Development and validation of oligonucleotide sets for sequencing, real time detection and differentiation of re-emerged sylvatic Dengue virus 2 strains

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Abstract

Dengue virus (DENV) is one of the most prevalent arboviral threats worldwide. The virus is associated with a high health and economic burden mainly in tropical and subtropical regions. Available molecular tools however fail to correctly serotype and sequence sylvatic DENV-2 (DENV-2/GVI) which in known to circulate in forests in West Africa and Malaysia. The recent emergence of human case linked to this virus variant in Southern Senegal raises concerns about the correct detection and

characterization of the virus for public health purposes. Here we develop and validate new sets of oligonucleotides to detect, discriminate and sequence DENV-2/GVI. Validations were carried out using epidemic DENV and sylvatic DENV-2 strains from the biobank of the WHO collaborating Center for Arboviruses and Haemorrhagic fevers. The presented approaches showed good performance to specifically detect sylvatic DENV-2 in both singleplex and multiplex PCR with other DENV serotypes respectively with a limit of detection of 68.85 and 133.21 RNA copies/reaction at 0.95 probability in a probit analysis. Additionally, developed tilling PCR primers yield a better genome coverage ranging from 93.9 to 95.1 % for all processed DENV-2/GVI strains both on Illumina and Nanopore platforms and outperform previous schemes to efficiently amplified DENV-2/GVI strains. In summary the developed oligonucleotides will contribute to improving DENV surveillance and genomic epidemiology in endemic areas.

Background

Dengue infection is a major public health threat worldwide, according to the World Health Organization up to third of the global population are at risk for dengue infection. With the observed upsurge of dengue cases there is an urgent need to enhance virus variant genomic surveillance. In contrast to South American and Asian regions genomic data about circulating DENV strains in Africa are scarce (1,2). African DENV sequences represent less than 1 % of global DENV Genbank entries (2). The real genetic diversity of circulating DENV strains in Africa is unknown (2–4).

In Senegal real time detection and subsequent genomic characterization of circulating DENV strains has been performed since 2017 from RT-qPCR DENV positive samples (4). This strategy lead to the identification of multifoci and multiserotype circulation of DENV around the country (4). DENV 1-4 circulation is expanding worldwide therefore serotyping of circulating viral variants is required for the purpose of reliable epidemiology and surveillance (5).

For this purpose, a plethora of systems is available (6,7), however since DENV as many other RNA viruses is prone to fast viral evolution and erosion of oligonucleotide target sites can occur (8).

In November 2021, we identified a DENV positive RNA sample from Sare Yoba (Kolda region) with a high viral load in a panDENV assay (< Ct 25) yielding no serotyping PCR amplification results using both the PSR dengue typing kit (Tibmolbiol) and the CDC dengue typing kit (9). Retrospective screening of a batch of DENV positive samples collected during an epidemic in the Kédougou area (Southern Senegal) lead to the same DENV serotyping failure (10).

It had previously been observed that tilling PCR based sequencing of previously untyped DENV positives using published DENV-2 primers schemes (11) tentatively lacked a good coverage across the DENV genome. This observation in addition to the fact that recently identified sylvatic DENV-2 strains caused a huge epidemic in southern Senegal (10) invoked the urgent need for sensitive and specific oligonucleotides for both molecular detection and sequencing of this re-emerging viral genotype.

Herein we evaluate the performance of newly designed RT-qPCR oligos (sensitivity and specificity) both in singleplex and in multiplex PCR. We additionally estimate the performance of specific Sylvatic DENV-2 tilling PCR primers, in comparison to previously published DENV-2 primer schemes.

