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Improved LNA probe-based assay for the detection of African and South American yellow fever virus strains

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ABSTRACT

Background: Real-time assays for Yellow fever virus (YFV) would help to improve acute diagnostics in outbreak investigations.
Objectives: To develop a real-time assay for YFV able to detect African and South American strains.
Study design: Three short probe (14–18 nt) formats were compared and a plasmid-transcribed RNA standard was used to test the performance of the assays. Additionally the new TaqM1 enzyme was tested.
Results: A locked nucleotide probe (LNA probe) performed best with an analytical sensitivity of 10 RNA molecules detected. 44 African and 10 South American strains were detectable. One South American strain from 1984 had a one-nucleotide deviation in the hybridisation sequence for which the LNA probe had to be adapted. Comparison of enzymes revealed that not all enzymes are suitable for LNA probes.
Conclusion: The developed LNA probe based YFV real-time PCR performed best in an enzyme mix and less efficient using multifunctional enzymes.

1. Background

Yellow fever (YF) is unknown in Asia but occurs in sub-Saharan Africa and tropical South America with an estimated case count of 200,000 and 30,000 deaths per year.[1,2] In the last two decades, a dramatic re-emergence of yellow fever was recognized in the two endemic regions in Africa and South America.

From 2000 to 2007, 49 different areas in 18 African countries reported yellow fever cases including 31 (63%) newly affected areas. The largest recent outbreak occurred in Abidjan, Côte d'Ivoire, in 2008, where an investigation after the initial three cases determined a high risk of an urban outbreak of yellow fever leading to a vaccination campaign reaching a total of 2.23 million people.[2]

In South America outbreaks occur as a consequence of the sylvatic YF transmission cycle. After a silence of almost 40 years YF cases appear on the increase spreading towards the south towards Southern Brazil, Paraguay and Argentina from the endemic northern and central Brazilian states. Recently a 4-fold increase of mortalities within 1 year amongst a not vaccinated rural population in the states of São Paulo and Rio Grande do Sul, was reported. The first case of a vertical transmission from mother to child was reported in the current outbreak there.[3,4]

Response to the detection and confirmation of index cases (1 of 3–7 subclinical cases) needs to be rapid, since especially in an urban setting the mosquito borne spread can be very efficient in a context of dense human populations with low vaccine coverage. Yellow fever virus (YFV) is the type species of the *Flaviviridae* and has a 11 kb +ssRNA genome. A thorough analysis of glycoprotein E and E-PrM coding region sequences recently described two African and two South American genotypes. The latter two appear to descend directly from the West-African genotypes and historically appear to have spread to South America by the slave trade and have dispersed by migration since.[5] A phylogenetic analysis of NS5/3′NCR region sequences of YFV strains isolated from different geographic regions, hosts and periods of time in Brazil described five distinct lineages. In the course of time the ancient 'Old-Para' lineage, seems to have been replaced by four other lineages (1A–D) actively circulating across the country and associated with the disease in regions epidemiologically considered as endemic and transition areas for the YFV transmission.[6,7]

The surveillance system for YF in Africa currently relies on a laboratory network for the detection of YFV-IgM by ELISA.[8] Genome detection would be a helpful tool for outbreak investigation teams

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since genome detection would fill the gap of the diagnostic window between onset of virus replication and generation of IgM in acute cases. Several PCRs and real-time PCRs for the detection of YFV have been described.[9–11]

Probe length can be limiting for real-time PCR design, as determining a signature of 25–30 conserved nucleotides (T_M 65–68 °C) in an alignment of highly variant sequences of a RNA virus is challenging. As an alternative shorter "Minor Grove Binder" (MGB) or Locked Nucleic Acid (LNA) probe (14–18 nt) types are available. The latter include nucleotides with a bridging methylene between 2′*O* and 4′*C* in the sugar moiety locking the structure into a rigid C3′-endo conformation, which provides superior hybridisation characteristics and enhanced biostability compared to conventional DNA nucleotides. LNA oligos are composed of either 100% LNA bases or a mixture of LNA and DNA bases.[12] The "Minor Groove-Binder" molecule (MGB) is a dihydrocyclopyrroloindole tripeptide (DPI_3) binding into the minor groove of the α-helix increasing the binding energy of a probe by the attachment to the 3′end of a Taqman probe (Minor Groove-Binder-Probe, MGB probe (Applied Biosystems))[13] or the 5′end of a Molecular Beacon (Quantiprobes, Q-probes (Qiagen), formerly Eclipse probes (*Epoch Bioscience*)).[14–16]

