GENE SEQUENCES FOR THE DETECTION OF CHIKUNGUNYA AND DENGUE FEVER

FIELD

The present invention relates to the fields of medicine, cell biology, molecular biology and genetics. More particular, the invention relates to methods of nucleic acid sequences suitable for use in detecting a pathogen or products thereof in a sample.

BACKGROUND

Dengue (DEN) is endemic in most parts of tropics and subtropics and chikungunya (CHIK) fever has emerged in many parts of the Indian Ocean islands, India, Italy and recently in Malaysia and Singapore.

A number of molecular assays have been described for detection of DENV and CHIKV separately. One assay for differential diagnosis of DEN and CHIK has recently been reported (Dash et al., 2008). Although this assay can detect both infections concurrently, the detection is done by conventional Ethidium Bromide stained gel-electrophoresis. Accordingly, it is incapable of distinguishing between genuine-detection and non-specific detections.

Our invention seeks to address these and other issues in the prior art.

SUMMARY

According to a 1st aspect of the present invention, we provide a method of detecting chikungunya and dengue nucleic acids in a biological sample, the method comprising the steps of: (a) an amplification step in which chikungunya and dengue nucleic acids are amplified by chikungunya primers and dengue primers in a nucleic acid amplification reaction; and (b) a detection step in which amplified nucleic acids from (a) are hybridised to a chikungunya nucleic acid probe and a dengue nucleic acid probe in a nucleic acid hybridisation reaction.

The amplified nucleic acids may be hybridised to a plurality of dengue nucleic acid probes. The dengue nucleic acid probes may be capable of binding to different dengue nucleic acids. The different dengue nucleic acid acids may each be characteristic of a dengue strain. The method may enable identification of a nucleic acid from a particular dengue strain in a biological sample.

The nucleic acid amplification reaction may comprise a polymerase chain reaction (PCR). The polymerase chain reaction may comprise a reverse transcription polymerase chain reaction (RT-PCR). It may comprise duplex RT-PCR.

The chikungunya and dengue probes may be immobilised. They may be immobilised on microspheres. The microspheres may comprise coded microspheres. This may be such that identification of the microsphere reveals the identity of a probe immobilised thereon.

The coded microspheres may comprise colour coded microspheres.

The amplified nucleic acids may be labelled with a detectable label. The method may comprise labelling chikungunya nucleic acid primers and dengue nucleic acid primers with a detectable label. The method may be such that amplification step (a) generates labelled amplified nucleic acids.

The detectable label may comprise anything whose presence is revealable. The detectable label may comprise as a signal generating means. The signal generating means may comprise biotin. It may comprise a biotin strepavidin-phycoerythin conjugate.

The method may compirse detecting detectable labels to detect amplified nucleic acids. The detected amplified nucleic acids may be bound to the microsphere. They may be bound to the microsphere by way of microsphere-immobilised chikungunya and dengue probes.

The method may further comprise identifying a microsphere to identify a probe immobilised thereon. This may enable the identity of a bound amplified nucleic acid to be determined.

The detectable label comprising a biotin strepavidin-phycoerythin conjugate may be detected by means of a first laser. The identity of a colour coded microsphere may be determined by means of a second laser. The first laser may be of a different wavelength to the second laser.

The detectable label may be detected at the same time, or at a different time as the identification of the colour coded microsphere. The detectable label may be detected simultaneously with the identification of the colour coded microsphere.

The chikungunya nucleic acid primers may consist of the nucleic acid sequences shown in Table D1.1. The dengue nucleic acid primers may consist of the nucleic acid sequences shown in Table D1.2, or both.

The chikungunya nucleic acid probes may consist of the nucleic acid sequences shown in Table D2.1. The dengue nucleic acid probes may consist of the nucleic acid sequences shown in Table D2.2.

There is provided, according to a 2nd aspect of the present invention, a method according to the 1st aspect of the invention for simultaneously detecting the presence of chikungunya and determining the strain of dengue in a biological sample.

We provide, according to a 3rd aspect of the present invention, a nucleic acid sequence as shown in Table D1.1, Table D1.2, Table D2.1 or Table D2.2, or a variant, homologue, derivative or fragment thereof.

As a 4th aspect of the present invention, there is provided a combination of two or more sequences according to the previous aspect of the invention. The combination may comprise two or more sequences set out in Table D1.1 and Table D1.2, or two or more sequences set out in Table D2.1 and Table D2.2, or both.

