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Development of a TaqMan real time reverse transcriptase PCR assay for detection of Zika, Yellow Fever and Dengue-4 viruses simultaneously in a single reaction

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Zika, yellow fever and dengue viruses are three important flaviviruses that are epidemic and endemic in Central Africa, Middle and South America, Southeast Asia and Pacific Islands. Since these three viral diseases present with similar symptoms and there is a possibility of multiple flaviviruses co-infection, it is important to develop an effective and quick diagnostic tool. Here, we designed and validated a one-step triplex real time reverse transcription polymerase chain reaction (RT-PCR) using TaqMan probes targeting NS5 region for detecting zika virus (ZIKV), dengue virus-4 (DENV-4) and yellow fever virus (YFV). The assay was highly specific for the target viral RNA corresponding to ZIKV, DENV-4 and YFV strains. The sensitivity of the assay was compared to one-step, RT-PCR, it showed a 100-fold higher sensitivity than RT-PCR with ZIKV and DENV and 10-fold higher sensitivity with YFV. In conclusion, the one-step, triplex, TaqMan, real time RT-PCR assay identified and distinguished ZIKV, DENV-4 and YFV by targeting single gene.

Key words: Zika, yellow fever, dengue, multiplex real time reverse transcription-polymerase chain reaction (RT-PCR).

INTRODUCTION

Zika virus (ZIKV), yellow fever virus (YFV) and dengue virus (DENV) are identified as members of the Flaviviridae family that are transmitted by arthropods. *Aedes aegypti* and *Aedes albopictus* mosquitoes are the common vectors in the transmission of ZIKV, DENV and YFV (Gubler, 1998; Musso et al., 2015). There is currently an overlap in the distribution and vectors transmitting ZIKV, DENV, and YFV. Accordingly, ZIKV is

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present in Central and South America, including the Caribbean region, Central Africa, Southeast Asia and Pacific Islands. DENV is also distributed in Central and South America, Middle Africa, Southeast Asia and Pacific islands. Yellow Fever is mainly distributed and is endemic in Middle Africa and South America. In these regions, *A. aegypti*, is prevalent which makes all of these three diseases geographically related (Bhatt et al., 2013; Ferguson et al., 2016; CDC, 2018; Kraemer et al., 2015; WHO, 2019a). Since 2018, there are reports of unvaccinated travelers, to areas with yellow fever outbreaks contracting yellow fever. Several of these travelers died (CDC, 2018). WHO estimated that half a million people with severe dengue need hospitalization annually, and with an estimated 2.5% case mortality, yearly (WHO, 2019b). The recent outbreak of ZIKV in Brazil has been connected directly to infant microcephaly. Since that, Arboviruses such as ZIKV and DENV are becoming global public health concerns due to increasing burden of disease and their rapid spread in nature (Petersen et al., 2016). Another concern that raised public health attention is reporting in Brazil that there was an increase in Guillain-Barré syndrome cases due to ZIKV infection. Thus, differentiation between Zika and other flaviviruses diseases becomes necessary (Styczynski et al., 2017).

Studies have shown that human or other non-human vertebrate, mammalian and avian hosts can be infected with more than one flaviviruses with a single bite (Dupont-Rouzeyrol et al., 2015; Le Coupanec et al., 2017; Chaves et al., 2018) which may cause misdiagnosis. In addition, the clinical signs of early stages of infection with ZIKV, DENV and YFV are similar (Monath, 2001; Pongsiri et al., 2012; Sharma and Lal, 2017; Wasserman et al., 2016) which brings some difficulties in diagnosis. Real-time PCR is a rapid and specific test that can be used for the detection of various viral pathogens. The TaqMan real-time PCR method has high specificity and sensitivity which can be adapted for multiplex with greatly enhanced speed of detection. Multiplex single-reaction detection using real time RT-PCR and loop mediated isothermal amplification to detect ZIKV, chikungunya (CHIKV) and DENV have been developed in 2016 and 2017 (Boga et al., 2019; Pabbaraju et al., 2016; Waggoner et al., 2016; Yaren et al., 2018). But these studies did not include yellow fever until a very recent report (Wu et al., 2018) developed a multiplex real time RT-PCR assay targeting envelope protein of ZIKV, 3-untranslated region of DENV and YFV and E1 of chikungunya virus. Pabbaraju et al. (2016) developed a multiplex real time RT-PCR assay targeting the non-structural 5 (NS5) region of ZIKV, non-structural protein 4 (nsP4) from CHIKV and 3-untranslated region (3-UTR) of DENV. Waggoner et al. (2016) also reported a triplex real time RT-PCR assay targeting NS4B region of ZIKV, nsP2 from CHIKV and 5-UTR of DENV.

