48   **1. Introduction**

49

50   Dengue, caused by a member of the family *Flaviviridae,* is the most common arboviral infection

51   worldwide. Globally, case incidence is thought to be 50-100 million per annum, though the true

52   number may be higher as not all infections are apparent and misclassification may occur in

2

53 regions with a high burden of febrile illness (Bhatt et al., 2013; Stanaway et al., 2016). Clinical

54 infections range widely in severity from asymptomatic to dengue haemorrhagic fever and death.

55 Other than supportive therapy no specific treatment is available and as yet there is no effective

56 vaccine universally recommended for those at risk (Aguiar et al., 2016; Halstead, 2017; World

57 Health Organization, 2017). A vaccine would need to prevent infection in the infection-naïve,

58 such as travellers to dengue-endemic regions, and neonates in endemic countries, as well as

59 those who have experienced primary infection. Recommendations for the only licensed vaccine

60 currently available (the Pasteur CYD-TDV) are limited to those aged 9-45, who are likely to have

61 previously been exposed to infection (Sridhar et al., 2018; Wilder-Smith et al., 2018).

62

63 Four serotypes co-circulate in most endemic countries, and these are genetically variable

64 (Costa et al., 2012). Initial infection with any serotype gives type-specific lifelong immunity,

65 however, prior infection with one serotype can predispose to a more serious episode on

66 subsequent infection with a different serotype (Halstead et al., 1970; Katzelnick et al., 2017).

67

68 Diagnosis of dengue is on clinical suspicion based on symptoms and travel history; in addition a

69 wide variety of laboratory methods are in current use. Blood samples may be analysed for

70 RNA, IgM and IgG or NS1 antigen; the presence of RNA and/or IgM suggesting acute infection.

71 Point of care diagnostic tests are also available, although the sensitivity of these tests when

72 performed in the field has been variable (Pang et al., 2017; Sa-ngamuang et al., 2018).

73 Molecular techniques are widely used, though the resource requirements for these tests mean

74 that their implementation may not be feasible in resource-limited settings. Molecular tests to

75 detect viraemic infections include qualitative/nested PCR, reverse-transcription quantitative

76 PCR (RT-qPCR) and isothermal amplification methods (Domingo et al., 2010; Poersch et al.,

77 2005). Some tests seek to differentiate serotypes for epidemiological purposes, while others

78 are intended to be pan-serotype. Serotyping of dengue infection has limited applicability in

3

79   immediate clinical management. However, epidemiological surveys are important to assess

80   whether a predominant serotype in a locale is supplanted, bringing with it the risk of secondary

81   infections and more severe consequences.

82

83   A widely cited pan-serotype RT-qPCR assay published in 2002 (Drosten et al., 2002) that is

84   apparently used by laboratories in many countries (Domingo et al., 2010) was identified in a

85   literature search and evaluated using archived samples. An initial failure to detect one of four

86   samples tested led to a closer inspection of this assay and, as a result, to its improvement

87   through minor groove binder (MGB) modification of its TaqMan probe (i.e. 5' hydrolysis probe).

88   The MGB-modified and un-modified probe (herein designated the 'original' probe) assays were

89   tested on a larger group of samples and two sequence mismatches between the 'original' probe

90   and dengue serotype 4 sequences were identified. The MGB modification was thus shown to

91   prevent false-negative results being generated with serotype 4 samples.

92

93

94   **2. Materials and Methods**

95

96   *2.1 Samples*

97   Samples #1-21: Archived EDTA-plasma samples from University College London Hospital that

98   had been submitted for arbovirus testing between January and August 2016 were reviewed. 21

99   samples that had been reported elsewhere as positive for dengue virus RNA were identified and

100   retrieved from storage at -20 °C The total storage time at -20 °C varied between 5 and 14

101   months prior to nucleic acid extraction (mean 10 months). Samples #22-32: Archived samples

102   were stored at -80 °C since 2012 (Samples #22, 26-30), 2016 (Samples #23-25) or 2018

103   (Samples #31-32). All samples from Brazil were processed at the Flavivirus Laboratory,

104   Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.