Methods

- ✓ **Sample selection and nucleic acid extraction**

For the purpose of this study 9 previously classified sylvatic DENV-2 strains and 28 epidemic dengue strains belonging to epidemic DENV 1-3 strains were retrieved from the WHO collaborating centre for arboviruses and haemorrhagic fever viruses biobank at Institut Pasteur de Dakar (IPD) this collection was supplemented with viral isolates from the newly identified contemporary sylvatic DENV-2 identified in Sare Yoba and

serum samples collected during DENV-2/GVI epidemic in Kédougou. Following the manufacturer`s recommendation, RNA was extracted from selected dengue samples (Table S3) using Qiagen Viral RNA mini kit (Qiagen, Hilden, Germany). Extracted RNA was eluted in a final volume of 60µl.

- ✓ **Design of the Sylvatic DENV-2 tilling PCR primers**

After the initial tiling PCR failure, we downloaded 15 closely related full genome sequences of a partial NS5 sequence of the newly identified sylvatic DENV-2 strain from Kolda based on previous BLAST results. The obtained dataset (*n = 15*) was aligned using MAFFT (12), manually curated using Aliview (13), trimmed to conserve coding polyprotein sequence. Using this curated dataset tilling PCR primers were designed using the primal scheme tool (https://primalscheme.com/) ; amplicon length was fixed to 900bp and amplicon overlap to 50 bp. All newly designed primers (Table 2) were ordered at the concentration of 5nM from Tibmolbiol (Berlin, Germany).

- ✓ **Tilling Multiplex-PCR amplifications: Newly designed scheme vs published DENV-2 scheme**

To sequence sylvatic DENV-2 strains viral RNAs were reverse transcribed into cDNAs using LunaScript RT SuperMix (NEB #E3010) as previously described (14). Briefly, 8 µL template RNA and 2 µL LunaScript RT SuperMix (New England Biolabs #E3010) were mixed and put in the following thermal conditions: 25 °C for 10 min, followed by 50 °C for 10 min and 85 °C for 5 min. The viral genome was amplified with either short 400 bp amplicon oligonucleotides previously published for DENV-2 (5) or long-range 900bp amplicon nucleotides designed during this study in addition to a previously available scheme using amplicon of the same length (11). The tiling PCR amplification reactions were performed in separate reactions. The cDNAs were also used for direct

amplification using primers at 10 µM and the Q5® High-Fidelity 2X Master Mix (New England Biolabs#M0494X) with the following thermal cycling protocol: 98 °C for 30 seconds, 35 cycles of: 95°C for 30 seconds, 65°C for 5 minutes, and a cooling step at 4°C.

- ✓ **Illumina / Nanopore sequencing and analysis**

Sequencing libraries were synthesized by tagmentation of the 400 and 900 bp amplicons using the Illumina DNA Prep kit and the IDT® for Illumina PCR Unique Dual Indexes following manufacturer's recommendations and as previously described (15). After a cleaning step with the Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN), libraries were quantified using a Qubit 3.0 fluorometer (Invitrogen Inc., Waltham, MA, USA) for manual normalization before pooling in the sequencer. Cluster generation and sequencing were conducted in an Illumina iSeq100 instrument with ISeq 100 i1 Reagent v2 (300-cycle). Additionally, a set of DENV-2/GVI human serum samples collected during surveillance in southern Senegal were sequences using Nanopore approach as previously described (14).

Raw fastq data were collected in fastq format from the Illumina machine and were analysed using CZID platform (http://czid.org,) accessed *on 18 July 2023*. Nanopore raw fastq data were analyzed using Genome detective tool as previously described (14).

After assembly fasta consensus files and associated assembly files (depth of coverage, sequencing statistics, assembly reports) were downloaded and analyzed.

- ✓ **Sylvatic DENV-2 RT-qPCR oligos design and ivRNA standard synthesis**

Newly obtained sylvatic DENV-2 sequences (n = 9) were supplemented with a backbone dataset containing fifteen sylvatic DENV-2 sequences and 13 epidemic dengue sequences to design primers and probe for the specific detection of sylvatic DENV-2 strains. The Obtained dataset was aligned using MAFFT, manually inspected

using Aliview (13) a conserved region of 79 bp among all used sylvatic DENV-2 sequences was identified and oligonucleotides were designed (Table 1) and checked for unspecific hybridization by BLAST (Basic Local Alignment Search Tool) (16).