2. Objective

To develop and optimise a real-time RT-PCR assay for the detection of YFV.

Table 1
Virus strains tested.

No. CRORA, Dakar	Isolation year	Host	Isolation site
1. FNV 281 ref. 1	1927	*Homo sapiens*	Dakar
2. Dak Ar Amt/7	1973	*Ae. africanus*	Touba (Ivory Coast)
3. Ar B 5656	1974	*Ae. africanus*	Bozo (Central African Republic)
4. Ar D 24553	1976	*Ae. furcifer-taylori*	Kedougou (Eastcentral Senegal)
5. Ar A 408/78	1978	*Ae. luteocephalus*	Burkina Faso
6. An D 26923	1978	*Erythrocebus patas*	Kedougou (Southeast Senegal)
7. Ar D 27797	1979	*Ae. aegypti*	Minteh Kunda (Gambia)
8. YF 10.5. 79 Ref. 2	1979	Reference strains	Reference strain
9. Mali no. 20	1987	*H. sapiens*	Mali
10. HD 47471	1987	*H. sapiens*	Rosso (Mauritania)
11. Ar D 99740	1993	*Ae. furcifer*	Kedougou (Southeast Senegal)
12. H D 117294	1995	Human	Koungheul (Central Senegal)
13. Ar D 114 891	1995	*Ae. aegypti*	Koungheul (Central Senegal)
14. Ar D 114 991	1995	*Ae. aegypti*	Koungheul (Central Senegal)
15. Ar D 114 988	1995	*Ae. furcifer*	Koungheul (Central Senegal)
16. Ar D 114987	1995	*Ae. luteocephalus*	Koungheul (Central Senegal)
17. Ar D 114896	1995	*Ae. aegypti*	Koungheul (Central Senegal)
18. Ar D 114970	1995	*Ae. aegypti*	Koungheul (Central Senegal)
19. Ar D 114972	1995	*Ae. aegypti*	Koungheul (Central Senegal)
20. Ar D 114988	1995	*Ae. furcifer*	Koungheul (Central Senegal)
21. Ar D 114989	1995	*Ae. metallicus*	Koungheul (Central Senegal)
22. H D 122030	1996	*H. sapiens*	Kaffrine (Central Senegal)
23. Ar D 122522	1996	*Ae. aegypti*	Kaffrine (Central Senegal)
24. Ar D 121 040	1996	*Ae. furcifer*	Kedougou (Southeast Senegal)
25. HA 016/97	1998	*H. sapiens*	Liberia
26. Ar D X	2000	*Aedes spec.*	Kedougou (Southeast Senegal)
27. Ar D 149213	2000	*Ae. luteocephalus*	Kedougou (Southeast Senegal)
28. Ar D 149214	2000	*Ae. furcifer*	Kedougou (Southeast Senegal)
29. Ar D 149215	2000	*Ae. vittatus*	Kedougou (Southeast Senegal)
30. Ar D 149887	2000	*Ae. taylori*	Kedougou (Southeast Senegal)
31. Ar D 149791	2000	*Ae. furcifer*	Kedougou (Southeast Senegal)
32. Ar D 149179	2000	*Ae. luteocephalus*	Kedougou (Southeast Senegal)
33. Ar D 149194	2000	*Ae. taylori*	Kedougou (Southeast Senegal)
34. Ar D 149815	2000	*Ae. furcifer*	Kedougou (Southeast Senegal)
35. Ar D 149170	2000	*Ae. luteo*	Kedougou (South Eastern Senegal)
36. SH A 032-02	2001	*H. sapiens*	Duékué (Ivory Coast)
37. Ar D 156029	2001	*Ae. furcifer*	Kedougou (Southeast Senegal)
38. Ar D 156583	2001	*Ae. taylori*	Kedougou (Southeast Senegal)
39. Ar D 156591	2001	*Ae. luteocephalus*	Kedougou (Southeast Senegal)
40. Ar D 156098	2001	*Ae. furcifer*	Kedougou (Southeast Senegal)
41. Ar D 156468	2001	*Ae. furcifer*	Kedougou (Southeast Senegal)
42. Ar D 157928	2001	*Ae. luteocephalus*	Kedougou (Southeast Senegal)
43. Ar D 157991	2001	*Ae. taylori*	Kedougou (Southeast Senegal)
44. Ar D 158832	2001	*Ae. furcifer*	Kedougou (Southeast Senegal)
IEC Belem			
1. H396226	1981	Human	Para state (Nothern Brazil)
2. H413820	1983	Human	Rondônia (Nothern Brazil)
3. H422973	1984	Human	Para state (Nothern Brazil)
4. H423602	1984	Human	Para (Nothern Brazil)
5. H463676	1987	Human	Para state Nothern Brazil)
6. H521244	1993	Human	Maranhão (Nothern Brazil)
7. AR630785	2000	*Hg. jantynomys*	Goiás State (Central of Brazil)
8. H629290	2000	Human	Bahia (Southeast Brazil)
9. H622205	2000	Human	Goiás State (Central of Brazil)
10. H655417	2002	Human	Roraima (Nothern Brazil)
11. H6861174	2004	Human	Roraima (Nothern Brazil)