The aim of the current study is to develop a one-step triplex real time RT-PCR targeting a different region, the nonstructural protein 5 gene region, for simultaneous detection of ZIKV, DENV and YFV in a single tube.

MATERIALS AND METHODS

NS5 genes of ZIKV, DENV-4 and YFV were identified using DNA Star software (DNASTAR, Inc., Madison, WI, USA) by alignment of various sequences of the NS5 genes of a series of Flaviviruses selected from the NCBI GenBank database. The primers and probes were designed using Beacon Designer® Table 1. The primers and probes were synthesized by integrated DNA technologies (USA).

The viruses used in this study are listed in Table 2. QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) was used to extract genomic RNA from reference viruses and spiked clinical samples according to the manufacturer's instructions.

Qiagen one-step RT-PCR kit (Qiagen, USA) was used to amplify 111, 123 and 145 bp of NS5 gene of ZIKV, DENV-4 and YFV, respectively. The thermal cycling conditions included an initial activation of reverse transcriptase at 50°C for 30 min, a nucleic acid denaturation at 95°C for 15 min, followed by 35 cycles at 94°C for 30 s, 45°C for 30 s and 72°C for 1 min and a final extension cycle at 72°C for 10 min. The PCR products were analyzed by agarose gel electrophoresis using 1.5% agarose at 85 voltage for 1 h. Virus-specific amplicons were detected by the three sets of newly designed primers specific for ZIKV, DENV and YFV and three previously published specific primers for ZIKV, DENV (Waggoner et al., 2016) and flavivirusses (Bhatnagar et al., 2012).

One-step TaqMan real-time RT-PCR reactions were performed using iTaq universal probe one-step RT-PCR kit (Bio-Rad, USA). Single TaqMan real time RT-PCR was done first in order to test the specificity of each of the primer sets together with the specific probes for each virus. TaqMan real-time RT-PCR were run in a 20 µl reaction tube containing 10 µl of iTaq universal probe reaction mix, 0.5 µl iScript transcriptase, 1 µl of each forward and reverse primers (10 pmol/ul each) and 1 µl of fluorogenic probes (5 pmol/ul), 1 µl of RNA template (2 pmol in 20 µl reaction volume) and 5.5 µl Nuclease-free water. The thermal cycler Bio-Rad CFX96 real time system was used and the reaction was started with the reverse transcription step at 50°C for 10 min. Then a DNA denaturation at 95°C for 3 min was followed by 40 cycles of PCR with denaturation at 95°C for 10 s, annealing extension and plate read at 60°C for 30 s. Each individual reaction of the three viruses and 3 sets of primers and probes were done in duplicate. In each reaction, CHIKV viral RNA (ATCC® VR3246SD™) was used as negative control and non-template controls were also included. For the triplex TaqMan real time RT-PCR, known concentration of the viral RNA of each virus were mixed, 3 sets of primers and probes were placed in one tube and the reaction run under the same thermal conditions mentioned earlier.

The specificities and sensitivities of the real-time RT-PCR tests for ZIKV, DENV and YFV, were evaluated by testing fluorescence-labeled, virus-specific, probes against a known viral RNA panel from ZIKV, PRVABC59 with HEX dye probes, DENV-4, H241 with TexRd-XN dye probes, and YFV, 17D with 56-FAM dye probes.

The detection threshold of real time RT-PCR and conventional RT-PCR assays was determined by using 10-fold serial dilutions of these viruses. All detection assays were done in duplicate. Thus, the viral RNAs were serially diluted as follows: from 2.0×10^4 to 2×10^0 copies/reaction (total RNA from 20 pg/rx to 2 fg/rx) of ZIKV and 4.6×10^4 to 6.4×10^0 copies/reaction (total RNA from 300 pg/rx to 30 fg/rx) of DENV-4. Since the copy number of YFV was unknown, dilutions were based only on mass of total RNA starting from 300 pg/reaction of YFV viral RNA down to 3 fg/rx. RNAs of 25

Table 1. Sequences of the primers and probes used in the study.

Virus	Primers/Probes	Sequence(5′-3′)
YFV	F	CGGTATCTTGAGTTTGAG
	R	CAGGTCTCTGATCACATA
	P	56-FAM/AATGCCTTC/ZEN/CACTCCTCCTCC/3IABKFQ
ZIKV	F	GTCAGAGAAAGTGACCAA
	R	TGTGCAAACCTATCATCA
	P	5HEX/ATCTCCACT/ZEN/GACTGCCATTCGT/3IABKFQ
DENV-4	F	AGCCATTTTCAAACTAACTTA
	R	GTTGGTGAATGTGTTCAA
	P	5TexRd-XN/ATGTTCCAACCTGTCCACTACCTC/3BHQ-2

*Genomic position are based on the following sequences: Yellow Fever virus 17D (GeneBank: X02700), Zika virus PRVABC59 (GeneBank: KU501215) and Dengue virus type 4 H241 (GeneBank: AY947539). YFV, Yellow fever virus; ZIKV, Zika virus; DENV, dengue virus; F, forward primer; R, reverse primer; P, probe.