4

105

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 100µl was available, which was supplemented with 100µl

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 Figure 1, synthesized by Sigma, Haverhill, UK); either a 6-carboxyfluorescein (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 (Figure 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 mins, 95 °C 20 secs, then 45 cycles of 95 °C 3 secs,

5

131   60 °C 1 min. Negative controls (H$_2$O) were run on every plate. Data were analysed using the

132   ABI 7500 software v2.0.6.

133

134   *2.5 Synthesis of RNA by* in *vitro transcription*

135   Transcripts used to generate the dengue RNA dilution series were synthesised using the T7

136   MegaScript kit (Life Technologies) and PCR amplicons as template, amplified from Sample #14

137   (serotype 2). RNA from this clinical samples was initially reverse transcribed using Superscript

138   IV and random hexamers (both Life Technologies). Amplified DNA template was produced by

139   nested PCR using primers EG273/EG250 then EG295/EG296 (Table 1) and Platinum SuperFi

140   polymerase (cat. no. 12351010, Life Technologies). Transcripts were cleaned up using the

141   MegaClear kit (Life Technologies) and the yield assessed using the Nanodrop One[C] (Thermo

142   Scientific, Paisley, UK).

143

144   *2.6 Sequencing*

145   RNA extracts were reverse transcribed using Superscript IV with reverse primer EG244 (Table

146   1) (Samples #2, #3, #6-17 and #19-21) according to the manufacturer's instructions. Where

147   amplification by nested PCR was initially unsuccessful, random hexamers were used for the

148   reverse transcription step (Samples #4 and #18). Nested PCR reactions were carried out on all

149   samples using the primers in Table 1 and Platinum SuperFi Taq polymerase according to the

150   manufacturer's instructions in 25 µl (first round) and 50 µl (second round) reactions. After the

151   second round, 5 µl PCR reaction product was analysed by agarose gel electrophoresis to

152   ensure unique bands of the correct size had been obtained, and DNA from the remaining 45 µl

153   reaction product was purified by the QIAquick PCR purification kit (Qiagen). Bi-directional

154   Sanger sequencing of the purified amplicon was carried out by Genewiz Ltd. (Essex, UK).

155

156   *2.7 Serotyping*

6

157   To assign serotype to Samples #1-21, the segment that was sequenced (corresponding to

158   10467-10660, numbering according to GenBank AF038403) was used as input for a nucleotide

159   BLAST search (www.ncbi.nlm.nih.gov/BLAST) and the serotype of the closest matched dengue

160   sequences from the database noted. None of the sequences from this study matched more

161   than one serotype. Samples #22-32 were assigned a serotype using the method described in

162   (Santiago et al., 2013).

163

164   *2.8 Sequence analyses to explore probe/target mismatches in serotype 4 samples*

165   Analysis of .ab1 files, alignment to template and inspection of sequences was carried out using

166   DNADynamo (Blue Tractor Software Ltd).

167

168   To create a multiple sequence alignment, all whole virus genome sequences from samples

169   taken from a human host, classified as dengue serotype 4 that included data between

170   nucleotides 10635 and 10701 (the qPCR amplicon from (Drosten et al., 2002), numbering

171   according to AF038403) were downloaded from the Virus Pathogen Database and Analysis

172   Resource (www.viprbrc.org), aligned using MUSCLE via the online interface, then opened in

173   Seqotron (Fourment and Holmes, 2016) for further analysis. This dataset comprised 192

174   genomes. The section encompassing the 'original' assay primers and probe was selected and

175   extraneous sequence data removed, as well as any genomes with missing data for this section.

176   The resulting FASTA file contained 147 sequences from geographically diverse locations. To

177   create Supplementary Table 1, the percent identity of each nucleotide at each position was

178   calculated using consensus mode in DNADynamo.

