

PRIMER PAIR, KIT AND METHOD FOR DETECTING ZIKA VIRUS

FIELD OF THE INVENTION

[0001] The present invention relates to primer pair, kit and method for detecting Flavivirus, and more particularly to primer pair, kit and method for detecting Zika virus.

BACKGROUND OF THE INVENTION

[0002] Zika virus is a mosquito-borne flavivirus that was first identified in Uganda in 1947 in monkeys. It was later identified in humans in 1952 in Uganda and the United Republic of Tanzania. The virus is transmitted between humans by virus-carrying Aedes mosquitoes through mosquito bites.

[0003] Before 2015, Zika outbreaks typically occurred in Africa, Southeast Asia, and the Pacific Islands. However, due to globalization, the most prominent outbreak which started in March 2015 in Brazil, soon spread throughout the Americas, Africa, Asia, and Europe. The symptoms of the disease range from asymptomatic to mild symptoms such as fever, rash, headache, joint pain, and conjunctivitis and muscle pain. Typically, the mild symptoms last for several days to a week and the infected people may not even realize they have been infected. However, recent findings suggest that Zika infection can cause neurological disorders such as Guillain-Barré syndrome and serious birth defects called congenital Zika syndrome, with the most prominent feature in severe microcephaly in newborns of infected mother.

[0004] At current time, two techniques, which are serological test and real-time reverse transcription polymerase chain reaction (real-time RT-PCR), are commonly used for the diagnosis of Zika. These techniques confirm Zika infections by the detection of specific antibodies for Zika and viral RNA,

respectively. Serological test, although being able to provide a definitive result ranging from 10 minutes to a few hours, this method involves the detection of IgM antibodies in the patient's serum which only appear a few days after the onset of the clinical symptoms (e.g. fever). In addition, the specificity of serological tests is questionable due to the possibility of false positives generated by cross reactions with IgMs for other arboviruses (especially if the patient has a previous history of infection from other flaviviruses). Real-time RT-PCR is a more recent diagnostics solution which addresses the shortcomings of the above-mentioned methods. Real-time RT-PCR enables a fast detection of the Zika virus genome, even in the initial phase of infection, as it is more sensitive. Chances of false positives are also lower due to higher specificity.

[0005] While the sensitivity and the specificity of Zika detection have been improved over the years, the speed offers by current real-time RT-PCR test still falls short as typical RT-PCR turnaround time is almost one and half hours. The current pressing need is to have a Point-of-Care diagnostic assay that provides rapid turnaround time for Zika detection which is sensitive and specific.

SUMMARY OF THE INVENTION

[0006] An object of the embodiment of the present invention is to provide a primer pair for detecting Zika virus with high sensitivity, high specificity, and reduced reaction time.

[0007] Another object of the embodiment of the present invention is to provide a kit for detecting Zika virus with high sensitivity, high specificity, and reduced reaction time.

[0008] An additional object of the embodiment of the present invention is to provide a method for detecting Zika virus with high sensitivity, high specificity, and reduced reaction time.

[0009] According to an aspect of the embodiment of the present invention, there is provided a primer pair for detecting Zika virus. The primer pair includes a forward primer including a sequence of SEQ ID NO: 1, and a reverse primer including a sequence of SEQ ID NO: 2.

[0010] In an embodiment, the forward primer and the reverse primer are used for real-time reverse transcription polymerase chain reaction.

[0011] According to another aspect of the embodiment of the present invention, there is provided a kit for detecting Zika virus. The kit includes a forward primer including a sequence of SEQ ID NO: 1, a reverse primer including a sequence of SEQ ID NO: 2, and a probe.

[0012] In an embodiment, the probe includes a sequence of SEQ ID NO: 3.

[0013] In an embodiment, the probe includes a sequence of the complementary sequence of SEQ ID NO: 3.

[0014] In an embodiment, the probe is labeled with a 5'-reporter dye and a 3'-quencher.