3. Study design

3.1. Virus strains, virus culture

Flaviviruses: YFV vaccine strain 17D (*), Dengue 1–4 (*), West Nile Virus 1, 2 (*, #), Tick-borne Encehalitis Virus (*), Japanese Encephaltis Virus (*), and Alphaviruses Venezuelan Equine Encephalitis Virus (*), Western Equine Encephalitis Virus (*) and Chikungunya Virus (#), O'nyong-nyong Virus (#) (*Pilaski collection, Göttingen, # CRORA, Dakar) were grown on VeroB4 cells in 95% DMEM, 5% foetal calf serum in 175 cm^2 flasks at 37 °C/5% CO_2. Additionally RNA was extracted from supernatants of African and South American strains (Table 1). Viral RNA was prepared from culture supernatants using RNeasy columns (Qiagen, Germany) according to the manufacturer's instructions and used to prepare RNA standards.

3.2. RNA standard

A standard covering the 5′end of the YFV genome to the 3′end of the capsid coding region was constructed using the primers YFV UP/DP (Table 2). *In vitro* transcription and quantification of transcribed RNA were performed as previously described[17] with an improved step for DNA digestion using the DNAfree kit (Ambion) instead of DNASE digestion and TRIZOL purification.

3.3. Real-time RT-PCR amplicon design

The amplicon for the detection of YFV was designed for African and South American strains in the 5′ untranslated region (UTR) of the YFV genome using 20 available sequences (AF09461, AY603338, AY640589, AY968064–AY968065, D14458, U52389/U52392/U52395/U52398, U52403–U52404/U52409/U52410/U52413/U52416/U52419/U52422/U54798, U89338). Primer T_M ranged between 58 °C and 60 °C. The T_M of the Taqman-MGB probe (5′FAM/3′TAMRA) was determined using the PrimerExpress software (Applied Biosystems). The (5′FAM/3′BBQ) tagged LNA probes were designed using the LNA-probe design window at http://lna-tm.com/.[12] The Eclipse probe was designed using the design window at http://www.qiagen.com/goto/assays.