179

180   **3. Results**

181

182   *3.1 A widely used pan-serotype RT-qPCR assay can yield false-negative results*

       7

183   In 2010, an international external quality assessment exercise for molecular diagnostics of

184   dengue was reported, in which participating laboratories were able to compare the performance

185   of their assays (Domingo et al., 2010). The most widely used published assay in that

186   international assessment was the RT-qPCR pan-serotype assay described by Drosten et al. in

187   2002 (Drosten et al., 2002). This assay was therefore selected for further evaluation. The

188   primer and probe binding sites for this assay are shown in Figure 1, and are located in the 3'

189   terminal non-coding region of the genome, where there is a high degree of sequence

190   homogeneity between serotypes. In order to encompass the genetic diversity of serotype 4

191   there are two reverse primers (R1 and R2) in the mix at a 1:1 ratio.

192

```
         10630                                                                        10706
           ↓                   F                        P              R1              ↓
                 GGATAGACCAGAGATCCTGCTGT        CAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATG
Serotype 1   CGCTGGGAGAGACCAGAGATCCTGCTGTCTCTACAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATGGTGCT
Serotype 2   CGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATGGTGCT
Serotype 3   CGCTGGGAGAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATGGTGCT
Serotype 4   CGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCGAGATGGATTGGTGTT
                                                                 GAGCGCCGCAAGATGGATTG
                                                                        R2
```

193

Figure 1. Representatives of the four dengue serotypes showing primer/probe binding

sites.

196   The sequences are: serotype 1 Hawaii (EU848545); serotype 2 New Guinea C (AF038403);

197   serotype 3 Philippines H87 (M93130); serotype 4 Philippines H241 (AY947539). F = forward

198   primer; P = probe; R1 = reverse primer 1; R2 = reverse primer 2. The genomic numbering

199   refers to AF038403 (serotype 2). The black shading represents the single nucleotide overlap

200   between the probe and the reverse primer.

201

202

203   Preliminary tests on four known RNA-positive samples (sample numbers #18 - #21)

204   unexpectedly gave one false-negative result. The properties of the primers and probe were

8

205   therefore investigated further. The melting temperatures (T_m) of the oligonucleotides were

206   calculated by several alternative methods as shown in Table 2. These calculations revealed

207   that the T_m of the probe was approximately the same as that of the primers. This was a

208   surprising finding given that TaqMan probe design guidelines state that the probe T_m should be

209   about 10 °C higher than that of the primers (Bustin, 2000). If the probe T_m is too low then stable

210   binding may not occur before displacement and hydrolytic cleavage by the DNA polymerase

211   during amplification. An inadequate T_m difference between that of the probe and that of the

212   primers would be likely to make probe hybridization unstable in the face of even minor

213   sequence mismatch.

214

215   *3.2 MGB-modified probe has optimised characteristics and reduces risk of false-negatives*

216   Heterogeneity in the bases upstream of the probe sequence, and the proximity of the reverse

217   primer downstream of the probe, meant that extending the probe to increase its T_m was not an

218   option. To optimise the assay we therefore elected to synthesise an alternative probe of

219   identical sequence and length but with a 3' minor groove binder (MGB) moiety. This stabilises

220   the duplex, increasing the T_m of the probe by between 10 and 20 °C, without lengthening it

221   (Garson et al., 2012). This alternative probe was synthesised (EG242) and the assay was

222   designated 'MGB-modified'.

223

224   To investigate whether adding the MGB modification to the probe made a difference to assay

225   characteristics (PCR efficiency and detection limit) a dilution series of a sample with a high viral

226   load (Sample #14) was prepared. Ten-fold serial dilutions were made and analysed by both the

227   MGB-modified and the 'original' assays in triplicate (Figure 2a). The fitted regression line for the

228   MGB-modified probe showed detection at approximately 1 C_q lower than the 'original' probe

229   across most of the dilution range (Figure 2a). The slopes, -3.33 and -3.17, representing a

9

230   nominal PCR efficiency of ~99.7 % and ~106.6 %, and the R squared values (0.998 and 0.994)

231   for the MGB-modified and 'original' assays respectively, were not significantly different. The

232   dilution series was repeated a second time with RNA transcript generated from Sample #14 to

233   assess the minimum copy number detectable by the two assays (Figure 2b). Again, fitted

234   regression lines for the MGB-modified probe showed detection at approximately 1 C_q lower than

235   the 'original' probe across most of the dilution range, equivalent to approximately a 2-fold

236   increase in sensitivity (Figure 2b). The limit of qualitative detection for both assays was

237   approximately 100 RNA copies per reaction, though the limit of quantitation was ~1000 copies.