[0015] In an embodiment, the forward primer, the reverse primer, and the probe are used for real-time reverse transcription polymerase chain reaction.

[0016] According to an additional aspect of the embodiment of the present invention, there is provided a method for detecting Zika virus, wherein the method includes amplifying nucleic acid from Zika virus using real-time reverse transcription polymerase chain reaction with a forward primer, a reverse primer, and a probe, wherein the forward primer includes a sequence of SEQ ID NO: 1, and the reverse primer includes a sequence of SEQ ID NO: 2.

[0017] In an embodiment, the probe includes a sequence of SEQ ID NO: 3.

[0018] In an embodiment, the probe includes a sequence of the complementary sequence of SEQ ID NO: 3.

[0019] In an embodiment, the probe is labeled with a 5'-reporter dye and a 3'-quencher.

[0020] In an embodiment, the nucleic acid from Zika virus is RNA.

[0021] In an embodiment, the method further includes sub-steps of converting a genomic RNA of Zika virus to a complementary DNA by reverse transcription, and amplifying and detecting the complementary DNA by real-time polymerase chain reaction with the forward primer, the reverse primer, and the probe.

[0022] It is contemplated that any embodiment of a method or composition described herein can be implemented with respect to any other method or composition described herein.

[0023] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

[0024] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternative are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or."

[0025] Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating specific embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] FIG. 1 shows the partial sequence of the envelope gene and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence of the envelope gene;

[0027] FIG. 2 shows the DNA sequences of the forward primer, the reverse primer, and the probe;

[0028] FIG. 3 shows the temperature profile of the real-time RT-PCR in the fast cycling mode;

[0029] FIG. 4 shows the results of the specificity analysis;

[0030] FIG. 5 shows the sigmoidal curve for estimation of the limit of detection (LoD);

[0031] FIG. 6 shows the amplification plots generated from different copies of Zika RNA; and

[0032] FIG. 7 shows the standard curve for the Zika assay generated from the results in FIG. 6.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0033] The present invention will now be described more specifically with reference to the following embodiments. It is to be noted that the following descriptions of the embodiments of this invention are presented herein for purpose of illustration and description only; it is not intended to be exhaustive or to be limited to the precise form disclosed.

[0034] The embodiment of the present invention utilizes real-time reverse transcription polymerase chain reaction (real-time RT-PCR), also called quantitative reverse transcription polymerase chain reaction (qRT-PCR), with probe-based detection for rapid, sensitive and specific diagnosis of Zika infections in humans. The real-time RT-PCR includes a first step of reverse transcription of converting the viral genomic RNA from Zika to a complementary DNA (cDNA)

and then a second step of real-time PCR whereby the cDNA is amplified with the primer pairs. Particularly, in the real-time RT-PCR, the specific forward primer, reverse primer, and probe hybridize to the cDNA target of Zika, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated, so that the resulting increase in fluorescence can be detected. In an embodiment, the reporter dye is FAM fluorescence, and the quencher is BHQ1 group.

[0035] Zika virus is a single-strand RNA virus having 10675 bases (Zika virus strain PRVABC59; NCBI Reference Sequence KU501215.1). In the present embodiment, the envelope (E) gene of Zika virus was selected as the target for the detection. FIG. 1 shows the partial sequence of the envelope gene (SEQ ID NO: 4, which is from position 2101 to position 2275 of KU501215.1 Sequence) and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence of the envelope gene. FIG. 2 shows the DNA sequences of the forward primer, the reverse primer, and the probe. The forward primer has the sequence of SEQ ID NO: 1 (5'- GGAGTGGCAGCACCATTG -3'), which starts at position 2181 and includes 18-mer. The reverse primer has the sequence of SEQ ID NO: 2 (5'- CAGGCTGTGTCTCCCAAGA -3'), which starts at position 2262 and includes 19-mer. The probe is a reverse probe and has the sequence of SEQ ID NO: 3 (5'- TTCTCTTGGCACCTCTCACAGTGGCTTCAA -3'), which starts at position 2237 and includes 30-mer. The forward primer and the reverse primer are predicted to generate an amplicon size of 82 base pairs.