Table 2. Types and sources of viruses used in the study.

Virus Name	Strain Designation	Source
*AIV-H1N1	A/Swine/IA/31	National Veterinary Services and laboratory
AIV-H2N3	A/Mallard/ALB/77	National Veterinary Services and laboratory
AIV-H3N6	A/MAL/ALB/331185	National Veterinary Services and laboratory
*AIV-H5N2	A/TY/CA/209092/02	National Veterinary Services and laboratory
*AIV-H7N2	A/CK/NY/273874/03	National Veterinary Services and laboratory
Avian Encephalomyelitis Virus	AE19 (11/77)	University of Connecticut
Avian Reovirus	S1133	University of Connecticut
Dengue-1	Hawaii	WRCEVA
Dengue-2	New Guinea C (NGC)	WRCEVA
Dengue-3	H 87	WRCEVA
Dengue-4	H241	WRCEVA
Dengue-4	P 75-215 (BC 126-97)	WRCEVA
Dengue-4	703	WRCEVA
Dengue-4	M 30153 - AC 36	WRCEVA
Dengue-4	UIS 497	WRCEVA
Dengue-4	703-4	WRCEVA
Dengue-4	H 241	WRCEVA
Dengue-4	BC 156-95	WRCEVA
Infectious bronchitis virus	Mass 41	Charles River Laboratory
Mayaro virus	BE H 383185	WRCEVA
Mayaro virus	BE AN 343102	WRCEVA
Newcastle disease virus	Lasota	University of Connecticut
Yellow fever virus	17 D (Vaccine strain)	WRCEVA
Zika virus	DAK AR 41667	WRCEVA
Zika virus	R 103451	WRCEVA
Zika virus	SJRP-HB-2016-1911 (2040081)	WRCEVA
Zika virus	MEX I- 44	WRCEVA
Zika virus	PRVABC 59	WRCEV
Zika virus	MR 766	WRCEVA
Chikungunya	181/25 (vaccine strain)	WRCEVA

*Low Pathogenic Avian influenza virus (AIV).