The combination may consist of each of the nucleic acid sequences shown in Table D1.1 and Table D1.2, or a variant, homologue, derivative or fragment thereof.

The combination may consist of each of the nucleic acid sequences shown in Table D2.1 and Table D2.2, or a variant, homologue, derivative or fragment thereof

The combination may consist of a combination consisting of each of the above combinations.

The nucleic acid or combination may comprise a labelled nucleic acid. The label may comprise biotin. It may comprise a biotin strepavidin-phycoerythrin conjugate.

The nucleic acid or combination may be in solution. It may comprise an immobilised nucleic acid. The nucleic acid may be immobilised on a microsphere. It may be immobilised on an array. The array may comprise a microarray.

We provide, according to a 5th aspect of the present invention, a combination consisting of each of the nucleic acid sequences shown in Table D1.1 and Table D1.2, or a variant, homologue, derivative or fragment thereof; and (b) each of the nucleic acid sequences shown in Table D2.1 and Table D2.2, or a variant, homologue, derivative or fragment thereof; in which the nucleic acids of (a) are in solution and the nucleic acids of (b) are immobilised on microspheres.

The present invention, in a 6th aspect, provides a kit for the detection of chikungunya or dengue in a patient, the kit comprising a nucleic acid or combination as set out above, together with instructions for use.

In a 7th aspect of the present invention, there is provided use of a nucleic acid sequence or a combination as set out above as a primer for nucleic acid amplification of a chikungunya or dengue gene or as a probe for nucleic acid hybridisation of a chikungunya or dengue gene.

According to an 8th aspect of the present invention, we provide a method of diagnosis of chikungunya or dengue in an individual, the method comprising obtaining a biological sample from the individual and detecting chikungunya and dengue nucleic acids therein by a method as set out above.

We provide, according to a 9th aspect of the invention, a method of treatment or prevention of chikungunya or dengue in an individual, the method comprising detecting chikungunya or dengue in an individual according to the 8th aspect of the invention and administering a suitable treatment or prophylactic to the individual.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984,

Oligonucleotide Synthesis: A Practical Approach, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; Using Antibodies : A Laboratory Manual : Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies : A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2), 1855. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9); and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Sensitivity of simultaneous CHIK and DENV assay.

Figure 2. Results from clinical samples obtained with this simultaneous assay, single CHIKV-Taqman assay and single DEN SYBR assay.

Figure 3 is a diagram showing the disposition of chikungunya and dengue primers in tubes for the purposes of an assay set out in the Examples.

Figure 4 is a diagram of a flow chart showing the detection protocol, as set out in the Examples.

DETAILED DESCRIPTION

We provide a nucleic acid sequence as shown in Table D1.1, Table D1.2, Table D2.1 or Table D2.2, or a variant, homologue, derivative or fragment thereof. A combination of two or more sequences is also provided. The nucleic acid sequence or combination may be used for detection of chikungunya and dengue or discrimination between strains of dengue, or both.

CHIKUNGUNYA AND DENGUE SEQUENCES

We describe a number of nucleic acid sequences. The sequences may be used for any means for which nucleic acid sequences are suitable. For simplicity, the nucleic acid sequences the subject of this disclosure may be referred to as "chikunguya sequences" and "dengue sequences".

The "chikunguya sequences" and "dengue sequences" may comprise a sequence corresponding to a portion of an chikungunya nucleic acid sequence or dengue nucleic acid sequence. For example, the chikunguya sequence could comprise a chikungunya gene. Similarly, the dengue sequence could comprise a dengue gene.

The chikungunya gene may comprise any gene from or of an chikungunya virus. The dengue gene may comprise a gene from or of an dengue virus. The sequences may further comprise other nucleic acid residues which have no such correspondence.

The chikungunya sequence may comprise a sequence set out in Table D1.1 or Table D1.2. The dengue sequence may comprise a sequence set out in Table D2.1 or Table 2.2. The chikungunya or dengue sequence may comprise a variant, homologue, derivative or fragment of any such sequence.

The chikungunya virus may comprise any strain of the chikungunya virus. The chikungunya virus may comprise the IND-63-WB1 strain (Gen Bank accession no. EF027140). The chikungunya virus may comprise any of the following: NC004162:D570/06 (Africa), EU244823:ITA07-RA1(Italy), EU037962:Wuerzburg(Germany), EF012359:D570/06 (Mauritius/island nation off the coast of the African continent), EF210157:DRDEHydISW06 (India), EF452493:AF15561 (Bangkok), AF369024:S27-African prototype (Africa), DQ443544: LR2006OPY1(Travelers Returning from Indian Ocean Islands) and AF490259: Ross (Ross/Scotland).