3.4. Real-time RT-PCR conditions

500 nM primers and 200 nM probes (Table 2, primers and LNA probes purchased from TIBMOLBIOL, Germany, MGB probe from Applied Biosystems, Eclipse probe from Qiagen, Germany), were used (i) with the LightCycler480 RNA Master Hydrolysis Probe kit (Roche) hereafter abbreviated as RK for use on the LightCycler480 (LC480, Roche) and LightCycler2.0 (LC2.0, Roche), RT at 63 °C/5 min, activation at 95 °C/5 min, 45 cycles of PCR at 95 °C/5 s, 60 °C/60 s, (ii) with the QuantiTect Virus Kit (Qiagen) hereafter abbreviated as QT for use on LC480 and LC2.0 and SmartCycler (SC, Cepheid), RT at 50 °C/5 min activation at 95 °C/15 min and 45 cycles of PCR at 95 °C/5 s, 60 °C/15 s (extension time was expanded to 60 s for the YFV Eclipse probe, 2 μg GP32 was added for the SC mix), and (iii) with TaqM1 in 50 mM Bicine (pH 8.2), 115 mM KOAc, 2.5 mM $MgCl_2$, 8% glycerol and 500 μM dNTPs, RT at 63 °C/5 min, denaturation 95 °C/1 min and 45 cycles of PCR at 95 °C/5 s, 60 °C/60 s.

Table 2
Primers for S-segments and for species-specific RT-PCR.

Name	Standard primer	TM	Location
YFV STD UP	agtaaatcctgtgtgctaattgagg	59.2	1–25
YFV STD DP	agccactgtgagtttcagca	58	465–481
	Detection primer and probes		
YFV FP c	attgaggtgcattggtctgc	60.2	19–38
YFV FP1	attgaggtgyattggtctgc	59.1	21–38
YFV FP2	tgctaattgaggtgyattggtc	61.4	14–35
YFV RP	gtcrgttctctgctaatcgctca	61	90–112
YFV RP2	gtcrrttctctgctaatcgctca	58.	92–110
YFV RP3	ctgagctttdcgaccagaca	60	120–139
YFV RP4	gtcrrttctctgctaatcgctca	60	90–112
YFV LNA 1	FAM-agttgctaggcaAtAAA-BBQ	58	45–61
YFV LNA 2	FAM-agttgctargcaAtAAA-BBQ	58	45–61
YFV MGB	6FAM-agttgctaggcaataaa - MGBNFQ	71.2	45–61
YFV Eclipse	MGB-EDQ-gagttgctaggcaata-FAM	65.5	44–59

All primers are given in 5′–3′ orientation. YFVSTD UP/DP: standard fragment upstream primer/downstream primer; FP: forward primer; RP: reverse primer; LNA: LNA-Taqman probe (LNA nucleotides are given in bold letters); MGB: Minor Groove-Binder-Taqman probe; BBQ: Black Berry Quencher; MGBNFQ: Molecular-Groove Binding Non-fluorescence Quencher; EDQ: Eclipse™ Dark Quencher.

3.5. Determination of sensitivity

The YFV RNA standard was tested in triplicate for each real-time mix and CT values were plotted against molecules detected and a semi-log regression was calculated. For exact determination a probit regression was performed using results of 10 replicates and the free software R (version 2.8, www.r-project.org).

4. Results

4.1. PCR design

To determine the best primer combination, all upstream and downstream primers were combined with each other and tested for efficiency ($E = 10^{-1/\text{slope}} - 1$) on the RNA standard range from 10^4 to 10^7 molecules using a SybrGreen-PCR. The performance of the primer combinations could be assigned to three groups (Fig. 1A). In group one the primer combinations led to very low slopes and therefore bad efficiencies. In group two primer combinations had much higher slopes and efficiencies but higher threshold cycle values (CT). Lower CT values in group two were taken as an additional indication for better efficiencies of the primer combinations. The primer combination YFV FP/RP was chosen as the optimal primer combination.