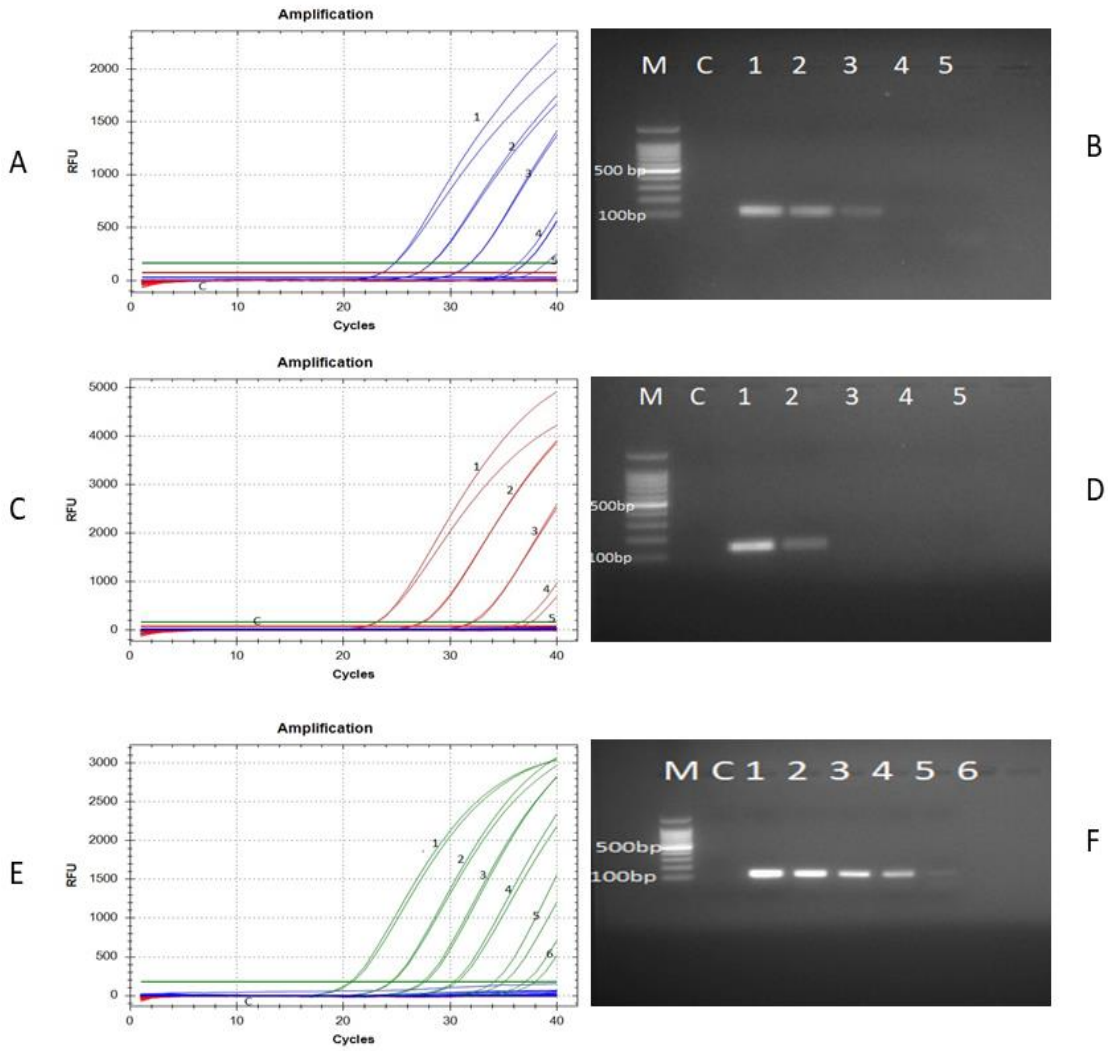

Figure 1. Comparative sensitivity of the TaqMan real time PCR and RT-PCR. (A) Sensitivity for detection of ZIKV viral RNA by TaqMan real time PCR; (B) Sensitivity for detection of ZIKV viral by RT-PCR [1: 20 pg/Rx; 2: 2 pg/Rx; 3: 200 fg/Rx; 4: 20 fg/Rx and 5: 2 fg/Rx]; (C) Sensitivity for detection of DENV-4 viral RNA by TaqMan real time RT-PCR (D) Sensitivity for detection of DENV-4 viral by RT-PCR [1: 300 pg/Rx; 2: 30 pg/Rx; 3: 3 pg/Rx; 4: 300 fg/Rx and 5: 30 fg/Rx]; (E) Sensitivity for detection of YFV viral RNA by TaqMan real time RT-PCR; and (F) Sensitivity for detection of YFV viral by RT-PCR [1: 300 pg/Rx; 2: 30 pg/Rx; 3: 3 pg/Rx; 4: 300 fg/Rx; 5: 30 fg/Rx and 6: 3 fg/Rx]; M: 100 bp DNA ladder and C: negative control.

different viruses reference included in Table 2 were tested for specificity by real time RT-PCR and conventional RT-PCR. In order to assess the detection ability of our assay in cases of ZIKV, DENV and YFV co-infections, the assay was performed by combining the RNA extracted from ZIKV, DENV-4 and YFV.

In order to evaluate the triplex TaqMan real-time RT-PCR using clinical specimens, heat-inactivated culture supernatant of seven viral strains (3 ZIKA, 3 DENV and 1 YFV) were diluted to four 10-fold serial dilutions in human serum provided by Innovative™ Research (single donor from a 42-year-old black female). Total nucleic acids were extracted from the spiked serum using QIAamp® viral RNA mini kit (Qiagen) in duplicate. After extraction, RNAs were tested by triplex TaqMan real time RT-PCR and one step RT-PCR assay to study the sensitivity of detection differences. Known ZIKV PRVABC59, DENG-4 H241 and YFV 17D viral RNA were used as positive controls.

RESULTS AND DISCUSSION

As shown in Figure 1, the detection limit of the single one step TaqMan real-time RT-PCR assay was determined using 10-fold serially diluted ZIKV PRVABC59, DENG-4 H241 and YFV 17D viral RNA and compared to conventional one step RT-PCR. The detection limits were calculated to be 2 fg/Rx (2×10° RNA copies/Rx) for ZIKV and 300 fg/Rx (4.6×10° RNA copies/reaction) for DENV-4 and the detection limit was 3 fg/reaction of YFV by TaqMan real tine RT-PCR. On the other hand, the detection limits by conventional one step RT-PCR were200 fg/Rx, 30 pg/Rx and 30 fg/Rx of ZIKV, DENV and YFV, respectively. A high correlation between Cq