The dengue virus may comprise any strain of the dengue virus. The dengue virus could comprise DEN virus type 1 strain D1/SG/06K2290DK1/2006 (EU081281). It may comprise any one or more of EU081281, EU081276, EU081261, NC_001477, AY762084, and M87512.

The dengue virus may comprise a type 2 strain D2/SG/05K3295DK1/2005 (EU081177). It may comprise any one or more of EU081177, NC_001474 , AF169680, M20558, AY858036, and AB122022.

The dengue virus may comprise a type 3 strain Singapore (AY662691). It may comprise any one or more of EU081225, EU081221, EU081197,NC_001475, AY66269, and EU081214.

The dengue virus may comprise a type 4 strain ThD4_0734_00 (AY618993). It may comprise any one or more of AY762085, AY947539, NC_002640, AY618993, AY152120, and AY762085..

The chikungunya virus or the dengue virus may comprise a human chikungunya or dengue virus or an animal chikungunya or dengue virus virus.

The chikungunya gene may include the E1 gene. The dengue gene may include the 5NS to 3'NTR coding and non-coding regions. It will be evident that where the term "gene" is used, it may comprise coding regions, non-coding regions or both.

The chikungunya and dengue sequences described here may be single stranded or double stranded.

The chikungunya and dengue sequences described here may in particular be capable of binding to a relevant chikungunya or dengue gene, as the case may be. The chikungunya or dengue gene may comprise DNA, RNA, mRNA, cDNA, etc, or a portion thereof. The chikungunya or dengue gene may include the E1 gene (chikungunya) or the 5NS to 3'NTR coding and non-coding regions (dengue), or both. Other genes may be detected as part of the process.

The sequences may be used to detect the presence of chikungunya or dengue nucleic acids in a sample. They may be used to detect the presence of chikungunya or dengue viruses or products thereof in a sample. They may be used to detect the presence of chikungunya or dengue viruses of particular strains in a sample. They may be used to discriminate between these strains.

The chikungunya and dengue sequences described here may in particular be capable of binding to a relevant chikungunya or dengue gene of an chikungunya or dengue strain. For example, the dengue sequences may be capable of discriminating between different dengue strains.

The dengue sequences may therefore in particular be capable of binding to multiple variants from different dengue strains. The dengue sequences may be such that they are capable of binding to certain gene variants and not others. The dengue sequences may be capable of binding to only one gene variant.

Thus, we disclose dengue sequences comprising nucleic acid sequences capable of binding to different 5NS to 3'NTR coding or non-coding region variants.

We disclose dengue sequences comprising nucleic acid sequences capable of binding to a dengue virus type 1 strain 5NS to 3'NTR coding or non-coding region variant. For example, the dengue sequence may comprise a nucleic acid sequence capable of binding to a dengue virus type 1 strain D1/SG/06K2290DK1/2006 (EU081281) 5NS to 3'NTR coding or non-coding region variant.

We disclose dengue sequences comprising nucleic acid sequences capable of binding to a dengue virus type 1 strain 5NS to 3'NTR coding or non-coding region variant. For example, the dengue sequence may comprise a nucleic acid sequence capable of binding to a dengue virus type 2 strain D2/SG/05K3295DK1/2005 (EU081177) 5NS to 3'NTR coding or non-coding region variant.

We disclose dengue sequences comprising nucleic acid sequences capable of binding to a dengue virus type 1 strain 5NS to 3'NTR coding or non-coding region variant. For example, the dengue sequence may comprise a nucleic acid sequence capable of binding to a dengue virus type 3 strain Singapore (AY662691) 5NS to 3'NTR coding or non-coding region variant.

We disclose dengue sequences comprising nucleic acid sequences capable of binding to a dengue virus type 1 strain 5NS to 3'NTR coding or non-coding region variant. For example, the dengue sequence may comprise a nucleic acid sequence capable of binding to a dengue virus type 4 strain ThD4_0734_00 (AY618993) 5NS to 3'NTR coding or non-coding region variant.

The dengue sequences may be used to discriminate between different strains of dengue virus. For example, they may be used to discriminate between dengue virus strain 1, dengue virus strain 2, dengue virus strain 3 and dengue virus strain 4.

