KIT AND METHOD FOR MULTIPLEX DETECTION OF CHIKUNGUNYA VIRUS AND ZIKA VIRUS

FIELD OF THE INVENTION

[0001] The present invention relates to multiplex detection of Chikungunya virus and Zika virus, and more particularly to kit and method for multiplex detection of Chikungunya virus and Zika virus.

BACKGROUND OF THE INVENTION

[0002] Chikungunya virus (CHIKV) is an arbovirus belonging to Alphavirus genus, and family Togaviridae. The virus is transmitted between humans by virus-carrying Aedes mosquitoes through mosquito bites. The virus was first isolated in 1953 from serum and Aedes mosquitoes.

[0003] Chikungunya typically occurs in Africa and Asia. However, due to climate change and globalization, outbreaks have been reported in Europe and Americas. The symptoms of the disease range from asymptomatic to extreme morbidity. Typically, the disease begins with an acute febrile phase of up to 40°C lasting from few days to a week. This is followed by a prolonged arthralgic disease that affects the joints of the extremities. The pain associated with CHIKV infection of the joints persists for weeks or months. The word "Chikungunya" is believed to derive from description in an African local dialect (Makonde language "that which bends up") of the contorted posture of patients suffering from extreme joint pain associated with this disease. Other nonspecific symptoms may include headache, conjunctivitis, digestive complaints and slight photophobia.

[0004] 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.

[0005] 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.

[0006] At current time, three techniques, which are virus isolation (for Chikungunya), serological test, and real-time reverse transcription polymerase chain reaction (real-time RT-PCR), are commonly used for the diagnosis of these viruses. These techniques confirm infections by the detection of virus, specific antibodies for virus, and viral RNA, respectively. Virus isolation requires the use of live virus in cell cultures and the observation for cytopathic effect (CPE) caused by the virus. The CPE is then confirmed by using CHIKV specific antiserum to neutralize the CPE. The major drawback to this diagnostic test is that it must be carried out in biosafety level 3 laboratories and it takes 1-2 weeks to be fully completed. Serological test, although being able to provide a definitive result ranging from 15 minutes to 2-3 days, involves the detection of IgM and IgG 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 diagnostic solution which addresses the shortcomings of the above-mentioned methods. Real-time RT-PCR enables a fast detection of the Chikungunya virus genome and 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.

[0007] While the sensitivity and the specificity of Chikungunya and 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 Chikungunya and Zika detection which is sensitive and specific.

SUMMARY OF THE INVENTION

[0008] An object of the embodiment of the present invention is to provide a multiplex detection of Chikungunya virus and Zika virus with high sensitivity, high specificity, and reduced reaction time.

[0009] Another object of the embodiment of the present invention is to provide a multiplex detection of Chikungunya virus and Zika virus to simultaneously detect Chikungunya virus and Zika virus in the same reaction.

[0010] According to an aspect of the embodiment of the present invention, there is provided a kit for multiplex detection of Chikungunya virus and Zika virus, including at least one of a first primer set specific to Chikungunya virus and a second primer set specific to Zika virus. The first primer set is selected from the group consisting of: (a) a forward primer including the nucleotide sequence of SEQ ID NO: 1 and a reverse primer including the nucleotide sequence of SEQ ID NO: 2; and (b) a forward primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 1 and a reverse primer

including a nucleotide sequence having at least 90% identity to SEQ ID NO: 2. The second primer set is selected from the group consisting of: (a) a forward primer including the nucleotide sequence of SEQ ID NO: 4 and a reverse primer including the nucleotide sequence of SEQ ID NO: 6; (b) a forward primer including the nucleotide sequence of SEQ ID NO: 5 and a reverse primer including the nucleotide sequence of SEQ ID NO: 6; (c) a first forward primer including the nucleotide sequence of SEQ ID NO: 4, a second forward primer including the nucleotide sequence of SEQ ID NO: 5, and a reverse primer including the nucleotide sequence of SEQ ID NO: 6; and (d) at least one forward primer including a nucleotide sequence having at least 90% identity to the sequence selected from the group consisting of SEQ ID NO: 4 and SEQ ID NO: 5, and a reverse primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 6.

[0011] In an embodiment, the kit further includes a probe specific to Chikungunya virus, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 3; (b) the nucleotide sequence complementary to SEQ ID NO: 3; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 3 or the nucleotide sequence complementary to SEQ ID NO: 3. The probe is labeled with a 5'-reporter dye and a 3'-quencher.

[0012] In an embodiment, the kit further includes a probe specific to Zika virus, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 7; (b) the nucleotide sequence complementary to SEQ ID NO: 7; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 7 or the nucleotide sequence complementary to SEQ ID NO: 7. The probe is labeled with a 5'-reporter dye and a 3'-quencher.