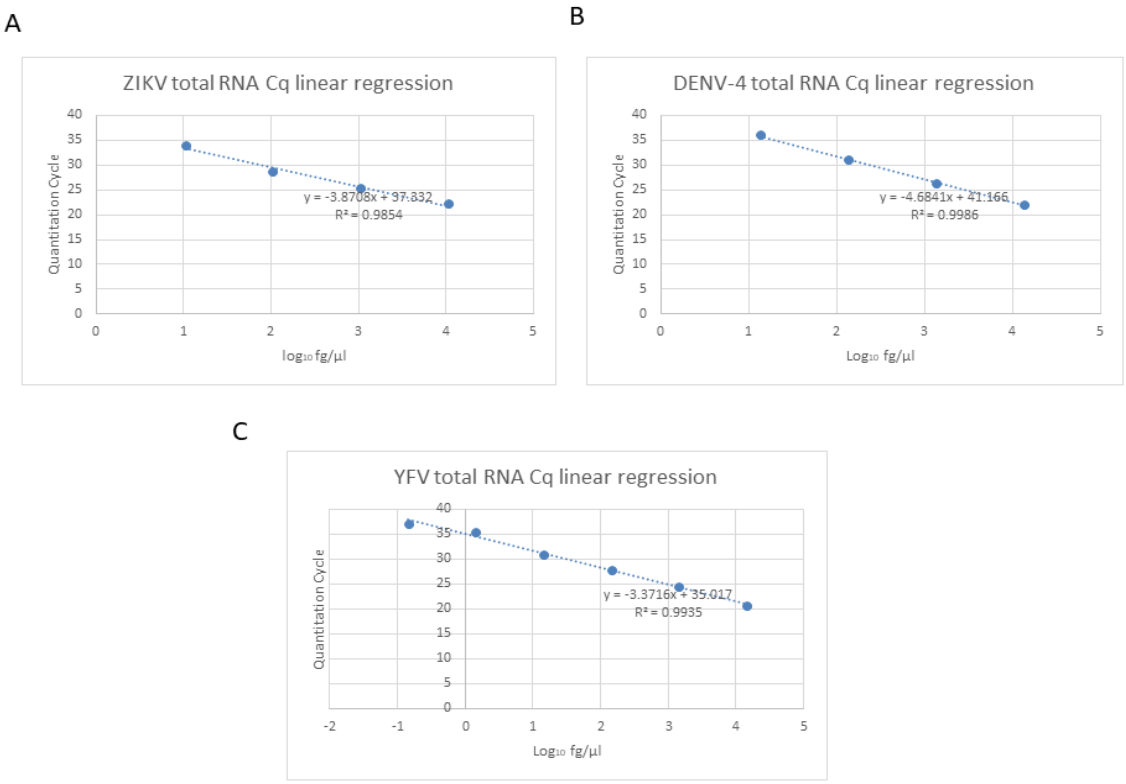

Figure 2. The linear regression of TaqMan real time RT-PCR for (A) ZIKV, (B) DENV-4 and (C) YFV.

and concentration was calculated for each of the three viruses under testing. Thus R^2 of 0.9854, 0.9986 and 0.9935 was calculated for ZIKV, DENV-4 and YFV, respectively as shown in Figure 2.

The RNAs of ZIKV, DENV-4 and YFV (Table 2) were amplified by the newly designed triplex TaqMan Real time RT-PCR to evaluate the cross-reactivity of the assay. Besides these RNAs, RNA of Mayaro viruses (MAYV), DEN-1, 2 and 3 and CHIKV were used as a representative member of alphavirus and flavivirus in the cross-amplification study. Furthermore, a panel of extra RNA viruses, including avian influenza virus H1N1, H2N3, H3N6, H5N2 and H7N2, avian encephalomyelitis virus, avian reovirus, infectious bronchitis virus and newcastle disease virus were also evaluated. Only Zika, Dengue-4 and yellow fever viruses showed positive results and no cross-reactivity was observed among other tested viruses.

Multiplex real time RT-PCR is rapid; sensitive that can greatly reduce the monetary and specially the time issues confronted with the other more traditional tests. Only few studies have been devoted to the development of multiplex real-time RT-PCR assay for detection of different flaviviruses such as DENV, ZIKV and CHIKV (Pabbaraju et al., 2016; Waggoner et al., 2016). Though, none of these studies included YFV in their assays until recently, Wu et al. (2018) reported the development of a multiplex real-time RT-PCR assay for DENV, ZIKV, YFV and chikungunya virus targeting 4 different genes. In this study, we have developed a new sensitive and specific one-step triplex TaqMan-RT-PCR targeting NS5 gene for detection of ZIKV, YFV and DENV-4 in serum and cell culture supernatants using specific Taqman probes in a single tube. Genes encoding NS5 are the most conserved regions in the coding region of the flavivirus genome based on sequence similarity analysis of NS5 (Chao et al., 2007; Fulop et al., 1993). BLAST analysis confirmed that the primer and probe sets that were used in the current study, were specific for the NS5 genes of ZIKv, DENV and YFV, respectively. Originally, the assay was carried out in single reactions, along with the testing of other closely related flaviviruses, such as DENV-1, DNV-2, DENV-3, mayaro virus and chikungunya virus, and other viruses. Only ZIKV, DENV-4 and YFV were properly detected by each single and multiplex assay, which show an appropriate diagnostic specificity of the assay.