238   Similar slopes, nominal PCR efficiencies and R squared values were seen (-3.26 and -3.24,

239   ~102.5 % and ~103.4 %, 1.000 and 0.999 for the MGB-modified and 'original' assays

240   respectively) (Figure 2b).

241

a.

b.

242
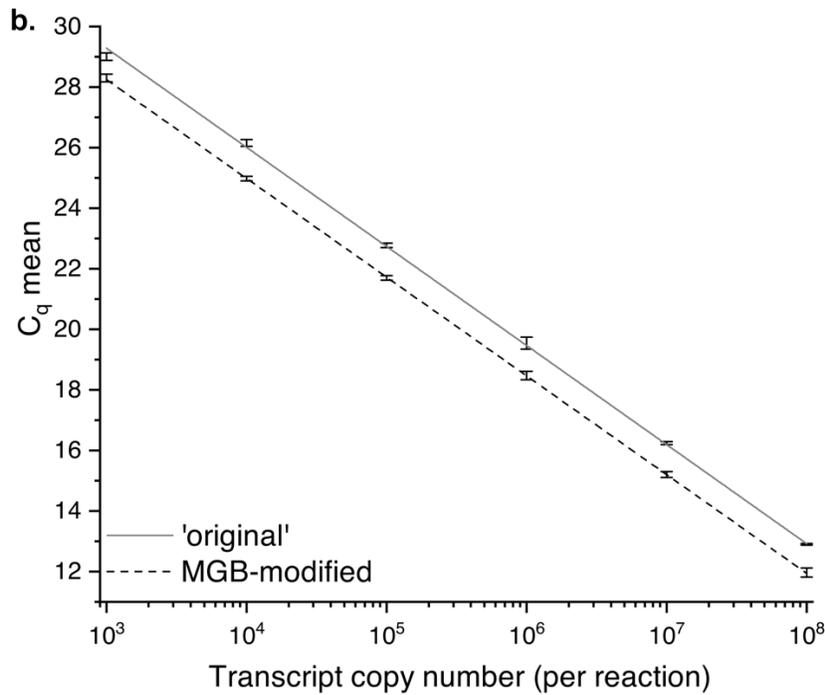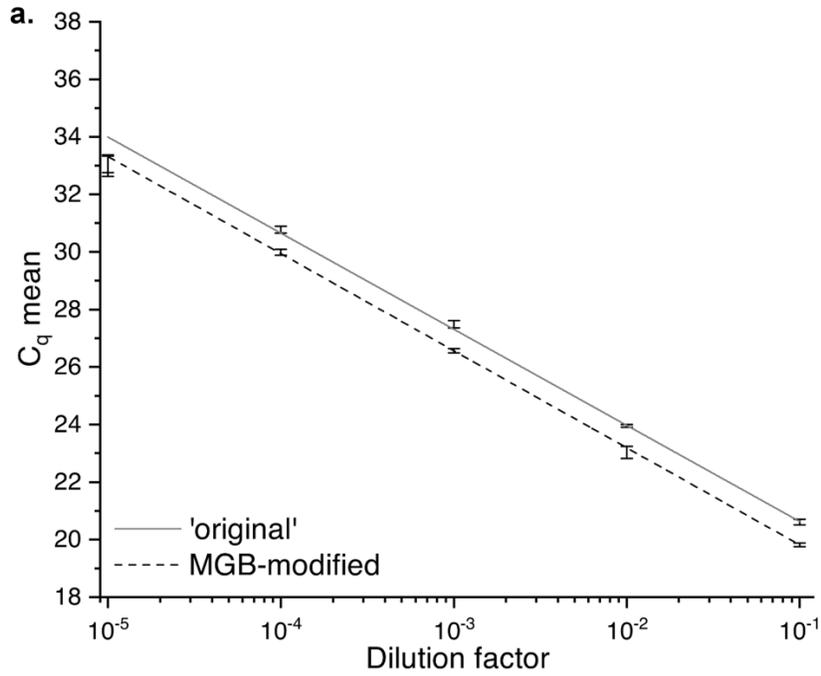
Figure 2. Amplification of samples generated from ten-fold serial dilutions.