[0013] In an embodiment, the kit further includes an internal control template, which is in vitro transcribed RNA of proA gene from *Pectobacterium carotovorum*.

[0014] In an embodiment, the kit further includes a control primer set specific to the proA gene, wherein the control primer set is selected from the group consisting of: (a) a forward primer including the nucleotide sequence of SEQ ID NO: 8 and a reverse primer including the nucleotide sequence of SEQ ID NO: 9; and (b) a forward primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 8 and a reverse primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 9.

[0015] In an embodiment, the kit further includes a probe specific to the proA gene, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 10; (b) the nucleotide sequence complementary to SEQ ID NO: 10; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 10 or the nucleotide sequence complementary to SEQ ID NO: 10. The probe is labeled with a 5'-reporter dye and a 3'-quencher.

[0016] According to another aspect of the embodiment of the present invention, there is provided a method for multiplex detection of Chikungunya virus and Zika virus, including the steps of: (a) contacting a biological sample with the aforesaid kit; (b) amplifying nucleic acids of Chikungunya virus and Zika virus, if present, from the biological sample; and (c) detecting a presence of amplified nucleic acids of Chikungunya virus and Zika virus.

[0017] In an embodiment, the method further includes a step of extracting nucleic acids from the biological sample before the step (a).

[0018] In an embodiment, the step (b) includes sub-steps of: (b1) converting viral genomic RNA to complementary DNA by reverse transcription;

and (b2) amplifying the complementary DNA by real-time polymerase chain reaction.

[0019] The use of the word “a” or “an” when used in conjunction with the term “comprising/including” 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.”

[0020] 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.”

[0021] 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

[0022] FIG. 1 shows the partial cDNA sequence of the nonstructural protein 1 gene of Chikungunya virus and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence;

[0023] FIG. 2 shows the partial cDNA sequence of the envelope gene of Zika virus and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence;

[0024] FIG. 3 shows the partial cDNA sequence of the proA gene of *Pectobacterium carotovorum* and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence;

[0025] FIGS. 4 and 5 show the results of the specificity analysis for Chikungunya and Zika, respectively;

[0026] FIGS. 6A and 6B show the co-detection results of Chikungunya and Zika;

[0027] FIG. 7 shows the amplification plots generated from different copies of Chikungunya genomic RNA;

[0028] FIG. 8 shows the standard curve for the Chikungunya detection generated from the results in FIG. 7;

[0029] FIG. 9 shows the amplification plots generated from different copies of Zika genomic RNA;

[0030] FIG. 10 shows the standard curve for the Zika detection generated from the results in FIG. 9;

[0031] FIG. 11 shows the comparison of the present multiplex assay to the two commercial Chikungunya detection kits; and

[0032] FIG. 12 shows the comparison of the present multiplex assay to the two commercial Zika detection kits.

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 present invention provides multiplex detection of Chikungunya virus and Zika virus, so as to simultaneously detect Chikungunya virus and Zika virus with high sensitivity, high specificity, and reduced reaction time. 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 Chikungunya and Zika infections in humans. The real-time RT-PCR includes a first step of reverse transcription of converting the viral genomic RNA (gRNA) from Chikungunya or Zika to complementary DNA (cDNA) and then a second step of real-time PCR whereby the cDNA is amplified with the primer pair specific to Chikungunya or Zika. Particularly, in the real-time RT-PCR, the specific forward primer, reverse primer, and probe hybridize to the cDNA target of Chikungunya or 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.

[0035] The embodiment of the present invention provides an assay kit for detecting Chikungunya virus and an assay kit for detecting Zika virus. The two assay kits may be independently used for detecting Chikungunya virus and Zika virus respectively, and may also be combined to be a multiplex detection kit for detecting Chikungunya virus and Zika virus simultaneously. The designs of the primers and probes in the assay kits are described in detail as follows.

[0036] Chikungunya virus is a single-strand RNA virus having 11826 bases (strain S27; NCBI Reference Sequence NC_004162.2). In the embodiment of the present invention, the nonstructural protein 1 (nsP1) gene of Chikungunya virus is selected as the target for the detection. The assay kit for detecting Chikungunya virus includes a forward primer and a reverse primer, and may additionally include a probe.

[0037] FIG. 1 shows the partial cDNA sequence of the nsP1 gene (SEQ ID NO: 11, which is from position 1051 to position 1225 of NC_004162.2 Sequence) of Chikungunya virus, and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence of the nsP1 gene. The

forward primer has the nucleotide sequence of SEQ ID NO: 1 (5'-GCGACCATTTGTGATCAAATGACC-3'), which starts at position 1082 and includes 24-mer. The reverse primer has the nucleotide sequence of SEQ ID NO: 2 (5'-GTTCTGCCGTTAACCACTATTCTCTG-3'), which starts at position 1191 and includes 26-mer. The probe is a reverse probe and has the nucleotide sequence of SEQ ID NO: 3 (5'-AGCTTCTGTGCATCCTCCGGCGTGAC-3'), which starts at position 1149 and includes 26-mer. The forward primer and the reverse primer are predicted to generate an amplicon size of 110 base pairs.