The chikungunya and dengue sequences may be used in any method for which nucleic acid sequences are generally suitable. The may in particular be used as primers or probes for the detection of chikungunya and dengue, for example as described above.

The chikungunya and dengue sequences may be used as nucleic acid primers. The primers may comprise one or more chikungunya and dengue sequences set out in Table D1.1 and Table D2.1.

The primers may be used for amplification of nucleic acids by any means known in the art. For example, PCR (polymerase chain reaction) may be employed. As another example, RT-PCR (reverse transcription – polymerase chain reaction) may be employed. Furthermore, duplex RT-PCR may be employed. Other amplification methods may also be used, for example, LCR (ligase chain reaction), dePCR (digital polymerase chain reaction), Long PCR, Quantitative End-Point PCR, Quantitative Real-Time PCR, Rapid Amplification of cDNA Ends (RACE), TMA, NASBA, etc. These methods are known in the art, and methods for employing the chikungunya and dengue sequences for use in such methods will be evident to the skilled reader.

The chikungunya and dengue sequences may be provided as combinations. For example, we disclose a combination of any two or more sequences set out in Table D1.1, Table D1.2, Table D2.1 and Table D2.2. The combination may comprise two sequences, corresponding to a forward primer and a reverse primer, as set out in Table D1.1 or Table D2.1. The combination of two sequences may include a forward primer for a particular gene and a reverse primer for that gene. Combinations of such pair-wise combinations are also provided.

The chikungunya and dengue sequences may be used as probes. The probes may comprise one or more chikungunya and dengue sequences set out in Table D1.2 and Table 2.2.

LABELLING

The amplification or hybridisation reaction may comprise binding between the chikungunya or dengue sequence and a target. The primers or probes may be labelled for this purpose. The amplified nucleic acids which are a product of the amplification reaction may be labelled.

The labelling may comprise any signal producing entity. The label may comprise a radioactive label, such as ^{32}P. The label may comprise a non-radioactive label such as

digoxigenin. The label may comprise a light emitting means. The label may comprise any suitable dye, such as a fluorescent dye. The label may comprise phycoerythrin.

The label may be attached to the probe, nucleic acid or primer by any suitable means. The attachment may be temporary, transient, semi-permanent or permanent. The attachment may be covalent, ionic, through hydrogen bonding, through Van der Waals forces, etc. The attachment may be chemical in nature. It may be direct, or mediated through an intermediary. A suitable binding agent may be attached to the entity to be labelled such as the probe, nucleic acid or primer, etc. A binding partner carrying the label may be attached to the entity to be labelled by being bound to the binding agent. For example, a biotin moiety may be attached to the entity to be labelled and strepavin bound to a label may be attached thereto. A nucleic acid to be labelled, such as an amplified nucleic acid, may have biotin attached thereto for this purpose by means of suitable biotin labelled primers, which allow the incorporation of the biotin moiety into the amplified nucleic acid as part of the amplification reaction.

The probe, primer, nucleic acid, etc may be bound to biotin, and strepavidin-phycoerythrin attached to the biotin to attach the phycoerythrin label to the probe, primer, nucleic acid, etc. A biotin labelled primer may be used to generate a biotin labelled amplified nucleic acid as a first step. A second step may comprise exposing the biotin labelled amplified nucleic acid to strepavidin-phycoerythrin. The binding of strepavidin to biotin effectively labels the amplified nucleic acid with a phycoerythrin label.

The signal may be detected by any suitable means known in the art. For example, a fluorescent label may be detected through excitation with light of a suitable excitation wavelength, and the emitted signal detected through various means, such as by eye, by scintillation counting, through microscopy, through film or digital photography, by photodetectors, etc. The signal may be quantified if necessary (e.g., for tracking productivity or progress of the reaction) by various means known in the art.

The chikungunya and dengue sequences may be used in solution for conventional nucleic acid hybridisation. The hybridisation may be performed under stringent hybridisation conditions or non-stringent hybridisation conditions. An example of a stringent hybridisation condition is 65°C and 0.1xSSC (1xSSC = 0.15 M NaCl, 0.015 M Na3 Citrate pH 7.0). A further example of a hybridisation condition is 48 mM phosphate Buffer, 1742.4 mM NaCl,

0.24% Tween 20, 4.8X Denhardt's solution, 2.4 nM probe or probes (e.g., 3 probes) – phosphate buffer comprises 12.96 mM KCl and 657.6 mM NaCl.