For sensitivity tests, all oligonucleotides and target in vitro RNA (ivRNA) were ordered from Tibmolbiol (Berlin, Germany). Transcribed RNA was delivered at a concentration of 10^{10} RNA molecules / per µl, according to the manufacturer's recommendations.

- ✓ **RT-qPCR conditions**

All RT-qPCR tests for specificity and detection assays were carried out in a final volume of 20µl reaction using the Lightmix 1Step enzyme (Tibmolbiol, Berlin, Germany) containing 15µl of mixture and 5µl of input RNA. All sensitivity assay in singleplex or multiplex were performed in a final reaction volume of 21µl as previously described (17).

- ✓ **RT-qPCR system performance evaluation in singleplex / multiplex format**

The sensitivity of the newly designed sylvatic DENV-2 RT-qPCR assay was determined using 10-fold dilutions of synthethized ivRNA (range from 10^5 to 10^0 copies per reaction) in five replicates as previously described (18). The specificity was evaluated by using extracted RNA from archived sylvatic DENV-2/GVI isolated retrieved from infected cells, and DENV 1-3 positive and sylvatic DENV-2/GVI positive human sera samples obtained from the ongoing syndromic surveillance program in Senegal. To validate the presence of DENV RNA all used RNA samples were tested in parallel with panDENV primers using the previously described protocol (17) .

- ✓ **Data analysis and representations**

The R software package was used to generate all figures and representations of the sequencing statistics and associated data retrieved from CZID website. The ggplot2 package was used to generate plot using functions *geom_point*, *geom_col*, *geom_pointrange* and *geom_line*. Probit analysis was done using *MASS* and *ggplot* packages.

Results

- ✓ **Identification of the Sylvatic DENV-2 strains**

A suspected sample from Sare Yoba (Kolda) in November 2021 was detected by panDENV PCR but failed to be properly serotyped by tiling PCR. To verify the sequence of the strain a partial NS5 gene was amplified from the patient serum sample RNA extract. BLAST analysis revealed that the detected dengue strain belongs to the sylvatic DENV-2 genotype (9).

- **DENV-2/GVI oligonucleotides primers sets**

A tilling PCR primer design approach allowed to generate a set of thirteen 900bp amplicons covering the coding region (CDS) of DENV-2/GVI (Table 2). The obtained oligonucleotides potentially allow to amplify overlapping PCR amplicons (overlap on average 50 bp) spanning the CDS of a wide panel of DENV-2/GVI strains. BLAST analysis of the individual oligonucleotides returned best hit sequences belonging to sylvatic DENV-2 genotype indicating a higher specificity for DENV-2/GVI. The table 2 summarise designed specific DENV-2/GVI tilling PCR primers set.

- **Sequencing human sera using Nanopore and Illumina technology**

The tilling oligonucleotides set was applied to generate viral genome sequences for 4 suspected (RT-qPCR serotyping failure) DENV-2/GVI human serum samples (panDENV Ct 21.09 to 30.93) using nanopore technology. This approach yielded 4 nearly complete genomes (depth of coverage ranging from 2406.9-7814.9 with a genome coverage ranging from 93.9 to 95.1%. This demonstrated that the newly designed tilling PCR oligonucleotides successfully determined the virus genome sequence from sera using both short read or long read sequencing technologies.