[0036] To ascertain the specificity of the primers and the probe for Zika, each of the primers and the probe listed in FIG. 2 was checked by NCBI BLAST against two databases including human genomic plus transcript and nucleotide collection, and the blast result shows that no other similar species have 100% same fragment

compared to both SEQ ID NO: 1 and SEQ ID NO: 3 of the embodiment of the present invention. However, SEQ ID NO: 2, shows 100% sequence similarity to Dengue serotype 3. Since the primers and probe work together as a set, even when the reverse primer (SEQ ID NO: 2) can bind to Dengue serotype 3's genomic material, the forward primer (SEQ ID NO: 1) and the reverse probe (SEQ ID NO: 3) will not bind to Dengue serotype 3's genomic material. That is to say, the set of primers and probe in the embodiment of the present invention will not generate a false positive with Dengue serotype 3, and is still specific in detecting the envelope gene of Zika virus. In other words, the specificity of the primers and the probe to Zika virus is quite high, and the primers and the probe may be used to amplify and detect the envelope gene of Zika virus. In an embodiment of the present invention, the primers and the probe can be only used to amplify and detect the envelope gene of Zika virus.

[0037] Therefore, the embodiment of the present invention provides a primer pair for detecting Zika virus, including the forward primer including a sequence of SEQ ID NO: 1 and the reverse primer including a sequence of SEQ ID NO: 2. The embodiment of the present invention also provides a kit for detecting Zika virus, including the forward primer including a sequence of SEQ ID NO: 1, the reverse primer including a sequence of SEQ ID NO: 2, and the probe including a sequence of SEQ ID NO: 3. On the other hand, the embodiment of the present invention also provides a method for detecting Zika virus, the method including amplifying nucleic acid from Zika virus using real-time reverse transcription polymerase chain reaction with the forward primer including a sequence of SEQ ID NO: 1, the reverse primer including a sequence of SEQ ID NO: 2, and the probe including a sequence of SEQ ID NO: 3. Particularly, the method further includes sub-steps of converting the viral genomic RNA of Zika virus to a complementary DNA (cDNA)

by reverse transcription, and amplifying and detecting the complementary DNA by real-time polymerase chain reaction with the forward primer including a sequence of SEQ ID NO: 1, the reverse primer including a sequence of SEQ ID NO: 2, and the probe including a sequence of SEQ ID NO: 3.

[0038] In some other embodiments of the present invention, since the primer pair is specific to Zika virus, all the sequence located between the forward primer and the reverse primer may be used as the probe sequence, and thus, the probe sequence is not limited to the aforesaid sequence. Further, the probe can be designed to hybridize to any strand of the DNA, so both the complementary sequences at the same location can be used as the probe sequence. Therefore, the complementary sequence of the aforesaid probe sequence may also be used as the probe sequence for detecting Zika virus. Accordingly, the embodiment of the present invention also provides a method for detecting Zika virus, the method including amplifying nucleic acid from Zika virus using real-time reverse transcription polymerase chain reaction with the forward primer including a sequence of SEQ ID NO: 1, the reverse primer including a sequence of SEQ ID NO: 2, and a probe including a sequence of the complementary sequence of SEQ ID NO: 3.