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

11

247   from 10^{-1} to 10^{-8}. The nominal PCR efficiency, R^2, and slope for the MGB-modified assay were

248   99.7 %, 0.998 and -3.33, and for the 'original' assay were 106.6 %, 0.994 and -3.17. b) Ten-fold

249   serial dilutions were made with RNA transcript generated from Sample #14 template. Between

250   10^0 and 10^8 copies per sample were tested; for both assays, only samples with 10^2 copies or

251   more yielded a positive result. The nominal PCR efficiency, R^2, and slope for the MGB-modified

252   assay were 102.5 %, 1.000 and -3.26, and for the 'original' assay were 103.4 %, 0.999 and -

253   3.24.

254

255   To compare the performance of the MGB-modified and 'original' assays on a wide variety of

256   samples, 32 archived samples reported elsewhere as dengue RNA-positive were tested.

257   Overall, 29 of 32 samples were found to be positive with the MGB-modified assay but only 21 of

258   32 with the 'original' assay. Sample #5 yielded one positive well out of three repeats and

259   Sample #25 yielded two positive wells out of three repeats with the MGB-modification but zero

260   of three were positive with the 'original' probe for both these samples. For every positive

261   sample, the C_q with the MGB-modified assay was approximately one cycle lower than generated

262   by the 'original' assay (Figure 3 and Table 3).

263

'original'
MGB-modified

undetectable above this line*
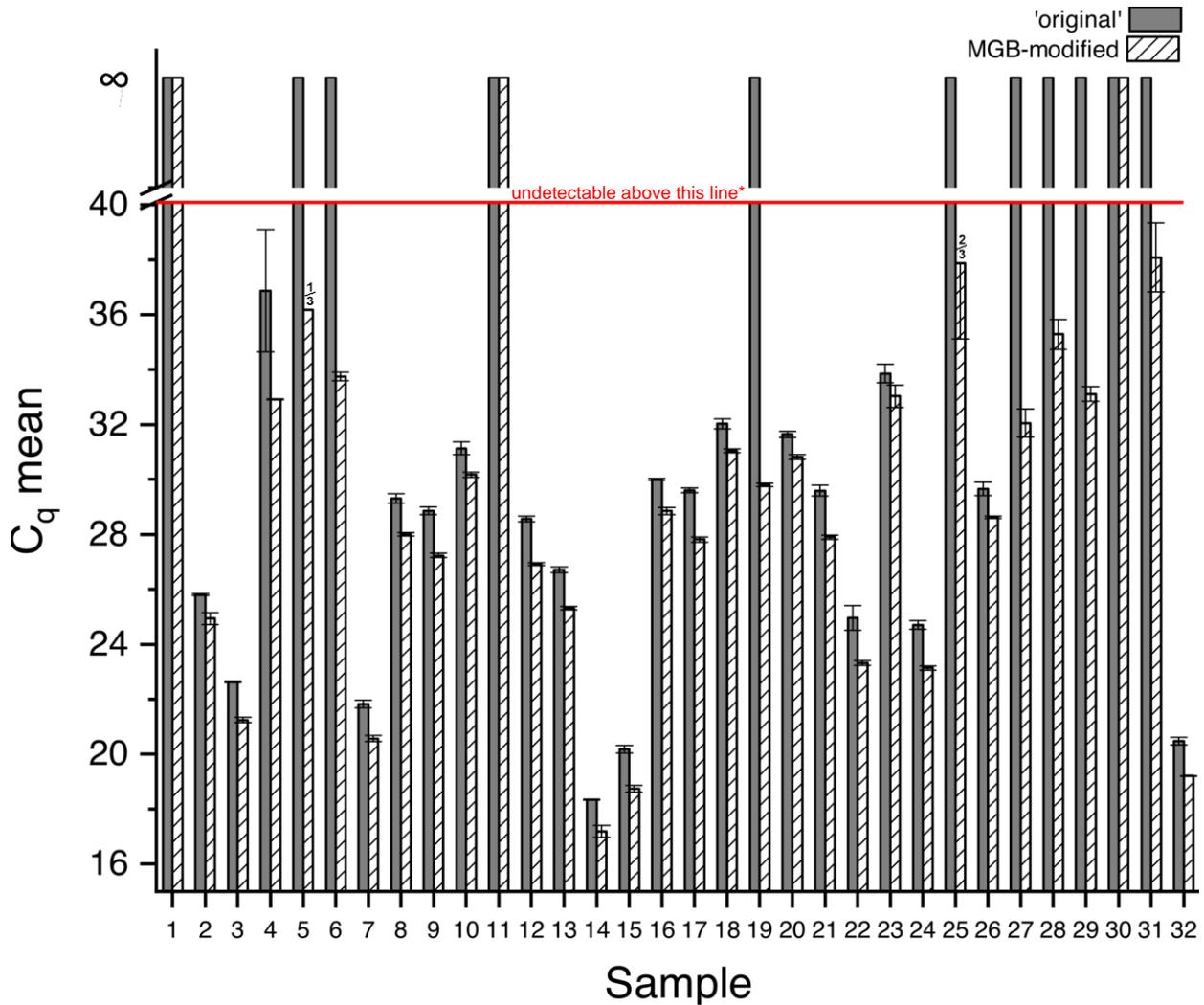
Figure 3. A comparison of C_q values obtained with 32 archived samples assayed by the MGB-modified and the 'original' assay.