Methods of using such nucleic acid sequence probes for the detection of target nucleic acid sequences are known in the art, and are described in for example, Maniatis et al.

The hybridisation may be performed with the chikungunya and dengue sequences in solution or immobilised. The chikungunya and dengue sequences may be immobilised, for example by being bound to a substrate.

The substrate may comprise a silicon base. The chikungunya and dengue sequences may be provided in a regular spaced conformation, for example in the form of an array. The array may comprise a microarray. Methods of producing nucleic acid arrays are known in the art.

The array may comprise one or more of the chikungunya and dengue sequences set out in Tables D1.1, D1.2, D2.1 and D2.2. The array may comprise a plurality of such sequences. The array may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60 or 61 etc such sequences. The array may comprise one or more control sequences.

The chikungunya and dengue sequences may be immobilised on separate entities, or on the same entity.

MICROSPHERES

The chikungunya and dengue sequences may be immobilised on particles, such as beads. The chikungunya and dengue sequences may be immobilised on microbeads or microspheres. Such beads, microbeads and microspheres etc are known in the art, are commercially available and are described extensively in the literature and below.

The beads, microbeads or microspheres etc will be referred to in the rest of this document for simplicity as "microspheres". They may be labelled in order that they may be tracked. Methods of labelling and types of labels will vary depending on the nature of the microsphere, etc, but the general principles described elsewhere in this document, and also in the literature, will assist in their choice.

Microspheres on which the chikungunya and dengue sequences may be immobilised include any suitable particle of any size. For example, the microspheres may comprise agarose beads, polyacrylamide beads, silica gel beads, etc.

The microspheres may be coded for identification. Any system of coding or marking may be employed, so long as it allows the de-coding and identification of a particular microsphere to take place. The coding may be by way of physical marking, or by other characteristics such as optical, physical, chemical, etc characteristics. The marking or coding may comprise colour coding. Thus, two different beads in any particular set may be distinguishable by colour. The colour coding may be decoded by any suitable means, such as by laser light.

The same or diferrent chikungunya and dengue sequences may be immobilised on different beads. For example, a first sequence may be immobilised on a first bead, and a second sequence may be immobilised on a second bead, the two beads having distinguishable characteristics, such as marking or coding.

For example, the microspheres may comprise Luminex xMAP microspheres, as described at www.luminexcorp.com/technology/index.html Such microspheres have a typical 5.6 micron size and comprise dyes attached thereon to provide for colour coding. De-coding of the colour coding of the microsphere enables the identification of an individual microsphere and hence the identity of the probe attached thereon. From this, the identity of a nucleic acid sequence bound or hybridised to the probe may be made known.

A description of the Luminex xMAP microsphere technology, from the Luminex website, is provided below:

"First, Luminex color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, xMAP technology allows multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely."

Microspheres, etc include those which are used for gel chromatography, for example, gel filtration media.

Examples include Sephadex, for example Sephadex G-10 having a bead size of 40-120 (Sigma Aldrich catalogue number 27,103-9), Sephadex G-15 having a bead size of 40-120μm (Sigma Aldrich catalogue number 27,104-7), Sephadex G-25 having a bead size of 20-50μm (Sigma Aldrich catalogue number 27,106-3), Sephadex G-25 having a bead size of 20-80μm (Sigma Aldrich catalogue number 27,107-1), Sephadex G-25 having a bead size of 50-150μm (Sigma Aldrich catalogue number 27,109-8), Sephadex G-25 having a bead size of 100-300μm (Sigma Aldrich catalogue number 27,110-1), Sephadex G-50 having a bead size of 20-50μm (Sigma Aldrich catalogue number 27,112-8), Sephadex G-50 having a bead size of 20-80μm (Sigma Aldrich catalogue number 27,113-6), Sephadex G-50 having a bead size of 50-150μm (Sigma Aldrich catalogue number 27,114-4), Sephadex G-50 having a bead size of 100-300μm (Sigma Aldrich catalogue number 27,115-2), Sephadex G-75 having a bead size of 20-50μm (Sigma Aldrich catalogue number 27,116-0), Sephadex G-75 having a bead size of 40-120μm (Sigma Aldrich catalogue number 27,117-9), Sephadex G-100 having a bead size of 20-50μm (Sigma Aldrich catalogue number 27,118-7), Sephadex G-100 having a bead size of 40-120μm (Sigma Aldrich catalogue number 27,119-5), Sephadex G-150 having a bead size of 40-120μm (Sigma Aldrich catalogue number 27,121-7), and Sephadex G-200 having a bead size of 40-120μm (Sigma Aldrich catalogue number 27,123-3).