The detection limit of the TaqMan real time RT-PCR assay was compared to the conventional RT-PCR assay, showing a 100-fold higher sensitivity of one step TaqMan real time RT-PCR than the conventional one step RT-PCR with ZIKV and DENV, whereas TaqMan real time RT-PCR showed 10-fold higher sensitivity than conventional RT-PCR with YFV.

MR 766 in this study is less detectable than other two closely related American Zika virus strain. When sequences are aligned, PRVABC59 show 10% nucleotide

Table 3. Results of Taqman real-time RT-PCR of the spiked samples in comparison to conventional RT-PCR.

Virus strain	Dilutions	Average Ct value	log10 of RNA fg/µL	RT-PCR results
		ZIKV		
PRVABC59	10^0	22.76	5.1	Positive
	10^{-1}	25.38	4.4	Positive
	10^{-2}	31.84	2.7	Negative
	10^{-3}	32.28	2.6	Negative
R 103451	10^0	18.94	6.1	Positive
	10^{-1}	22.33	5.2	Positive
	10^{-2}	25.28	4.4	Positive
	10^{-3}	28.22	3.7	Positive
MR 766	10^0	35.4	1.8	Negative
	10^{-1}	N/A	N/A	Negative
	10^{-2}	N/A	N/A	Negative
	10^{-3}	N/A	N/A	Negative
		DENV-4		
703-4	10^0	22.1	5.4	Positive
	10^{-1}	26.59	4.4	Positive
	10^{-2}	30.41	3.6	Positive
	10^{-3}	32.82	3.1	Positive
H 241	10^0	18.75	6.1	Positive
	10^{-1}	21.58	5.5	Positive
	10^{-2}	28.05	3.0	Positive
	10^{-3}	33.05	4.1	Positive
BC 156-95	10^0	25.48	4.6	Positive
	10^{-1}	29.61	3.83	Positive
	10^{-2}	32.6	3.1	Positive
	10^{-3}	35.29	2.6	Negative
		YELLOW FEVER		
YFV 17D	10^0	23.02	4.9	Positive
	10^{-1}	30.32	2.7	Positive
	10^{-2}	31.2	2.4	Positive
	10^{-3}	35.4	1.2	Positive

change compare to MR 766. Thirty eight amino acids are mutated and randomly distributed in NS5 region. This may be the reason that influences the sensitivity when detecting this much earlier isolated African strain (Staufta et al., 2016). If the multiplex real time RT-PCR cannot effectively detect this strain, other assistant detecting method such as ELISA or PRNT50 could be applied for further confirmation in diagnostic process or with another specifically designed probe.

In order to test whether the TaqMan real-time RT-PCR assay could be used to detect infection among patients, negative human serum specimens were spiked with heat inactivated supernatant 10 fold serially diluted cultures of ZIKV, DENV-1 and YFV. Serum samples were extracted in duplicate, and were tested by TaqMan real-time RT-PCR and conventional RT-PCR tests. TaqMan real-time RT-PCR was able to detect viral RNA in spiked serum samples with higher sensitivity than RT-PCR. Estimated ZIKV concentration ranged from 2.6 to 5.0 log10 fg/µl for the PRVABC59, from 3.6 to 6.0 log10 fg/µl for R 103451 strain and only 1.8 log10 fg/µl for MR 766. Estimated concentration of DENV-4 ranged from 3.1 to 5.4 log10 fg/µl for the 703-4 strain, from 3.0 to 6.1 log10 fg/µl for the H 241strain and from 2.6 to 4.6 log10 fg/µl for the BC 156-95 strain. Whereas the estimated concentration for YFV 17D strain ranged from 1.2 to 4.8 log10 fg/µl (Table 3). All amplification by TaqMan real time-RT-PCR was linear for each tested strain (R2 for each linear regression>0.99).