4.2. Comparison of probe formats, sensitivity

The YFV RNA standard was used to directly compare the performance of the individual probe types on the LC480. As shown in Fig. 1B, the three probe formats performed differently in terms of sensitivity. The LNA assay had a sensitivity of 10^2 molecules detected and a calculated efficiency of 1. Although the MGB probe and the LNA probe had identical sequences, the MGB probe detected the YFV RNA standard at a 2 $\log_{10}$ lower sensitivity than the LNA probe. The Eclipse probe design differed from the two other probes by one additional nucleotide at the 5′end and two missing nucleotides at the 3′end and performed comparable to the MGB probe. The final YFV LNA assay using the QT mix showed a sensitivity of 10 molecules detected on LC480 and SC. A probit model regression predicted the concentration at which a 95%-proportion of positive hits are achieved to be 13.4 copies per assay on the LC480 (Fig. 3B).

To assess the sensitivity of the YFV LNA real-time assay, RNA of 44 African YFV isolates of the CRORA collection at the Institut Pasteur de Dakar was tested. The isolates from 1976 to 2001, from mosquitoes and humans, cover the sylvatic and the urban transmission cycle in West Africa. Additionally, the RNA of 10 South American isolates from the collection of the Instituto Evandro Chagas, Belem, was tested. The assay detected the RNA of all but one strain. It failed to detect strain BeH422973 isolated in 1984, from a non-fatal human case in northern Brazil. The amplified target amplicon fragment was detectable on a 3% agarose gel, cut out and