Table 3. Statistics of sequenced DENV-2/GVI human sera using Nanopore sequencing approach

Sample ID	Total_reads	Mapped_reads	Depth_avg	% genome coverage
SH381907	142000	110463	7814.9	95.1
SH356683	183000	68490	2406.9	94.4
SH356692	143000	84220	3435.9	93.9
SH356702	144000	70756	2572.3	93.9

- **Sequencing viral isolates using Illumina technology**

Additionally, the tilling oligonucleotides set was applied to determine the sequence of 9 DENV-2/GVI genomes (panDENV Ct 21.80 to 35.63) from archived virus isolate samples using Illumina sequencing. For all samples sequencing yielded enough reads (depth of coverage 944.83-2000) to obtain good quality genomes. At a cut off threshold of above 10X the highest genome coverage was 94.23 - 99.35 fold (Table 4).

Table 4. Statistics of sequenced DENV-2/GVI isolates using Illumina sequencing approach

Sample ID	Total reads	Mapped reads	Depth Avg	% Genome coverage
316	156112	144838	1637.43	94.28
317	124168	104655	1196.81	94.26
320	193352	183018	2070.60	94.27
323	178548	168496	1904.38	94.73
324	193764	176460	2000.40	94.28
325	198286	43023	491.52	94.23
328	222396	134694	1523.50	94.28
329	140622	133187	1519.14	94.26
381907	86972	79052	944.83	99.35

Compared to previously available DENV-2 tiling PCR schemes the newly designed DENV-2/GVI tilling PCR primers yield the highest and most uniform genome depth across the genome. For all cell culture isolated strains, the mean depth of coverage ranges from between 491.52 to 2070.6 -fold using the new DENV-2/GVI schemes while this coverage is lower when using already published tilling PCR primers for DENV-2 (Figure 1 / Table S2).

For all tested strains the obtained coverage is above the 10X coverage cut-off widely accepted to call base during the genome assembly process.