[0038] Zika virus is a single-strand RNA virus having 10675 bases (strain PRVABC59; NCBI Reference Sequence KU501215.1). In the embodiment of the present invention, the envelope (E) gene of Zika virus is selected as the target for the detection. The assay kit for detecting Zika virus includes at least one forward primer and a reverse primer, and may additionally include a probe.

[0039] FIG. 2 shows the partial cDNA sequence of the envelope gene (SEQ ID NO: 12, which is from position 2101 to position 2275 of KU501215.1 Sequence) of Zika virus, and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence of the envelope gene. The first forward primer has the nucleotide sequence of SEQ ID NO: 4 (5'-GGAGTGGCAGCACCATTG-3'), which starts at position 2181 and includes 18-mer. The second forward primer has the nucleotide sequence of SEQ ID NO: 5 (5'-GGAGTGGCAGTACCATTG-3'), which also starts at position 2181 and includes 18-mer. The reverse primer has the nucleotide sequence of SEQ ID NO: 6 (5'-CAGGCTGTGTCTCCCAAGA-3'), which starts at position 2262 and includes 19-mer. The probe is a reverse probe and has the nucleotide sequence of SEQ ID NO: 7 (5'-TTCTCTTGGCACCTCTCACAGTGGCTTCAA-3'), which starts at position 2237 and includes 30-mer. The forward primer, either with SEQ ID NO: 4 or

SEQ ID NO: 5, and the reverse primer are predicted to generate an amplicon size of 82 base pairs.

[0040] The second forward primer with SEQ ID NO: 5 has one nucleotide change compared to the first forward primer with SEQ ID NO: 4, and the first forward primer and the second forward primer may be used to detect different strains of Zika virus. In an embodiment, the assay kit for detecting Zika virus includes the first forward primer with SEQ ID NO: 4 and the reverse primer with SEQ ID NO: 6. In an additional embodiment, the assay kit for detecting Zika virus includes the second forward primer with SEQ ID NO: 5 and the reverse primer with SEQ ID NO: 6. In a further embodiment, the assay kit for detecting Zika virus includes the first forward primer with SEQ ID NO: 4, the second forward primer with SEQ ID NO: 5, and the reverse primer with SEQ ID NO: 6.

[0041] To ascertain the specificity of the primers and the probe for Chikungunya, each of the primers and the probe (SEQ ID NO: 1 to 3) was checked by NCBI BLAST against two databases including human genomic plus transcript and nucleotide collection. The blast result shows that no other similar species have 100% same fragment compared to each of the primers and the probe of the embodiment of the present invention. The result demonstrates that the specificity of the primers and the probe to Chikungunya virus is quite high, and the primers and the probe may be used to amplify and detect the nsP1 gene of Chikungunya virus. In an embodiment of the present invention, the primers and the probe can be only used to amplify and detect the nsP1 gene of Chikungunya virus.

[0042] To ascertain the specificity of the primers and the probe for Zika, each of the primers and the probe (SEQ ID NO: 4 to 7) was checked by NCBI BLAST against two databases including human genomic plus transcript and nucleotide collection. The blast result shows that no other similar species have

100% same fragment compared to SEQ ID NO: 4, SEQ ID NO: 5, and SEQ ID NO: 7 of the embodiment of the present invention. However, SEQ ID NO: 6 shows 100% sequence similarity to Dengue serotype 3. Since the primers and the probe work together as a set, even when the reverse primer (SEQ ID NO: 6) can bind to Dengue serotype 3's genomic material, the forward primer (SEQ ID NO: 4 or 5) and the reverse probe (SEQ ID NO: 7) 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.

[0043] Moreover, the embodiment of the present invention further provides an assay kit for detecting an internal control template, which is in vitro transcribed RNA (IVT-RNA) of proA gene from *Pectobacterium carotovorum* (strain SCRI121; NCBI Reference Sequence HM157163.1). The assay kit for detecting proA internal control includes a forward primer and a reverse primer, and may additionally include a probe.

[0044] FIG. 3 shows the partial cDNA sequence of the proA gene (SEQ ID NO: 13, which is from position 291 to position 490 of HM157163.1 Sequence), and the annealing locations of the forward primer, the reverse primer, and the probe on the sequence of the proA gene. The forward primer has the nucleotide sequence of SEQ ID NO: 8 (5'-CTGCTGGTCAATCAACGTATCG-3'), which starts at position 301 and includes 22-mer. The reverse primer has the nucleotide sequence of SEQ ID NO: 9 (5'-GGTCGTTAGAAAGCCATTCATCG-3'), which starts at position

478 and includes 23-mer. The probe is a forward probe and has the nucleotide sequence of SEQ ID NO: 10 (5'-TCCATGCCAGTCCTTCAGCGATGCCTTA-3'), which starts at position 377 and includes 28-mer. The forward primer and the reverse primer are predicted to generate an amplicon size of 178 base pairs.