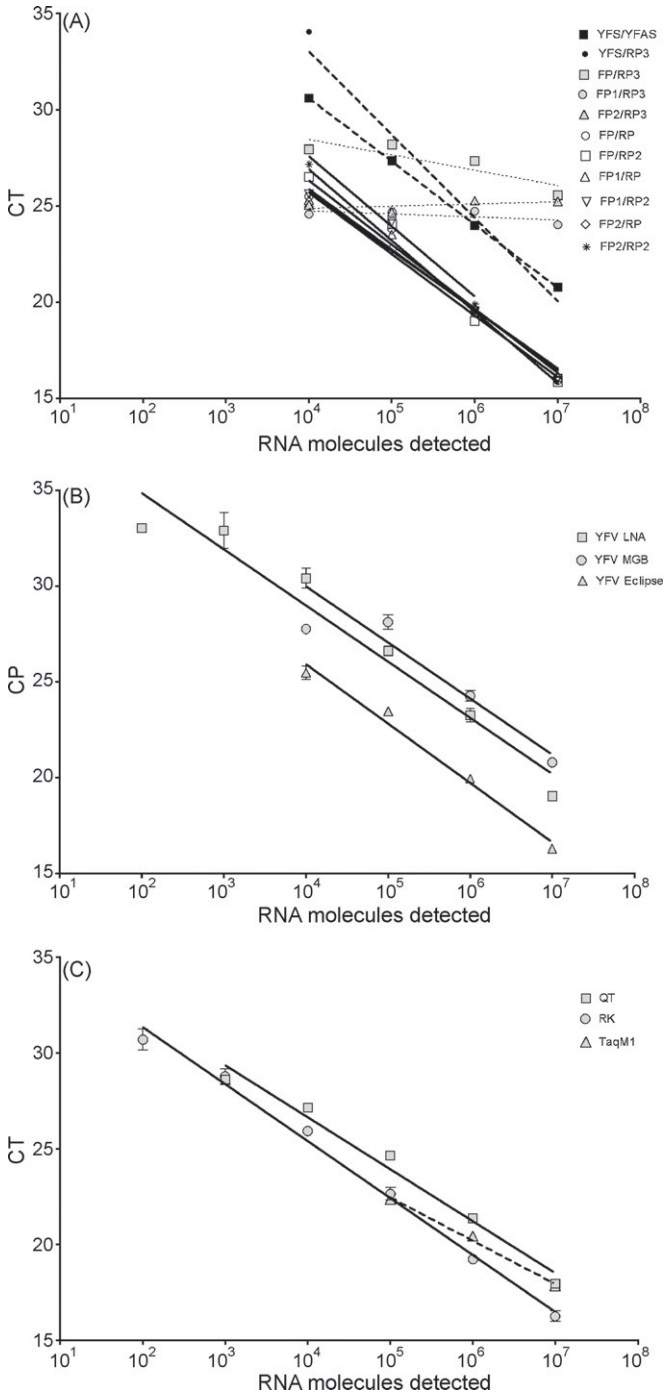

Fig. 1. Real-time assay performance. (A) Efficacy testing of primers using SyberGreen, light dotted lines group 1 (low efficiency), dashed lines group 2 (high efficiency late CT values), group 3 dark lines (high efficiency early CT values). (B) Comparison of the performance of three probe types. (C) Comparison of the performance of TaqM1, Tth (RK) and enzyme mix (QT). The regression lines were calculated from triplicate data sets. The data sets are given as mean values with standard deviation.

subjected to cycle sequencing. A one-nucleotide change in the target sequence of the probe was identified (G>A). The adapted probe YFV LNA2 (Fig. 2) was able to detect the RNA of this strain.

4.3. Specificity

None of the Flaviviruses and Alphaviruses listed in material and methods were cross detected by the YFV assay.

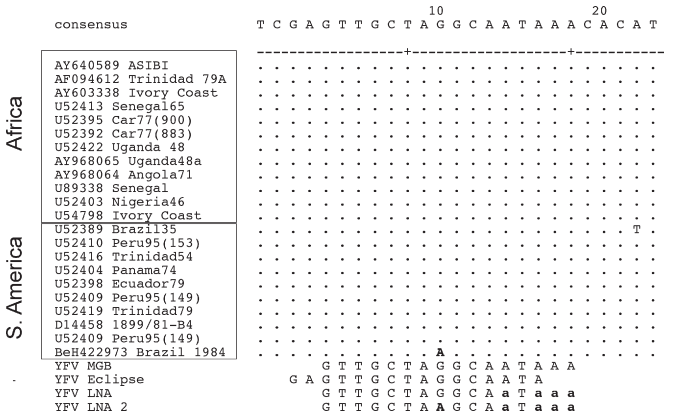

Fig. 2. Hybridisation sequences of YFV-assay probes. Dots in the alignment represent homologies to the consensus sequence shown in the top line. Aberrant nucleotides are spelled out. The lower case "a" represents LNA nucleotides in the LNA-probe sequences. BeH422973 Brazil 1984 represents the sequence found in the strain not detected by probe YFV LNA.

4.4. Performance of TaqM1

TaqM1 is a newly evolved enzyme with reverse transcription and polymerisation activity including a 5′ nuclease activity,[18] which make it ideal for real-time RT-PCR. In order to test the ability of this new enzyme to perform real-time PCR with LNA probes, we tested it with the newly developed YFV LNA assay. In initial tests we had determined an ideal RT activity at 63 °C and RT-PCR activity in a slightly alkaline bicine buffer.[19] The performance of TaqM1 was tested in comparison to the performance of the QuantiTect Virus Kit (QT) and the Roche kit (RK). Further analysis showed that the 15 s elongation time of the 2-step PCR protocol used in the YFV-assay development with the QT had to be elongated to 60 s for both the TaqM1 and the RK. Degradation of the LNA probe was not optimal below 45 s elongation time. As a whole, however the combination of two enzymes in the QT performed better than the multifunctional enzymes Tth (RK) and TaqM1. In fact the performance of the TaqM1 enzyme was much lower than the performance of the enzymes in the QT and the RK.

5. Discussion

Nucleic detection assays for RNA viruses need constant updating as sequences become available.[20] This study presents an updated real-time assay for the detection of YFV.[10,11] We used short LNA probes, MGB probes and Eclipse probes (14–18 nt) to be able to cover a wide range of sequence variants using a small conserved signature sequence.