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.

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

13

275 on serotype 2, GenBank AF038403) was amplified by nested PCR and sequenced for 19 of

276 Samples #1-21, yielding the serotype data shown in Table 3. Two samples, samples #1 and #5,

277 did not yield amplicons for sequencing. Samples #22-32 were serotyped by the molecular

278 assay described in (Santiago et al., 2013) (Table 3). The samples that were undetectable with

279 the 'original' assay but were detectable in triplicate with the MGB-modified assay (i.e. Samples

280 #6, #19, #27, #28, #29 and #31) were all serotype 4, suggesting that this serotype (an early

281 evolutionary breakaway from the lineage that led to serotypes 1-3, (Costa et al., 2012)) may be

282 particularly poorly detected by the 'original' version of this assay. To understand why the

283 'original' probe without the MGB modification may not have detected these samples, the region

284 covering the qPCR amplicon was sequenced, corresponding to 10479-10701 (numbering

285 according to GenBank AF038403) from Samples #6 and #19. These data were appended to

286 the sequencing data generated for serotyping (10467-10660, Table 3) and are shown in Figure

287 4. Sample #14, a serotype 2 sample which was detected by both the MGB-modified and

288 'original' probes was also sequenced in this region to provide a control for comparison. The

289 sequencing clearly demonstrated two mismatches in the probe binding region, for serotype 4

290 samples only. The destabilizing effect of these mismatches would have been negated by the