[0045] In an embodiment, the kit for multiplex detection of Chikungunya virus and Zika virus includes the assay kit for detecting Chikungunya virus, the assay kit for detecting Zika virus, and the assay kit for detecting proA internal control. The primers and the probes in the multiplex detection kit are summarized in Table 1.

Table 1

SEQ ID NO	**Sequence (5' → 3')**	**Primer/Probe Name**	**Orientation**
1	GCGACCATTTGTGATCAAATGACC	CHIK_nsP1_1082_1105_F	Forward Primer
2	GTTCTGCCGTTAACCACTATTCTCTG	CHIK_nsP1_1191_1166_R	Reverse Primer
3	AGCTTCTGTGCATCCTCCGGCGTGAC	CHIK_nsP1_1149_1124_P	Reverse Probe
4	GGAGTGGCAGCACCATTG	ZIKA_E_2181_2198_F1	Forward Primer
5	GGAGTGGCAGTACCATTG	ZIKA_E_2181_2198_F2	Forward Primer
6	CAGGCTGTGTCTCCCAAGA	ZIKA_E_2262_2244_R	Reverse Primer
7	TTCTCTTGGCACCTCTCACAGTGGCTTCAA	ZIKA_E_2237_2208_P	Reverse Probe
8	CTGCTGGTCAATCAACGTATCG	ProA_301_322_F	Forward Primer
9	GGTCGTTAGAAAGCCATTCATCG	ProA_478_456_R	Reverse Primer
10	TCCATGCCAGTCCTTCAGCGATGCCTTA	ProA_377_404_P	Forward Probe

[0046] In some embodiments, the primers and the probes of the present invention are not limited to the ones having sequences totally the same as SEQ ID NO: 1 to 10. It is noted that the hybridizing sequences need not have perfect complementarity to provide stable hybrids. In many situations, stable hybrids will form where fewer than about 10% of the bases are mismatches. Further, there may be genetic variations in particular Chikungunya virus strains or Zika virus strains. Therefore, the primers and the probes have sequences with at least

90% identity to SEQ ID NO: 1 to 10 may possess similar specificity to the original ones and thus can also be used for multiplex detection of Chikungunya virus and Zika virus.

[0047] In some embodiments, since the corresponding pair of the forward primer and the reverse primer are specific to Chikungunya virus (SEQ ID NO: 1 and 2), Zika virus (SEQ ID NO: 4 to 6), or proA internal control (SEQ ID NO: 8 and 9), all the sequences 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 probe sequences (SEQ ID NO: 3, 7 and 10). 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 sequences of the aforesaid probe sequences (SEQ ID NO: 3, 7 and 10) may also be used as the probe sequences for detecting Chikungunya virus, Zika virus, and proA internal control, respectively.

[0048] Therefore, the embodiment of the present invention provides a kit for multiplex detection of Chikungunya virus and Zika virus, which includes at least one of a first primer set specific to Chikungunya virus and a second primer set specific to Zika virus. The first primer set is selected from the group consisting of: (a) a forward primer including the nucleotide sequence of SEQ ID NO: 1 and a reverse primer including the nucleotide sequence of SEQ ID NO: 2; and (b) a forward primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 1 and a reverse primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 2. The second primer set is selected from the group consisting of: (a) a forward primer including the nucleotide sequence of SEQ ID NO: 4 and a reverse primer including the nucleotide sequence of SEQ ID NO: 6; (b) a forward primer including the nucleotide sequence of SEQ ID NO: 5 and a reverse primer including the

nucleotide sequence of SEQ ID NO: 6; (c) a first forward primer including the nucleotide sequence of SEQ ID NO: 4, a second forward primer including the nucleotide sequence of SEQ ID NO: 5, and a reverse primer including the nucleotide sequence of SEQ ID NO: 6; and (d) at least one forward primer including a nucleotide sequence having at least 90% identity to the sequence selected from the group consisting of SEQ ID NO: 4 and SEQ ID NO: 5, and a reverse primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 6.

[0049] In an embodiment, the kit for multiplex detection of Chikungunya virus and Zika virus further includes a probe specific to Chikungunya virus, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 3; (b) the nucleotide sequence complementary to SEQ ID NO: 3; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 3 or the nucleotide sequence complementary to SEQ ID NO: 3.

[0050] In an embodiment, the kit for multiplex detection of Chikungunya virus and Zika virus further includes a probe specific to Zika virus, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 7; (b) the nucleotide sequence complementary to SEQ ID NO: 7; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 7 or the nucleotide sequence complementary to SEQ ID NO: 7.