In support of previous comparative probe format studies in real-time PCR,[21,22] this study indicates that LNA probes are also a good alternative to MGB probes in real-time RT-PCR. In this particular assay the LNA probe showed superior sensitivity than the MGB probe formats. This confirms studies that described higher precision, accuracy, and reproducibility down to low numbers of detected molecules for LNA-probe assays compared to other real-time PCR formats.[23–25] The reasons for the higher sensitivity may stem from the increased thermal stability of duplex DNA conveyed by the LNA nucleotides in combination with an increased PCR efficiency of the shorter probe due to reduced amplification interference.[26,27] On the LC480 there appears to be a CT dip at 10 molecules detected, which is not pronounced on the SC (Fig. 3A) indicating platform-dependent non-linear amplification at low copy numbers.

Probes of this reduced length are more sensitive to mismatching and, as already shown for MGB probes,[28] can miss a strain due

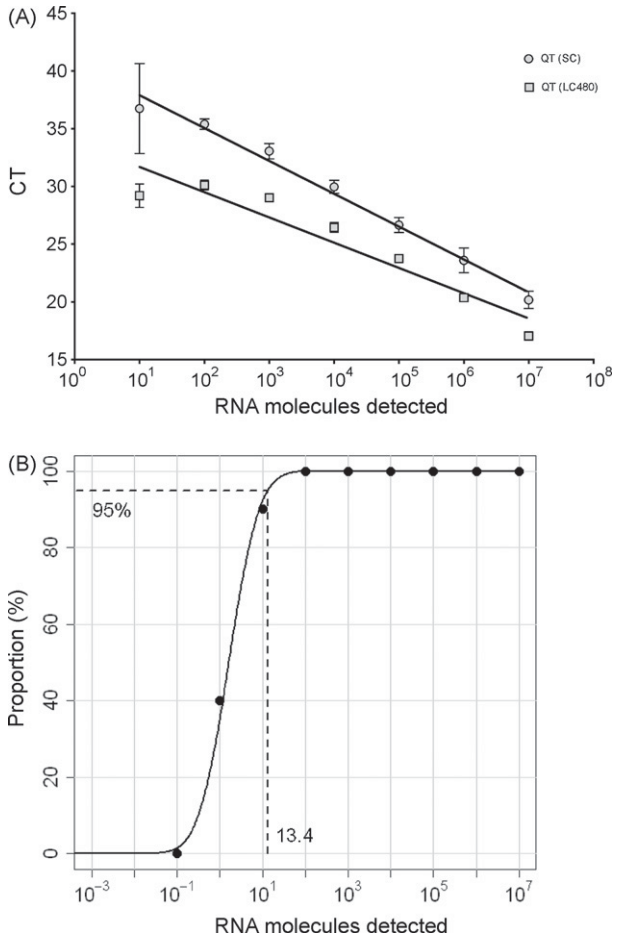

Fig. 3. (A) Sensitivity of QT mix assay on the LC480 and SC. The regression lines were calculated from triplicate data sets. The data on the SC were achieved by the addition of GP32 to the QT mix. The data sets are given as mean values with standard deviation. (B) Probit regression of 10 data sets for the QT mix on LC480.

to one-nucleotide mismatch in the target sequence of the probe. The discovery of the mismatch in the sample strain of the Para state YFV isolate from 1984 however also stresses the point that assays are only as good as their sensitivity testing, i.e. testing isolates from strain collections is absolutely imperative for a sound assay especially for RNA viruses.[29,30]

The novel TaqM1 enzyme performs well with Taqman probes.[19] Our results show that multifunctional enzymes (Tth, TaqM1) capable of combined RT-PCR are less efficient in degrading LNA probes and need some improvement. The 5′ nuclease activity of Taq polymerases (QT) is sufficient for the degradation of LNA probes.

In comparison to published YFV real-time assay[10,11] the YFV LNA-probe real-time assay described here has excellent analytical sensitivity as shown by probit analysis, in terms of YFV strain spectrum covered. Altogether, the YFV LNA-probe assay, QT, and mobile SC combine into a sound mobile nucleic acid detection assay for YFV, for use in field-work in Africa and South America.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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