291 MGB moiety in the modified probe.

292

```
DENV-14 CCATGGAAGCTGTACGCATGGCGTAGTGGACTAGCGGTTAGAGGAGACCCCTCCCTT-AC----AAATCGCAGCAACAATGGGGGCCC-AAGGTGAGATGAAGCTGTAGTCTCACTGGAAGGAC
 DENV-6 CCATGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTCCCATCACTGACAAAACGCAGCAAAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTTCTGGTGGAAGGAC
DENV-19 CCATGGAAGCTGTACGCGTGGCATATTGGACTAGCGGTTAGAGGAGACCCCTCCCATCACTGACAAAACGCAGCAAAAAAGGGGGCCCGAAGCCAGGAGGAAGCTGTACTTCTGGTGGAAGGAC


                                                  ↓                    ↓   ↓
 DENV-14 TAGAGGTTAGAGGAGACCCCCCCGAAATAAAAAACAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCCTCAGCATCATTCCAGGCACAGAACGCCAGAAAATGGAATG
  DENV-6 TAGAGGTTAGAGGAGACCCCCCAACACAAAAA-CAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTG
 DENV-19 TAGAGGTTAGAGGAGACCCCCCAACACAAAAA-CAGCATATTGACGCTGGGAAAGACCAGAGATCCTGCTGTCTCTGCAACATCAATCCAGGCACAGAGCGCCGCAAGATGGATTG
                                                  F                    P          R1 & R2
```

293

294 **Figure 4. Sequence alignment of Samples #6, #14 and #19**

295 The amplicon covering the section 10467-10701 (numbering according to AF038403) including

296 binding sites for the primers and probe is shown for Samples #6, #14 and #19. A section

297 between 10479-10701 was amplified, sequenced and appended to data from 10467-10660,

14

298   used to serotype all samples (Table 3).   Samples #6 and #19 are serotype 4; Sample #14 is the

299   high titre sample used to generate Figure 2 and is serotype 2. Mismatches to the assay primers

300   and probe are shown with arrows and highlighted in bold. Outside of the primer/probe binding

301   regions (delineated by horizontal lines), sequence differences between the samples are also

302   highlighted in bold. There are two different reverse primers (R1 and R2 mixed 1:1) therefore

303   although there are five variant sites between the two serotypes in this region, all have ~100%

304   homology with one of the reverse primers.

305

306   *3.4 Two mismatches in probe binding site are present in all available serotype 4 sequences*

307   We investigated whether the 'original' probe mismatches with serotype 4 samples found in this

308   study are also found in previously sequenced samples from around the globe. A multiple

309   sequence alignment was prepared from all available dengue serotype 4 genomes from the Virus

310   Pathogen Database and Analysis Resource (www.viprbrc.org) with sequence data covering the

311   entire forward primer and reverse primer binding region (n = 147 sequences). This

312   demonstrated that the two mismatches found in the probe-binding region of Samples #6 and

313   #19 in this study are in fact universally present in all available dengue serotype 4 genomes

314   (Supplementary Table 1).

315

316   *3.5 No false positive signals are seen in dengue-negative samples*

317   To ensure that the MGB-modified probe maintained its specificity and did not yield false positive

318   results with other members of the family *Flaviviridae*, 16 samples from patients with hepatitis C

319   and 22 samples from patients with Zika virus were tested *in vitro* by qPCR. None of the

320   samples tested by qPCR yielded false-positive results. An *in silico* analysis using NCBI Primer-

321   BLAST also failed to find any cross-reactivity with the human genome, or with the genomes of

322   other members of the *Flaviviridae* including the following viruses: hepatitis C, chikungunya, Zika

323   and yellow fever.

15

324

4. Discussion

326

327 The incidence of dengue is slowly rising worldwide and vigilance is increasingly required in non-

328 endemic countries where dengue is imported by returning travellers (Neumayr et al., 2017;

329 Stanaway et al., 2016). As yet, there is no vaccine that can be universally recommended and

330 no cure, and morbidity and mortality remains high in countries that can least afford the dual

331 burdens of healthcare costs and loss of human capital. A clear and accurate understanding of

332 incidence is key to obtaining a full epidemiological picture of the disease, and accurate

333 diagnostic tools are essential. In this study, we have shown how the sensitivity of a pan-

334 serotype RT-qPCR assay, employed to diagnose viraemic dengue infection can be significantly

335 improved by MGB modification of the TaqMan hydrolysis probe. Dengue serotype 4 comprises

336 a significant minority of infections worldwide, and therefore the diagnostic tools chosen by

337 laboratories must reliably detect this serotype. In the quality assessment study of 2010

338 (Domingo et al., 2010), samples of dengue serotype 4 were included at a level of 10^5 genome

339 equivalents per mL, two orders of magnitude higher than the minimum detection requirement to

340 achieve an 'acceptable' classification in this exercise. Despite this relatively high level, 14/46

341 laboratories failed to classify the serotype 4 sample as dengue positive. Several pan-serotype

342 dengue assays proposed in the literature include more than one primer (or probe) to ensure

343 detection of dengue serotype 4 (Alm et al., 2014; Dumoulin et al., 2008; Santiago et al., 2013).