Sepharose beads, for example, as used in liquid chromatography, may also be used. Examples are Q-Sepharose, S-Sepharose and SP-Sepharose beads, available for example from Amersham Biosciences Europe GmbH (Freiburg, Germany) as Q Sepharose XL (catalogue number 17-5072-01), Q Sepharose XL (catalogue number 17-5072-04), Q Sepharose XL (catalogue number 17-5072-60), SP Sepharose XL (catalogue number 17-5073-01), SP Sepharose XL (catalogue number 17-5073-04) and SP Sepharose XL (catalogue number l 17-5073-60) etc.

CHIKUNGUNYA AND DENGUE SEQUENCES

289	CHI226U	CTGYCAAATAGCAACAAACCCGGTAA
290	CHI226L	CATCGAATGCACCGCACACTT

Table D1.1. Chikungunya Primers

284	DEN1U1	GCATGGGGTAGCAGACTAGTGGTTA
317	DEN2U2	AGCCTGTAGCTCCACCTGAGAAGGTGTAAAA
316	DEN3U4	ACTGTCAGGCCACCTTAAGCCACAGTA
307	DEN4U3	GCCAATAATGGGAGGCGTAA
288	DEN-L	GATCTCTGGTCTYTCCCAGCGTCAATA

Table D1.2. Dengue Primers

293	CHIKV226P	CCCTACGGCGCAGTTCAYCGCTCTTACCGGG

Table D2.1. Chikungunya Probes

315	DEN1P3	GCTTCCCCTGGTGTTGGGCCCCGCT
332	DEN2P2	ATCTCACCTTGGGCCCCCRTTGTTGCTGCGA
310	DEN3P2	CGGTTTGCTCAAACCGTGGCTTTGGGGCCT
299	DEN4P2	CAGCTTCCGTGGCGCATGGCCTCCCTGGG

Table D2.2. Dengue Probes

Table D1.1 may include the specific examples of Chikungunya Primers set out below in (1). Thus, a reference to Table D1.1 may comprise a reference to the sequences shown in Table D1.1 above, as well as the sequences set out in (1) below.

(1) Chikungunya virus (CHIKV)

289:CHI226U:(5'-3'):/5Bio/CTGYCAAATAGCAACAAACCCGGTAA, Y(C/T)

290:CHI226L:(5'-3'):CATCGAATGCACCGCACACTT

Table D12 may include the specific examples of Dengue Primers set out below in (2). Thus, a reference to Table D1.2 may comprise a reference to the sequences shown in Table D1.2 above, as well as the sequences set out in (2) below.

(2) Dengue virus (DENV)

284:DEN1U1:(5'-3'):/5Bio/GCATGGGGTAGCAGACTAGTGGTTA

317:DEN2U2:(5'-3'):/5Bio/AGCCTGTAGCTCCACCTGAGAAGGTGTAAAA

316:DEN3U4:(5'-3'):/5Bio/ACTGTCAGGCCACCTTAAGCCACAGTA

307:DEN4U3:(5'-3'):/5Bio/GCCAATAATGGGAGGCGTAA

288:DEN-L:(5'-3'):GATCTCTGGTCTYTCCCAGCGTCAATA, Y(C/T)

Table D2.1 may include the specific examples of Chikungunya Probes set out below in (3). Thus, a reference to Table D2.1 may comprise a reference to the sequences shown in Table D2.1 above, as well as the sequences set out in (3) below.

(3) Probe for CHIKV

293:CHIKV226P:(5'-3'):/5H2N-C6/CCCTACGGCGCAGTTCAYCGCTCTTACCGGG, Y(C/T)

Table D2.2 may include the specific examples of Dengue Probes set out below in (4). Thus, a reference to Table D2.2 may comprise a reference to the sequences shown in Table D2.2 above, as well as the sequences set out in (4) below.