The results confirmed that the current Triplex RT-PCR

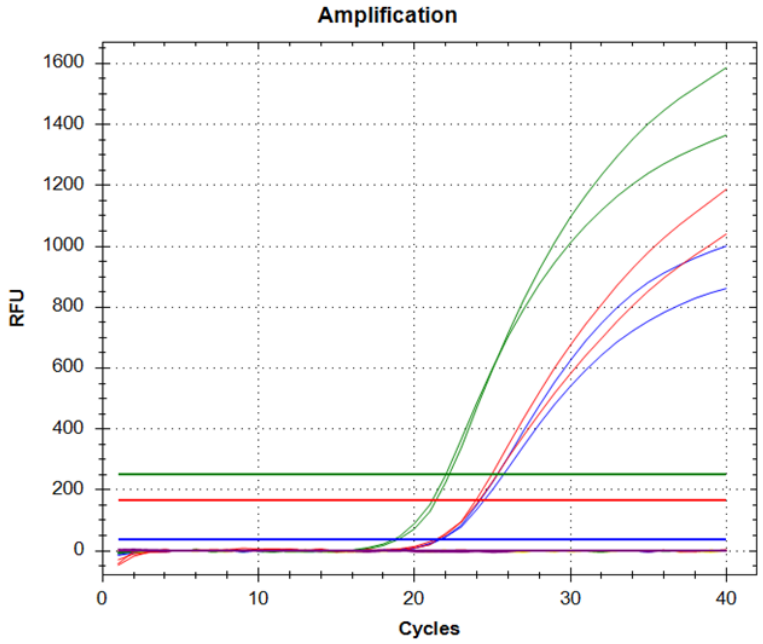

Figure 3. Amplification plot of the Triplex TaqMan real time RT-PCR showed detection of ZIKV, DENV-4 and YFV viral RNA in single tube. Blue: ZIKV with HEX dye probes, Red: DENV-4 with TexRd-XN dye probes, Green: YFV with 6-FAM dye probes.

test can simultaneously detect the three viruses in a single tube (Figure 3).

Given the concern for ZIKV, DENV and YFV emergence and only a few multiplex real-time PCR tests that have been assessed and reported in the literature, the current assay should provide a useful diagnostic tool for detection and differentiation between these three flaviviruses in infected patients. Current assay was able to detect and differentiate between ZIKV, DENV and YFV targeting single gene of flaviviruses. Mayaro and chikungunya viruses are alphaviruses that have genetic similarity to ZIKV, DENV and YFV and also they are etiologic agents responsible for human febrile illness (Schmaljohn and McClain, 1996). Mayaro virus now is affecting the whole Western Hemisphere after the chikungunya and zika virus emerging in 2013 to 2014 and become the modern concern (Hotez and Murray, 2017). Our novel assay could not detect mayaro and chikungunya viruses, which indicated the specificity and usefulness as a diagnostic tool of our test for falviviruses. Similar multiplex tests for detection of alphaviruses are required. In the future, multiplex TaqMan real-time RT-PCR can be expanded to detect and differentiate between DENV-1 and DENV-4.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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REFERENCES

Bhatnagar J, Blau DM, Shieh, WJ, Paddock CD, Drew C, Liu L, Jones T, Patel M, Zaki SR (2012). Molecular detection and typing of dengue viruses from archived tissues of fatal cases by rt-PCR and sequencing: diagnostic and epidemiologic implications. The American Journal of Tropical Medicine and Hygiene 86(2):335-340.

Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI (2013). The global distribution and burden of dengue. Nature 496(7446):504-507.

Boga JA, Alvarez-Arguelles ME, Rojo-Alba S, Rodríguez M, de Oña M, Melón S (2019). Simultaneous detection of Dengue virus, Chikungunya virus, Zika virus, Yellow fever virus and West Nile virus. Journal of Virological Methods 268:53-55.

Centers for Disease Control and Prevention (CDC) (2018). https://wwwnc.cdc.gov/travel/notices/alert/yellow-fever-brazil

Chao D, Davis BS, Chang GJ (2007). Development of Multiplex Real-Time Reverse Transcriptase PCR Assays for Detecting Eight Medically Important Flaviviruses in Mosquitoes. Journal of Clinical Microbiology 45(2): 584-589.

Chaves BA, Orfano AS, Nogueira PM, Rodrigues NB, Campolina TB, Nacif-Pimenta R, Pires ACAM, Júnior AB, Paz ADC, Vaz EBDC, Guerra MDGVB, Silva BM, de Melo FF, Norris DE, de Lacerda MVG, Pimenta PFP, Secundino NFC (2018). Coinfection with Zika Virus (ZIKV) and Dengue Virus Results in Preferential ZIKV Transmission by Vector Bite to Vertebrate Host. The Journal of Infectious Diseases 218(4):563-571.

Dupont-Rouzeyrol M, O'Connor O, Calvez E, Daurès M, John M, Grangeon JP (2015). Gourinat AC.Co-infection with Zika and dengue viruses in 2 patients, New Caledonia, 2014. Emerging Infectious Diseases 21(2):381-382.

Ferguson NM, Cucunubá ZM, Dorigatti I, Nedjati-Gilani GL, Donnelly CA, Basáñez MG, Nouvellet P, Lessler J (2016). EPIDEMIOLOGY. Countering the Zika epidemic in Latin America. Science 353:aag0219.

Fulop L, Barrett AD, Phillpotts R, Martin K, Leslie D, Titball RW (1993). Rapid identification of flaviviruses based on conserved NS5 gene sequences. Journal of Virological Methods 44(2-3):179-188.