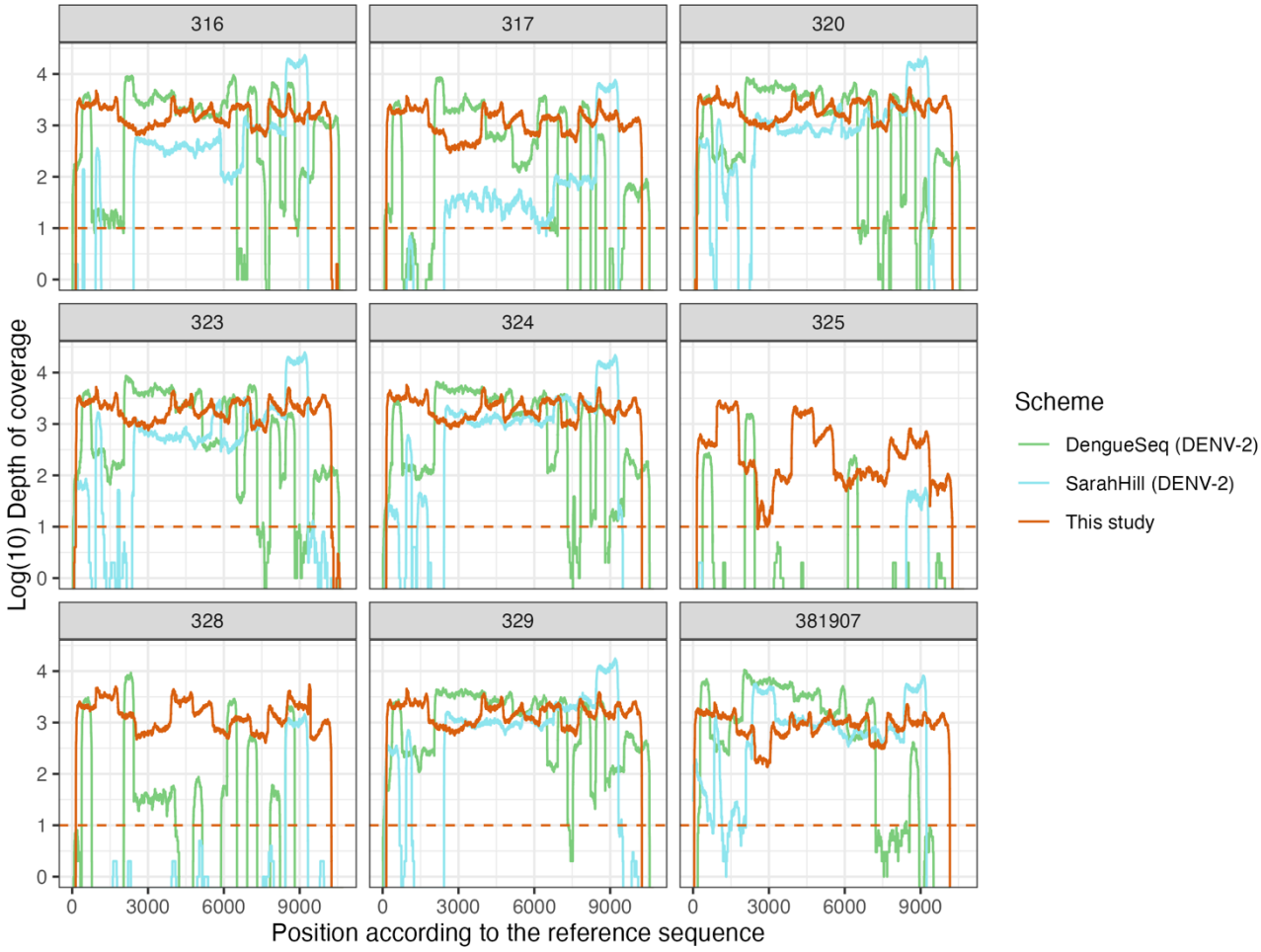

Figure 1. Comparison of depth of coverage across DENV-2/GVI strains according 03 primer scheme system

- **Sensitivity and Specificity of newly designed Sylvatic DENV-2 RT-qPCR primers**

To test the analytical sensitivity of the DENV-2/GVI real time PCR, ivRNA detections was linear over 4 $\log_{10}$ steps ranging from 10^5 (mean Ct 22) to 10^2 (mean Ct 31.24) molecules detected in singleplex PCR (Figure 2). In contrast the mean Ct values ranged from 24.45 - 32.57 at 10^5 and 10^2 copies/µl respectively in multiplex PCR. According to probit analysis the LOD at 95 % probability was respectively 68.46 and 133.21 for singleplex and multiplex formats (Figure 3).