(4) Probes for DENV

315:DEN1P3:(5'-3'):/5H2N-C6-/GCTTCCCCTGGTGTTGGGCCCCGCT

332:DEN2P2:(5'-3'):/5H2N-C6-/ATCTCACCTTGGGCCCCCRTTGTTGCTGCGA, R(A/G)

310:DEN3P2:(5'-3'):/5H2N-C6-/CGGTTTGCTCAAACCGTGGCTTTGGGGCCT

299:DEN4P2:(5'-3'):/5H2N-C6-/CAGCTTCCGTGGCGCATGGCCTCCCTGGG

CHIKUNGUNYA AND DENGUE NUCLEIC ACID SEQUENCES

The chikungunya and dengue sequences may comprise polynucleotides. Where the context admits, the terms "chikungunya sequence" and "dengue sequence" should be taken to comprise any specific sequence disclosed in this document, for example, a sequence set out in Table D1.1 or D2.1 or a sequence set out in Table D1.2 or Table D2.2, as well as any variants, fragments, derivatives and homologues of such specific nucleic acid sequences.

As used here in this document, the terms "polynucleotide", "nucleotide", and nucleic acid are intended to be synonymous with each other. "Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases

include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells. "Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

It will be understood by a skilled person that numerous different polynucleotides and nucleic acids can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides described here to reflect the codon usage of any particular host organism in which the polypeptides are to be expressed.

Chikungunya and dengue sequences and variants, fragments, derivatives and homologues may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of this document, it is to be understood that the polynucleotides may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of interest.

The terms "variant", "homologue" or "derivative" in relation to a nucleotide sequence include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence. The variants, homologues or derivatives may code for a polypeptide having biological activity.

As indicated above, with respect to sequence homology, there may be at least 50 or 75%, such as at least 85% or at least 90% homology to the sequences shown in the sequence listing herein. There may be at least 95%, such as at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A sequence comparison program which may be used may comprise the GCG Wisconsin Bestfit program described

above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

We further describe nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences may be at least 15 nucleotides in length, such as at least 20, 30, 40 or 50 nucleotides in length.

The term "hybridization" as used herein may include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction technologies.

Polynucleotides capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, such as at least 80 or 90% for example at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, such as at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides.

The term "hydrizable" may include "selectively hybridizable", i.e., that the polynucleotide used as a probe is used under conditions where a target polynucleotide is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other polynucleotides present, for example, in the cDNA or genomic DNA library being screening. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, such as less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ^{32}P.

Hybridization conditions are based on the melting temperature (Tm) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

We describe nucleotide sequences that can hybridise to the chikungunya and dengue nucleic acids, fragments, variants, homologues or derivatives disclosed here under stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3 Citrate pH 7.0).

Where the polynucleotide is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the methods and compositions described here. Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included.

Polynucleotides which are not 100% homologous to the chikungunya or dengue sequences disclosed here but which are also included can be obtained in a number of ways. Other variants of the sequences may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. For example, chikungunya and dengue homologues may be identified from other individuals, or other species. Further recombinant chikungunya and dengue sequence variant nucleic acids and polypeptides may be produced by identifying corresponding positions in the homologues, and synthesising or producing the molecule as described elsewhere in this document.

In addition, other viral/bacterial, or cellular homologues of chikungunya and dengue sequences particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to a chikungunya or dengue sequence nucleic acid. Such homologues may be used to design chikungunya and dengue sequence nucleic acids, fragments, variants and homologues. Mutagenesis may be carried out by means known in the art to produce further variety.

Sequences of chikungunya and dengue sequence homologues may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal or non-

animal species, such as viral, microbial or fungal species, and probing such libraries with probes comprising all or part of any of the chikungunya or dengue sequence nucleic acids, fragments, variants and homologues, or other fragments of chikungunya or dengue sequence nucleic acids under conditions of medium to high stringency.

Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences disclosed here.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the chikungunya and dengue sequence nucleic acids. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences. It will be appreciated by the skilled person that overall nucleotide homology between sequences from distantly related organisms is likely to be very low and thus in these situations degenerate PCR may be the method of choice rather than screening libraries with labelled fragments of the chikungunya or dengue sequence sequences.

In addition, homologous sequences may be identified by searching nucleotide and/or protein databases using search algorithms such as the BLAST suite of programs.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences, for example, chikungunya or dengue sequence nucleic acids, or variants, homologues, derivatives or fragments thereof. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The polynucleotides described here may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 8, 9, 10, or 15, such as at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term "polynucleotides" as used herein.