[0039] The primer pair and the probe in the embodiment of the present invention are optimized for the rapid detection of Zika virus through real-time RT-PCR. The real-time RT-PCR is conducted in a fast cycling mode. FIG. 3 shows the temperature profile of the real-time RT-PCR in the fast cycling mode. In an embodiment, the fast cycling mode is herein defined as a real-time RT-PCR is conducted where the temperature profile is configured as: (1) Hold stage for reverse transcription at 42°C for 5 minutes, followed by (2) Hold Stage for initial denaturation and enzyme activation at 95°C for 30 seconds, followed by 40 cycles

of (3) PCR stage for denaturation at 95°C for 1 second and annealing/extension at 60°C for 5 seconds. In this fast cycling mode, considerable time is saved for the following four aspects. First, time is saved with having a short annealing/extension time of 5 seconds, while present commercially available competing products generally require 60 seconds. Second, time is saved with having a shorter denaturation time of 1 second, while the present commercially available competing products generally require 15 seconds. Third, the reverse transcription time is shortened as 5 minutes, while the present commercially available competing products generally require 20 minutes. Fourth, the initial denaturation and enzyme activation time is shortened as 30 seconds, while the present commercially available competing products generally require 2 minutes.

[0040] Accordingly, the time saved in the fast cycling mode with the primer pair and the probe in the embodiment of the present invention results in at least a 50% shorter overall reaction time than that of the standard RT-PCR reaction on the same type of thermal cycler, so the embodiment of the present invention is capable of detecting Zika in less than 30 minutes. In other words, the primer pair and the probe in the embodiment of the present invention allow for the rapid detection of Zika through real-time RT-PCR by optimizing design for reduced denaturation/annealing/extension timing per cycle, reduced reverse transcription time and reduced initial denaturation time, thus resulting in at least 50% shorter overall reaction time. All these were achieved without compromising on the sensitivity and specificity of Zika detection.

[0041] The following describes an example of the method for detecting Zika virus of the embodiment of the present invention, and the reaction mixture is shown in Table 1. The real-time RT-PCR was performed using the fast cycling mode described above with a reaction mixture including Takara's One Step

PrimeScript™ RT-PCR Kit (Perfect Real Time; #RR064A), 500 nM of the forward primer (SEQ ID NO: 1), 500 nM of the reverse primer (SEQ ID NO: 2), and 200 nM of the probe (SEQ ID NO: 3) in a total volume of 20 µL. The reaction mixture is however not limited to the above mentioned enzymes and buffer system.

Table 1

	real-time RT-PCR components	Volume (µl)	Concentration
TaKaRa's Kit	2X One Step RT-PCR Buffer III	10	1X
	TaKaRa Ex Taq HS	0.4	2.5 U/µl
	PrimeScript RT enzyme Mix II	0.4	-
	RNase Free dH ₂ O	5.8	-
	Forward Primer (10 µM)	1	500 nM
	Reverse Primer (10 µM)	1	500 nM
	Probe (10 µM)	0.4	200 nM
	Genomic RNA Sample	1	-
		Total volume: 20 µl	

[0042] To further experimentally confirm the specificity of the primer pair and the probe for Zika detection, the primer pair and the probe were checked for cross-reactivity with a few common mosquito-borne viruses, including Dengue viruses (serotypes 1 to 4) and Chikungunya virus. FIG. 4 shows the results of the specificity analysis, wherein CHIK, DEN1 to DEN4, and ZIKA means genomic RNA samples from Chikungunya virus, Dengue virus serotypes 1 to 4, and Zika virus, respectively, and NTC means no template control. From FIG. 4, it is clear that no amplification was observed except when Zika genomic RNA was the sample used. That is to say, no cross-reactivity was found, thus confirming the

specificity of the primer pair and the probe in targeting the envelope gene of Zika virus only.

[0043] The embodiment of the present invention is capable of detecting down to just a few copies of Zika genomic RNA per reaction, in less than 30 minutes. In the sensitivity assay, the samples containing 1, 3, 5, 8, 10 and 15 copies of Zika genomic RNA per sample were tested in 6 replicates, and the cut-off for a positive detection was set at Ct value 38. As a result, the fractions of positive detection for the samples containing 1, 3, 5, 8, 10, and 15 copies per sample were 1/6, 2/6, 4/6, 5/6, 6/6 and 6/6, respectively. The measured fractions were fitted to a sigmoidal curve for estimation of the limit of detection (LoD) as shown in FIG. 5, wherein at 95% confidence, LoD was 9.36 copies of genomic RNA per reaction. That is to say, the primer pair and the probe of the embodiment of the present invention are capable of detecting down to 10 copies of Zika genomic RNA per reaction, which reveals high sensitivity.