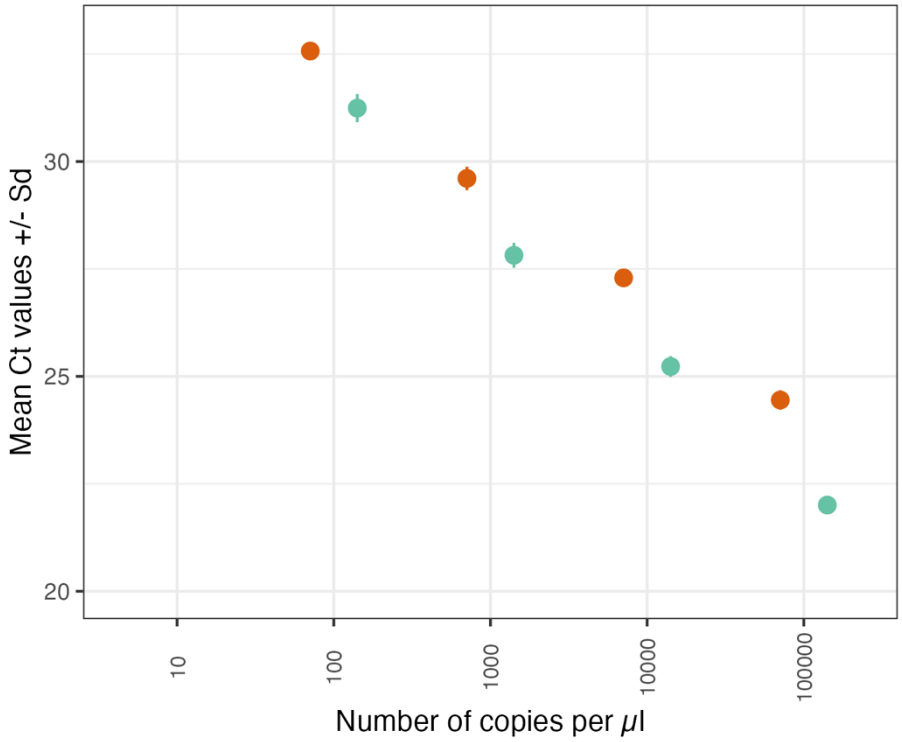

Figure 2. LOD comparison of DENV-2/GVI RT-qPCR in singleplex vs multiplex PCR. Green dot represents singleplex reaction while darkorange highlight multiplex reactions

In terms of analytical specificity, the newly designed DENV-2/GVI RT-qPCR primers detect DENV-2/GVI only without any cross amplifications of any other DENV1-4 types (*Table S3*). Additionally, for all performed paired singleplex - multiplex assays

on DENV-2 / GVI strains used during the validation the delta Ct values were below ± 1.41 except for two serum samples (Figure S1 / Figure S2).