[0051] In an embodiment, the kit for multiplex detection of Chikungunya virus and Zika virus further includes an internal control template, which is in vitro transcribed RNA of proA gene from *Pectobacterium carotovorum*. Accordingly, the kit for multiplex detection of Chikungunya virus and Zika virus further includes a control primer set specific to the proA gene, wherein the control primer set is selected from the group consisting of: (a) a forward primer

including the nucleotide sequence of SEQ ID NO: 8 and a reverse primer including the nucleotide sequence of SEQ ID NO: 9; and (b) a forward primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 8 and a reverse primer including a nucleotide sequence having at least 90% identity to SEQ ID NO: 9. The kit for multiplex detection of Chikungunya virus and Zika virus additionally includes a probe specific to the proA gene, wherein the probe includes a nucleotide sequence selected from the group consisting of: (a) the nucleotide sequence of SEQ ID NO: 10; (b) the nucleotide sequence complementary to SEQ ID NO: 10; and (c) a nucleotide sequence having at least 90% identity to SEQ ID NO: 10 or the nucleotide sequence complementary to SEQ ID NO: 10.

[0052] The specific primers and the probes are designed to hybridize and amplify the target genes. Therefore, the embodiment of the present invention also provides a method for multiplex detection of Chikungunya virus and Zika virus. The method includes the steps of: (a) contacting a biological sample with the aforesaid kit; (b) amplifying nucleic acids of Chikungunya virus and Zika virus, if present, from the biological sample; and (c) detecting a presence of amplified nucleic acids of Chikungunya virus and Zika virus.

[0053] In an embodiment, the method further includes a step of extracting nucleic acids from the biological sample before the step (a).

[0054] In an embodiment, the step (b) includes sub-steps of: (b1) converting viral genomic RNA to complementary DNA by reverse transcription; and (b2) amplifying the complementary DNA by real-time polymerase chain reaction.

[0055] The probe used in the real-time polymerase chain reaction 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. For multiplex detection of

different target genes, the different probes for detecting Chikungunya virus, Zika virus, and proA internal control may be labeled with different reporter dyes having distinguishable fluorescent colors, so as to facilitate observing the detection results. In an embodiment, the reporter dyes are respectively selected from the group consisting of ROX, FAM, HEX, Cy5, TET, and Texas Red, but not limited thereto. In an embodiment, the quencher is a dark quencher, such as Iowa black FQ quencher or Black Hole Quencher, but not limited thereto.

[0056] The primers and the probes in the embodiments of the present invention are optimized for the rapid multiplex detection of Chikungunya virus and Zika virus through real-time RT-PCR. The real-time RT-PCR is conducted in a fast cycling mode. Table 2 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 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 enzyme activation at 95°C for 10 seconds, followed by 45 cycles of (3) PCR stage for denaturation at 95°C for 5 seconds and annealing/extension at 60°C for 18 seconds. Therefore, the total real-time RT-PCR turnaround time in the embodiments of the present invention adds up to about 23 minutes. While in the commercially available competing products, it requires 20 minutes for reverse transcription, 2 minutes for enzyme activation, 45 cycles of PCR stage with 15 seconds for denaturation, 45 seconds for annealing, and 15 seconds for extension, and thus the total real-time RT-PCR turnaround time is about 77 minutes.

Table 2

	Step	Time	Temperature
	Reverse Transcription	5 min	42°C
	Enzyme Activation	10 sec	95°C
Cycling x 45	Denaturation	5 sec	95°C
	Annealing/Extension	18 sec	60°C

[0057] In the fast cycling mode of the embodiment of the present invention, considerable time is saved for the following four aspects. First, time is saved with having a short annealing/extension time of 18 seconds, while the commercially available competing products generally require 60 seconds. Second, time is saved with having a shorter denaturation time of 5 seconds, while the commercially available competing products generally require 15 seconds. Third, the reverse transcription time is shortened as 5 minutes, while the commercially available competing products generally require 20 minutes. Fourth, the enzyme activation time is shortened as 10 seconds, while the commercially available competing products generally require 2 minutes.

[0058] Accordingly, the time saved in the fast cycling mode with the primers and the probes in the embodiments of the present invention results in at least a 70% shorter turnaround time than that of the standard RT-PCR reaction on the same type of thermal cycler, and the embodiments of the present invention are capable of detecting Chikungunya virus and Zika virus in less than 30 minutes. In other words, the primers and the probes in the embodiments of the present invention allow for the rapid multiplex detection of Chikungunya virus and Zika virus through real-time RT-PCR by optimizing design for reduced denaturation/annealing/extension time per cycle, reduced reverse transcription time and reduced enzyme activation time, thus resulting in at least 70% shorter overall reaction time. All these were achieved without compromising on the sensitivity and specificity of Chikungunya and Zika detection.