344 Interestingly, the modification to the (Drosten et al., 2002) assay proposed by Dumoulin et al. is

345 the addition of a second probe identically located but with two nucleotides changes to map

346 exactly the dengue serotype 4 consensus sequence (Dumoulin et al., 2008). When the

347 (Drosten et al., 2002) assay was originally published in 2002, MGB-probes were only just

348 becoming available; their widespread use not common until a few years later. It is therefore

16

349 appropriate to update this assay using technological advances that have become available

350 since that time.

351

352 The MGB modification acts as a molecular clamp, increasing the affinity of the probe for its

353 complementary sequence and hence increasing the T_m. In this way, the qualities of the probe

354 can be modified without changing sequence or length. This has been previously shown to make

355 assays more tolerant of mismatches between the probe and target sequence and can raise the

356 T_m of the probe to around 10 °C higher than the T_m of the primers (Garson et al., 2012; Nolan et

357 al., 2006). While the effect on detection of the serotype 4 samples was dramatic in terms of

358 preventing false-negatives, the detection of other serotypes was not impaired, and indeed,

359 appeared to be improved by a mean of about one cycle compared to the non-MGB probe.

360 There is therefore a clear case for laboratories that use the 'original' assay to switch to using an

361 MGB-modified probe, which will increase reliability of detection of serotype 4 samples without

362 adversely affecting detection of serotypes 1, 2 and 3 or compromising specificity.

363

364 In terms of the travel history of patients from whom Samples #1-21 derived, more than half had

365 been to Thailand or Indonesia (including Bali), in line with previous data from travellers from

366 Europe with dengue (Neumayr et al., 2017) (Supplementary Table 2). Samples #22-32 were

367 from patients resident in Brazil. Out of the 32 total samples, seven were found to be serotype 4

368 and of the 21 European samples, two were found to be serotype 4. This is also broadly in line

369 with sentinel European laboratory data that found this serotype to be the least commonly

370 isolated (Neumayr et al., 2017). Both of the sequenced viruses isolated from these samples

371 had two mismatches with the probe (out of 20 nucleotides), in common with all serotype 4

372 sequences available in a public database that covered this section of the genome. Whilst these

373 mismatches might not prevent very highly viraemic samples from being detected, they would be

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374   likely to cause a false-negative result to be returned if the level of viraemia was moderate or

375   low.

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377   Although all archived samples from UCLH analysed here had been reported as dengue RNA-

378   positive when tested as fresh samples, they had been stored at -20 °C for up to 14 months

379   (average of 10 months) and due to clinical requirements, had been subjected to freeze thaws

380   before commencing the present study. The Brazilian samples were stored at -80 °C after

381   collection. We speculate that the storage at -20 °C , and/or a reduced input level of sample

382   through both reduced extraction volume and reduced sample input to the RT-qPCR assay, may

383   account for the failure of both the modified and the 'original' assays to detect Samples #1, #11,

384   and #30 and for the difficulty in obtaining amplicons for sequencing from Samples #1 and #5.

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386   **5. Conclusion**

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388   In conclusion, we have shown that an MGB-modification of a TaqMan probe from a widely used

389   RT-qPCR assay can improve the sensitivity of the assay for dengue serotype 4 samples, which

390   contain two mismatches with respect to the probe sequence. In light of this, we would suggest

391   that laboratories that use the 'original' assay to detect dengue viraemia use the MGB-modified

392   probe to avoid false-negative results from infections with dengue serotype 4.

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