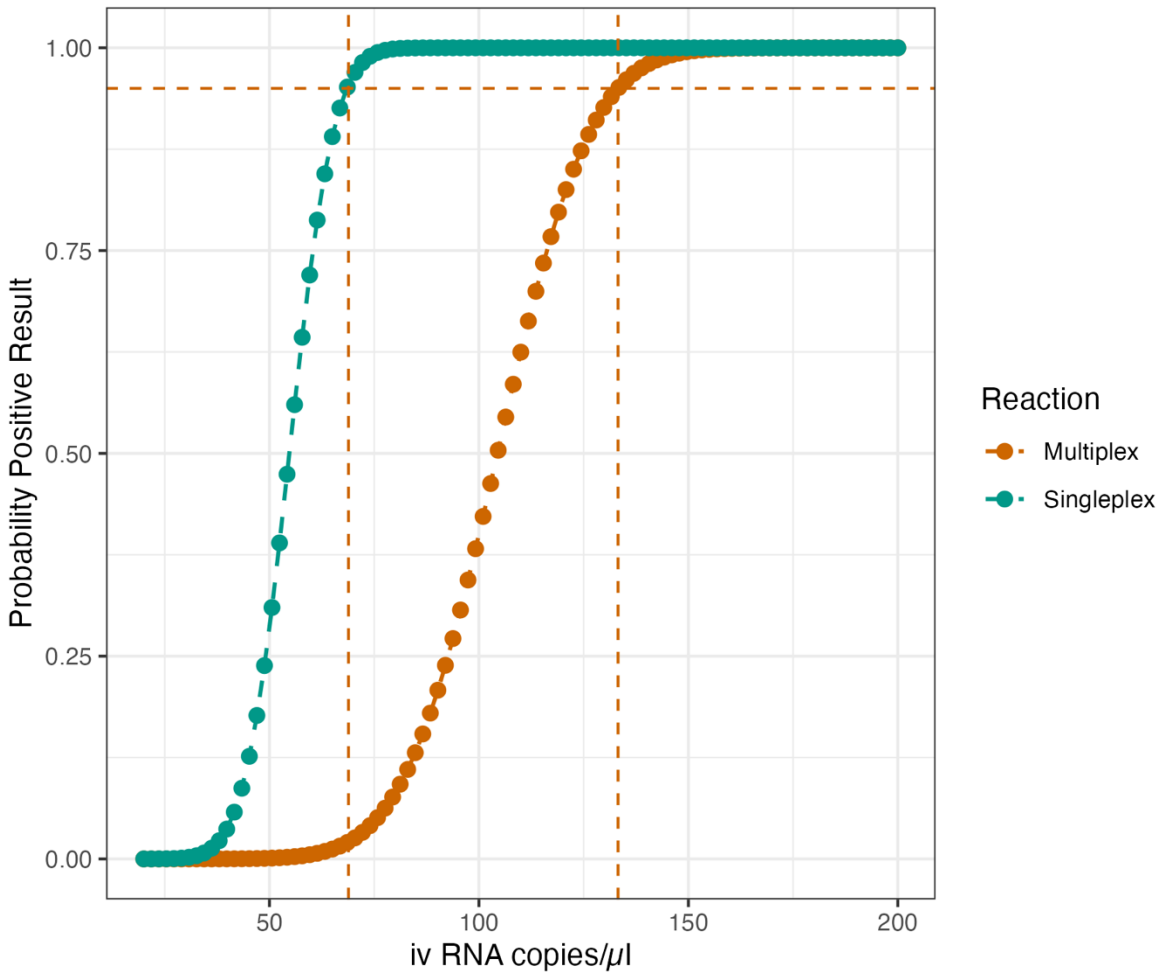

Figure 3. Probit regression analysis of DENV-2/GVI RT-qPCR. The analysis was based on serial dilutions of DENV-2/GVI standard RNA molecules in 5 replicates at each concentration. Both singleplex (green) and multiplex (dark orange) result curves were plotted. The probability of positive result is plotted against the concentration of DENV-2/GVI RNA molecules. The 95% detection limit is indicated with the dashed lines.

Discussion

In recent years DENV has becoming a major public health threat as exemplified by recorded outbreaks worldwide (19).

Most of these outbreaks where linked to urban epidemic DENV 1-4 strains (4,20–22). Nevertheless, beside these well-known circulating epidemic strains, sylvatic DENV-2 circulation was noticed in the past in West Africa in Nigeria and Cote d'Ivoire but mainly in Southern Senegal in the Kédougou area (*Diallo 2003, Kedougou*) and in Malaysia in Asia (23). Due to few associations with outbreaks and the restriction of their circulation to West Africa and Malaysia the viral strains associated to the sylvatic cycle are less studied (24). In Senegal in 2020 a huge DENV outbreak was described in the Kédougou region. following serotyping failure using the CDC dengue typing kit (10), in depth virological investigations revealed that the outbreak was caused by DENV-2/GVI (10) a virus genotype last reported in 2009 in Spain from a tourist visiting Guinea Bissau (25). The well-known continuous evolution of RNA viruses due to the generic lack of polymerase proofreading activity leads to the emergence of viral variants and may lead to the erosion of target regions routinely used molecular detection systems (8). Following the failure to serotype re-emerged DENV-2/GVI in southern Senegal by RT-qPCR, we developed and validated a new set of oligonucleotides to detect, discriminate and sequence DENV-2/GVI strains by tilling amplicon-based approach. Recent studies principally during the SARS-COV 2 pandemic pointed out the importance of genomic surveillance to track high public health impactful pathogens (26,27). The DENV-2/GVI tilling amplicon scheme composed of 36 oligonucleotides organized in two pools was developed and validated as previously described for emerging pathogens genomic sequencing (5,28,29). Theses oligos allowed to obtain nearly complete genomes at coverage percentages ranging between 93.9 and 95.1 directly from extracted RNA from sera samples of patients infected with DENV-2/GVI in southern Senegal (Kolda and Kédougou) (9,10) using the nanopore platform (Table 3).

The high quality genome yield (coverage > 93) obtained using the nanopore strategy from human sera RNA extracts has the potential to strengthen DENV genomic surveillance in remote areas (sylvatic environment) where Illumina sequencing cannot be efficiently implemented (30).