[0059] The following describes an example of the multiplex detection of Chikungunya virus and Zika virus according to the embodiments of the present invention, and the reaction mixture is shown in Table 3. 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), 200 nM of the probe (SEQ ID NO: 3), 500 nM of the forward primer (SEQ ID NO: 4), 500 nM of the forward primer (SEQ ID NO: 5), 500 nM of the reverse primer (SEQ ID NO: 6), 200 nM of the probe (SEQ ID NO: 7), 250 nM of the forward primer (SEQ ID NO: 8), 250 nM of the reverse primer (SEQ ID NO: 9), 100 nM of the probe (SEQ ID NO: 10), 1000 copies of the proA internal control IVT-RNA, and suitable amount of the extracted RNA sample in a total volume of 20 μL. The reaction mixture is however not limited to the above mentioned enzymes and buffer system.

Table 3

	real-time RT-PCR components	Volume (μl) per reaction	Concentration per reaction
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 dH2O	-	-
	SEQ ID NO: 1	-	500 nM
	SEQ ID NO: 2	-	500 nM
	SEQ ID NO: 3	-	200 nM
	SEQ ID NO: 4	-	500 nM
	SEQ ID NO: 5	-	500 nM
	SEQ ID NO: 6	-	500 nM
	SEQ ID NO: 7	-	200 nM
	SEQ ID NO: 8	-	250 nM
	SEQ ID NO: 9	-	250 nM
	SEQ ID NO: 10	-	100 nM
	ProA Internal Control IVT-RNA	-	1000 copies of IVT-RNA
	Extracted RNA Sample	-	-
		Total volume: 20 μl	

[0060] To further experimentally confirm the specificity of the primers and the probes for Chikungunya and Zika detection in the multiplex assay, the primers and the probes were checked for cross-reactivity with a few common mosquito-borne viruses, including Dengue viruses (serotypes 1 to 4), and the experiment was performed with high genomic RNA amounts (between 10^6 to 10^7 gRNA copies per reaction). FIGS. 4 and 5 show the results of the specificity analysis for Chikungunya and Zika, respectively, 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. At the Chikungunya detection fluorescence channel, as shown in FIG. 4, no amplification was observed except when Chikungunya genomic RNA is the sample used. Also, at the Zika detection fluorescence channel, as shown in FIG. 5, no amplification was observed except when Zika genomic RNA is the sample used. That is to say, no cross-reactivity was found, thus confirming the specificity of the multiplex assay.

[0061] The embodiments of the present invention are capable of detecting down to just a few copies of Chikungunya genomic RNA per reaction in the multiplex assay, in less than 30 minutes. The reactions containing 1, 3, 5, 8, 10 and 15 copies of Chikungunya genomic RNA per reaction were analyzed 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 reactions containing 1, 3, 5, 8, 10 and 15 copies per reaction were 3/6, 5/6, 5/6, 6/6, 6/6 and 6/6, respectively. In other words, for 8, 10, and 15 copies per reaction, all the 6 replicates were able to detect Chikungunya. Therefore, the limit for detection (LoD) of Chikungunya in the multiplex assay was predicted to be around 8 to 10 copies per reaction, which reveals high sensitivity. Further, a LoD confirmation run was done with 10 copies of Chikungunya genomic RNA per reaction at 20

replicates. All 20 replicates showed a positive detection, thus confirming the LoD to be 10 copies per reaction (based on >95% confidence with 20/20 detection).

[0062] The embodiments of the present invention are capable of detecting down to just a few copies of Zika genomic RNA per reaction in the multiplex assay, in less than 30 minutes. The reactions containing 1, 3, 5, 8, 10 and 15 copies of Zika genomic RNA per reaction were analyzed 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 reactions containing 1, 3, 5, 8, 10 and 15 copies per reaction were 3/6, 2/6, 5/6, 3/6, 6/6 and 6/6, respectively. In other words, for 10 and 15 copies per reaction, all the 6 replicates were able to detect Zika. Therefore, the limit for detection (LoD) of Zika in the multiplex assay was predicted to be around 10 to 15 copies per reaction, which reveals high sensitivity. Further, a LoD confirmation run was done with 10 copies of Zika genomic RNA per reaction at 20 replicates. 19 out of 20 replicates showed a positive detection, thus confirming the LoD to be 10 copies per reaction (based on 95% confidence with 19/20 detection).