Gubler DJ (1998). Dengue and dengue hemorrhagic fever. Clinical Microbiology Reviews 11:480-496.

Hotez PJ, Murray KO (2017). Dengue, West Nile virus, chikungunya, Zika-and now Mayaro?. PLOS Neglected Tropical Diseases 11:e0005462.

Kraemer MUG, Sinka ME, Duda KA, Mylne AQ, Shearer FM, Barker CM, Moore CG, Carvalho RG, Coelho GE, Van Bortel W, Hendrickx G, Schaffner F, Elyazar IR, Teng HJ, Brady OJ, Messina JP, Pigott DM, Scott TW, Smith DL, Wint GR, Golding N, Hay SI (2015). The global distribution of the arbovirus vectors Aedes aegypti and Ae. albopictus. eLife 4:e08347.

Le Coupanec A, Tchankouo-Nguetcheu S, Roux, P, Khun H, Huerre M, Morales-Vargas R, Enguehard M, Lavillette D, Missé D, Choumet V (2017). Co-Infection of Mosquitoes with Chikungunya and Dengue Viruses Reveals Modulation of the Replication of Both Viruses in Midguts and Salivary Glands of Aedes aegypti Mosquitoes. International Journal of Molecular Sciences 18(8):1708.

Monath TP (2001). Yellow fever: an update. The Lancet Infectious Disease 1(1):11-20.

Musso D, Cao-Lormeau VM, Gubler DJ (2015). Zika virus: following the path of dengue and chikungunya? Lancet 386:243-244.

Pabbaraju K, Wong S, Gill K, Fonseca K, Tipples GA, Tellier R (2016). Simultaneous detection of Zika, chikungunya and dengue virusesby a multiplex real-time RT-PCR assay. Journal of Clinical Virology 83:66-71.

Petersen LR, Jamieson DJ, Powers AM, Honein MA (2016). Zika virus. The New England Journal of Medicine 374:1552-1563.

Pongsiri P, Praianantathavorn K, Theamboonlers A, Payungporn S, Poovorawan Y (2012). Multiplex real time RT–PCR for detecting chikungunya virus and dengue virus. Asian Pacific Journal of Tropical Medicine 5(5):342-346.

Schmaljohn AL, McClain D (1996). Alphaviruses (Togaviridae) and Flaviviruses (Flaviviridae). In Medical Microbiology (ed. Baron, S.) (University of Texas Medical Branch at Galveston.

Sharma A, Lal SK (2017). Zika virus: transmission, detection, control, and prevention. Frontiers in Microbiology 8:110.

Staufta CB, Gorbatsevych O, Cello J, Wimmera E, Futcher B (2016). Comparison of African, Asian, and American Zika Viruses in Swiss Webster mice: Virulence, neutralizing antibodies, and serotypes. bioRxiv https://doi.org/10.1101/075747

Styczynski AR, Malta JMAS, Krow-Lucal ER, Percio J, Nóbrega ME, Vargas A, Lanzieri TM, Leite PL, Staples JE, Fischer MX, Powers AM, Chang GJ, Burns PL, Borland EM, Ledermann JP, Mossel EC, Schonberger LB, Belay EB, Salinas JL, Badaro RD, Sejvar JJ, Coelho GE (2017). Increased rates of Guillain-Barré syndrome associated with Zika virus outbreak in the Salvador metropolitan area, Brazil. PLOS Neglected Tropical Diseases 11(8):e0005869.

Waggoner JJ, Gresh L, Mohamed-Hadley A, Ballesteros G, Davila MJ, Tellez Y, Sahoo MK, Balmaseda A, Harris E, Pinsky BA (2016). Single-reaction multiplex reverse transcription PCR for detection of Zika, chikungunya, and dengue viruses. Emerging Infectious Diseases 22(7):1295-1297.

Wasserman S, Tambyah PA, Lim PL (2016). Yellow fever cases in Asia: primed for an epidemic. International Journal of Infectious Diseases 48:98-103.

World Health Organization (WHO) (2019a). https://www.who.int/csr/don/09-january-2019-yellow-fever-nigeria/en/

World Health Organization (WHO) (2019b). https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue

Wu W, Wang J, Yu N, Yan,J, Zhuo Z, Chen M, Su X, Fang M, He S, Zhang S, Zhang Y, Ge S, Xia N (2018). Development of multiplex real-time reverse-transcriptase polymerase chain reaction assay for simultaneous detection of Zika, dengue, yellow fever, and chikungunya viruses in a single tube. Journal of Medical Virology 90(11):1681-1686.

Yaren O, Alto BW, Bradley KM, Moussatche P, Glushakova L, Benner SA (2018). Multiplexed isothermal amplification based diagnostic platform to detect zika, chikungunya, and dengue 1. Journal of Visualized Experiments 13:133.