We also compared the in-vitro performance of the newly designed DENV-2/GVI scheme against two published schemes on DENV-2/GVI isolates listed in table S1 using the Illumina sequencing technology. The newly designed primer scheme allowed to retrieve 9 DENV-2/GVI genomes (08 archived and 01 contemporary strain) with a coverage ranging from 94.23 to 99.45 % similar to those obtained by Vogels and colleagues during their validation steps (5).

In contrast both previously published dengue primer schemes yielded a genome coverage below 90.00 ranging from 10.89 to 89.94% for the DengueSeq scheme (5) and from 8.20 to 85.65 % for SaraHill's scheme (5). Whereas the new DENV-2/GVI scheme yielded a depth of coverage consistency throughout the genome (Figure 1), the lower percentage of genome coverage of the other schemes was marked by frequent drop outs across the genome of tested DENV-2/GVI strains (Figure 1 blue line and green line) indicating a lack of performance of previous schemes to reliably sequence sylvatic DENV-2 genotypes. The newly developed scheme, like other amplicon-based approaches, therefore provides more sensitive, lower cost and higher throughput sequencing of DENV-2 variants than the other schemes.

Despite the importance of genome sequencing in understanding virus epidemiology (5,31) sequencing capabilities show heterogeneity worldwide and especially in remote areas (Africa) (32). Additionally sequencing is costly and labour intensive limiting its widespread use (33). For dengue virus surveillance reliable identification of virus variants is key to mitigate the virus burden and design tailored countermeasures (5). As an alternative to sequencing DENV-2/GVI strains the developed DENV-2/GVI RT-qPCR assay yield a LOD of 68.85 and 133.21 RNA molecules detected respectively for

singleplex and multiplex reactions ,which is in the range of other available dengue RT-qPCR serotypes discrimination tool (7).

In all tested DENV-2/GVI RNA samples the delta Ct between multiplex and singleplex reaction Ct values was < 1.41 defined as threshold for natural variation in RT-qPCR reactions (34) except for two serum samples (Figure S2). This confirm that the performance of the RT-qPCR system is similar in both singleplex and multiplex format. The higher delta CT values observed for the two serum samples could be linked inhibiting substances present in the sample that can influence RT-qPCR reactions (35). Indeed DENV infection can alter many organs including causing liver pathogenesis (36). Bile produced by hepatocytes from cholesterol can impact PCR efficiency (37) and it is well known that the level of bile acid can increase during dengue infection (38).

Conclusion

In summary a newly developed set of oligonucleotides allowed sensitive and specific identification, and reliable nearly complete genome characterization of DENV-2/GVI strains. An additionally developed specific RT-qPCR yielded comparable results in both singleplex and/multiplex format providing a reliable tool to strengthen dengue serotypes / genotypes surveillance. Deployed in the field it can improve cost-effectiveness for rapid on-site detection and differentiation of circulating DENV strains among Human-Monkey and arboreal mosquitoes in sylvatic environments.

Both tools may improve surveillance of DENV in sylvatic environments in Africa to help provide insights on the true genetic diversity of circulating DENV variants in the continent.

Data Availability Statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure
The funders were not involved in study design, data collection, analysis, publishing decisions, manuscript preparation, editing, approval, or decision to publish.

Conflicts of Interest
The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Table S1: Metadata of used DENV-2/GVI strains during this study
Table S2: Comparison of genome coverage on DENV-2/GVI strains according to used
TableS3: Results tests Singleplex vs Multiplex design assay on Urban and Sylvatic dengue strains
Figure S1: Comparison of Ct values of DENV-2/GVI RT-qPCR in singleplex and multiplex against used DENV-2/GVI strains. The left panel is for used viral isolates and right panel for human sera.
Figure S2: Ct values differences of developed DENV-2/GVI in Singleplex and multiplex on DENV-2/GVI used during this study. The dashed line represent the cut-off proposed by *Niesters et al (34)* to express the natural variation during RT-qPCR reaction.

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