[0044] The embodiment of the present invention also allows the quantitation of Zika RNA, in less than 30 minutes. FIG. 6 shows the amplification plots generated from different copies of Zika RNA. The samples had serially diluted copies (10 to 10^7 copies per reaction) of *in vitro* transcribed Zika RNA in duplicate to determine the linearity of the Zika assay when the primer pair and the probe of the embodiment of the present invention were used. FIG. 7 shows the standard curve for the Zika assay generated from the results in FIG. 6. Based on the standard curve, the efficiency of the amplification was 105% with an R^2 value of 0.99. As the R^2 value was close to the theoretical optimum of 1.0, this Zika assay could be expanded to a quantitative assay to estimate the amount of Zika genomic RNA for Point-of-Care testing.

[0045] In conclusion, the embodiment of the present invention provides the primer pair, kit, and method for detecting Zika virus using real-time RT-PCR with specific primer pair and probe. The primer pair, kit and method of the embodiment of the present invention have the advantage of high sensitivity, and allow the detection of low copies of Zika RNA. The primer pair, kit and method of the embodiment of the present invention further have advantage of high specificity, which is able to differentiate Zika virus from other mosquito-borne viruses, and this is important in order to select an appropriate treatment for Zika infections. Further, the primer pair, kit, and method of the embodiment of the present invention have the advantage of reduced reaction time, and are capable of detecting Zika in less than 30 minutes. Thus, the present invention offers a possible solution as a Point-of-Care molecular diagnostic test for Zika.

[0046] While the invention has been described in terms of what is presently considered to be the most practical and preferred embodiments, it is to be understood that the invention needs not be limited to the disclosed embodiment. On the contrary, it is intended to cover various modifications and similar arrangements included within the spirit and scope of the appended claims which are to be accorded with the broadest interpretation so as to encompass all such modifications and similar structures.

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WHAT IS CLAIMED IS:

1. A primer pair for detecting Zika virus, comprising a forward primer consisting of SEQ ID NO: 1, and a reverse primer consisting of SEQ ID NO: 2.
2. The primer pair according to claim 1, wherein the forward primer and the reverse primer are used for real-time reverse transcription polymerase chain reaction.
3. A kit for detecting Zika virus, comprising a forward primer consisting of SEQ ID NO: 1, a reverse primer consisting of SEQ ID NO: 2, and a probe.
4. The kit according to claim 3, wherein the probe consists of SEQ ID NO: 3.
5. The kit according to claim 3, wherein the probe consists of the complementary sequence of SEQ ID NO: 3.
6. The kit according to claim 3, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.
7. The kit according to claim 3, wherein the forward primer, the reverse primer, and the probe are used for real-time reverse transcription polymerase chain reaction.
8. A method for detecting Zika virus, the method comprising amplifying nucleic acid from Zika virus using real-time reverse transcription polymerase chain reaction with a forward primer, a reverse primer, and a probe, wherein the forward primer consists of SEQ ID NO: 1, and the reverse primer consists of SEQ ID NO: 2.
9. The method according to claim 8, wherein the probe consists of SEQ ID NO: 3.
10. The method according to claim 8, wherein the probe consists of the complementary sequence of SEQ ID NO: 3.

11. The method according to claim 8, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.
12. The method according to claim 8, wherein the nucleic acid from Zika virus is RNA.
13. The method according to claim 8 further comprising sub-steps of:
 - converting a genomic RNA of Zika virus to a complementary DNA by reverse transcription; and
 - amplifying and detecting the complementary DNA by real-time polymerase chain reaction with the forward primer, the reverse primer, and the probe.