[0063] The embodiments of the present invention allow the co-detection of both Chikungunya and Zika in the same real-time RT-PCR reaction. In the multiplex assay, two reactions were analyzed, one of which contained 100 copies of Chikungunya genomic RNA and 100 copies of Zika genomic RNA, and the other of which contained 10 copies of Chikungunya genomic RNA and 10 copies of Zika genomic RNA. FIGS. 6A and 6B show the co-detection results of Chikungunya and Zika. As shown in FIGS. 6A and 6B, the multiplex assay can detect both Chikungunya and Zika when 100 copies of Chikungunya genomic RNA and 100 copies of Zika genomic RNA were both present in the reaction. When 10 copies of Chikungunya genomic RNA and 10 copies of Zika genomic RNA were both present in the reaction, the multiplex assay can detect

Chikungunya in 2 out of 2 replicates and Zika in 1 out of 2 replicates. Therefore, the multiplex assay has the capability of detecting both Chikungunya and Zika at the same time, even when the virus RNA is at low amounts.

[0064] The embodiments of the present invention allow the quantitation of Chikungunya genomic RNA, in less than 30 minutes. FIG. 7 shows the amplification plots generated from different copies of Chikungunya genomic RNA. The samples had serially diluted copies (10^7 to 10 copies) of Chikungunya genomic RNA per reaction to determine the linearity of the multiplex assay in detecting Chikungunya when the primers and the probes of the embodiments of the present invention were used. FIG. 8 shows the standard curve for the Chikungunya detection generated from the results in FIG. 7. Based on the standard curve, the efficiency of the amplification was 102% with an R^2 value of 1.00. As the R^2 value was at the theoretical optimum of 1.0, the multiplex assay could be expanded to a quantitative assay to estimate the amount of Chikungunya RNA for Point-of-Care testing.

[0065] The embodiments of the present invention allow the quantitation of Zika genomic RNA, in less than 30 minutes. FIG. 9 shows the amplification plots generated from different copies of Zika genomic RNA. The samples had serially diluted copies ($2x10^6$ to 20 copies) of Zika genomic RNA per reaction to determine the linearity of the multiplex assay in detecting Zika when the primers and the probes of the embodiments of the present invention were used. FIG. 10 shows the standard curve for the Zika detection generated from the results in FIG. 9. Based on the standard curve, the efficiency of the amplification was 104% with an R^2 value of 1.00. As the R^2 value was at the theoretical optimum of 1.0, the multiplex assay could be expanded to a quantitative assay to estimate the amount of Zika RNA for Point-of-Care testing.

[0066] The multiplex assay for detecting Chikungunya and Zika of the embodiment of the present invention is further compared with two commercial

Chikungunya detection kits or two commercial Zika detection kits. The experiments were performed in triplicate with low amounts of virus RNA, i.e. 10 or 100 copies of genomic RNA per reaction. FIG. 11 shows the comparison of the present multiplex assay to the two commercial Chikungunya detection kits. FIG. 12 shows the comparison of the present multiplex assay to the two commercial Zika detection kits.

[0067] As shown in FIG. 11, when low amounts of Chikungunya RNA is added into the reaction, the average Ct values for 100 copies of genomic RNA of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 31.673, 34.055, and 33.196, respectively, and the average Ct values for 10 copies of genomic RNA of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 34.415, 37.617, and 36.461, respectively. The present multiplex assay is able to detect Chikungunya at an earlier Ct, and thus, the present multiplex assay outperforms the two commercial Chikungunya detection kits in detecting low amounts of Chikungunya RNA. Further, the total real-time RT-PCR turnaround times of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 23 minutes, 77 minutes, and 62 minutes, respectively. Therefore, the present multiplex assay also took a significantly shorter time to complete the real-time RT-PCR as compared to the commercial kits.

[0068] As shown in FIG. 12, when low amounts of Zika RNA is added into the reaction, the average Ct values for 100 copies of genomic RNA of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 31.953, 33.711, and 32.101, respectively, and the average Ct values for 10 copies of genomic RNA of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 35.983, 36.304, and 35.480, respectively. The present multiplex assay is able to detect Zika at an earlier or comparable Ct, and thus, the present multiplex assay is better than or comparable to the two

commercial Zika detection kits in detecting low amounts of Zika RNA. Further, the total real-time RT-PCR turnaround times of the present multiplex assay, the commercial kit 1, and the commercial kit 2 are 23 minutes, 77 minutes, and 62 minutes, respectively. Therefore, the present multiplex assay also took a significantly shorter time to complete the real-time RT-PCR as compared to the commercial kits.

[0069] In conclusion, the embodiments of the present invention provide the kit and method for multiplex detection of Chikungunya virus and Zika virus using real-time RT-PCR with specific primers and probes. The kit and method have the advantage of being able to simultaneously detect Chikungunya virus and Zika virus with high sensitivity and high specificity. The kit and method allow the detection of low copies of Chikungunya and Zika RNA, and can also be used for quantitative detection. The kit and method further have the advantage of reduced reaction time without compromising on specificity or sensitivity. Therefore, the present invention provides a rapid, sensitive and specific multiplex detection of Chikungunya virus and Zika virus simultaneously, so as to treat infections and prevent transmissions effectively.

[0070] 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.

SEQUENCE LISTING

<110> Delta Electronics Int¡¦l (Singapore) Pte Ltd

<120> KIT AND METHOD FOR MULTIPLEX DETECTION OF CHIKUNGUNYA VIRUS AND ZIKA VIRUS

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taacgacctg aacgtcacgc 200

WHAT IS CLAIMED IS:

1. A kit for multiplex detection of Chikungunya virus and Zika virus, comprising a first primer set specific to Chikungunya virus and a second primer set specific to Zika virus,

wherein the first primer set comprises

a forward primer consisting of the nucleotide sequence of SEQ ID NO: 1 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 2,

wherein the second primer set is selected from the group consisting of:

(a) a forward primer consisting of the nucleotide sequence of SEQ ID NO: 4 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6;

(b) a forward primer consisting of the nucleotide sequence of SEQ ID NO: 5 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6; and

(c) a first forward primer consisting of the nucleotide sequence of SEQ ID NO: 4, a second forward primer consisting of the nucleotide sequence of SEQ ID NO: 5, and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6.

2. The kit according to claim 1 further comprising a probe specific to Chikungunya virus, wherein the probe consists of

the nucleotide sequence of SEQ ID NO: 3 or

the nucleotide sequence complementary to SEQ ID NO: 3.

3. The kit according to claim 2, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.

4. The kit according to claim 1 further comprising a probe specific to Zika virus, wherein the probe consists of

the nucleotide sequence of SEQ ID NO: 7 or

the nucleotide sequence complementary to SEQ ID NO: 7.

5. The kit according to claim 4, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.

6. The kit according to claim 1 further comprising an internal control template, which is in vitro transcribed RNA of proA gene from *Pectobacterium carotovorum*.

7. The kit according to claim 6 further comprising a control primer set specific to the proA gene, wherein the control primer set comprises

a forward primer consisting of the nucleotide sequence of SEQ ID NO: 8 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 9.

8. The kit according to claim 7 further comprising a probe specific to the proA gene, wherein the probe consists of

the nucleotide sequence of SEQ ID NO: 10 or

the nucleotide sequence complementary to SEQ ID NO: 10.

9. The kit according to claim 8, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.

10. A method for multiplex detection of Chikungunya virus and Zika virus, comprising the steps of:

(a) contacting a biological sample with the kit of claim 1;

(b) amplifying nucleic acids of Chikungunya virus and Zika virus, if present, from the biological sample; and

(c) detecting a presence of amplified nucleic acids of Chikungunya virus and Zika virus.

11. The method according to claim 10 further comprising a step of extracting nucleic acids from the biological sample before the step (a).

12. The method according to claim 10, wherein the step (b) comprises sub-steps of:

(b1) converting viral genomic RNA to complementary DNA by reverse transcription; and

(b2) amplifying the complementary DNA by real-time polymerase chain reaction.

13. The method according to claim 10, wherein the kit further comprises a first probe specific to Chikungunya virus and a second probe specific to Zika virus,

wherein the first probe consists of

the nucleotide sequence of SEQ ID NO: 3 or

the nucleotide sequence complementary to SEQ ID NO: 3,

wherein the second probe consists of

the nucleotide sequence of SEQ ID NO: 7 or

the nucleotide sequence complementary to SEQ ID NO: 7.

14. The method according to claim 13, wherein each of the first probe and the second probe is labeled with a 5'-reporter dye and a 3'-quencher.

15. The method according to claim 14, wherein the first probe and the second probe are labeled with different 5'-reporter dyes.

16. The method according to claim 10, wherein the kit further comprises an internal control template, which is in vitro transcribed RNA of proA gene from *Pectobacterium carotovorum*.

17. The method according to claim 16, wherein the kit further comprises a control primer set specific to the proA gene, which comprises

a forward primer consisting of the nucleotide sequence of SEQ ID NO: 8 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 9.

18. The method according to claim 17, wherein the kit further comprises a probe specific to the proA gene, which consists of

the nucleotide sequence of SEQ ID NO: 10 or

the nucleotide sequence complementary to SEQ ID NO: 10.

19. The method according to claim 18, wherein the probe is labeled with a 5'-reporter dye and a 3'-quencher.

20. A kit for detection of Chikungunya virus comprising a primer set, wherein the primer set comprises

a forward primer consisting of the nucleotide sequence of SEQ ID NO: 1 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 2.

21. A kit for detection of Zika virus comprising a primer set selected from the group consisting of:

(a) a forward primer consisting of the nucleotide sequence of SEQ ID NO: 4 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6;

(b) a forward primer consisting of the nucleotide sequence of SEQ ID NO: 5 and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6; and

(c) a first forward primer consisting of the nucleotide sequence of SEQ ID NO: 4, a second forward primer consisting of the nucleotide sequence of SEQ ID NO: 5, and a reverse primer consisting of the nucleotide sequence of SEQ ID NO: 6.