Polynucleotides such as a DNA polynucleotides and probes may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector

Polynucleotides or primers may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S, enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers and may be detected using by techniques known *per se*. Polynucleotides or primers or fragments thereof labelled or unlabeled may be used by a person skilled in the art in nucleic acid-based tests for detecting or sequencing polynucleotides in the human or animal body.

Such tests for detecting generally comprise bringing a biological sample containing DNA or RNA into contact with a probe comprising a polynucleotide or primer under hybridising conditions and detecting any duplex formed between the probe and nucleic acid in

the sample. Such detection may be achieved using techniques such as PCR or by immobilising the probe on a solid support, removing nucleic acid in the sample which is not hybridised to the probe, and then detecting nucleic acid which has hybridised to the probe. Alternatively, the sample nucleic acid may be immobilised on a solid support, and the amount of probe bound to such a support can be detected. Suitable assay methods of this and other formats can be found in for example WO89/03891 and WO90/13667.

Tests for sequencing nucleotides, for example, the chikungunya or dengue sequence nucleic acids, involve bringing a biological sample containing target DNA or RNA into contact with a probe comprising a polynucleotide or primer under hybridising conditions and determining the sequence by, for example the Sanger dideoxy chain termination method (see Sambrook *et al.*).

Such a method generally comprises elongating, in the presence of suitable reagents, the primer by synthesis of a strand complementary to the target DNA or RNA and selectively terminating the elongation reaction at one or more of an A, C, G or T/U residue; allowing strand elongation and termination reaction to occur; separating out according to size the elongated products to determine the sequence of the nucleotides at which selective termination has occurred. Suitable reagents include a DNA polymerase enzyme, the deoxynucleotides dATP, dCTP, dGTP and dTTP, a buffer and ATP. Dideoxynucleotides are used for selective termination.

The chikungunya or dengue sequence variant polypeptide sequence may be used as a therapy in any form, such as in an isolated form. The term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature. In one aspect, the sequence is in a purified form. The term "purified" means that the sequence is in a relatively pure state – e.g. at least about 90% pure, or at least about 95% pure or at least about 98% pure.

METHODS OF DETECTING CHIKUNGUNYA AND DENGUE

The chikungunya and dengue sequences described here may be used for detection of chikungunya and dengue virus (or nucleic acids therefrom) in a sample. An example follows.

The detection may comprise a two step process.

In a first step, amplification is carried out. Here, the chikungunya and dengue nucleic acids may be amplified by chikungunya primers and dengue primers in a nucleic acid amplification reaction. The amplification reaction may comprise for example a polymerase chain reaction (PCR) such as reverse transcription polymerase chain reaction (RT-PCR) or duplex RT-PCR.

The sample may be processed to extract nucleic acids, such as messenger RNA. The mRNA may be reverse transcribed to cDNA. The cDNA may be amplified using the chikungunya and dengue sequences as primers. Pairwise combinations of primers, such as comprising forward and reverse primers for a particular gene variant may be used. The sequences set out in Table D1.1 and Table D1.2 may be employed for bulk amplification of any chikungunya and dengue gene sequences present in the sample.

The amplified nucleic acids are labelled with a detectable label. This may be achieved, as described above, by labelling chikungunya nucleic acid primers and dengue nucleic acid primers with a detectable label. In such a way, the amplification step generates labelled amplified nucleic acids. The label may comprise biotin or a biotin strepavidin-phycoerythin conjugate.

In a second step, detection is carried out. Here, amplified nucleic acids from the amplification reaction are hybridised to a chikungunya nucleic acid probe and a dengue nucleic acid probe in a nucleic acid hybridisation reaction.

The amplified nucleic acids may be detected by solution hybridisation using one ore more of the chikungunya and dengue sequences as probes. The presence of binding between an chikungunya and dengue probe sequence and its target (i.e., an amplified chikungunya and dengue gene or nucleic acid or portion thereof) as detected in the hybridisation may be used as an indicator of the presence of an chikungunya and dengue in the sample.

Thus, specific strain detection of chikungunya and dengue strains may be performed by for example detecting binding between specific chikungunya and dengue probe sequences and their targets. This may be achieved for example by performing separate hybridisations and detecting binding of certain probe sequences and not others. Alternatively, or in addition, the chikungunya and dengue sequence probes may be immobilised for example in a spaced array to facilitate detection and discrimination of specific binding to certain probe sequences. Such

methods of spotting nucleic acids on an array and array hybridisation and detection of binding are known in the art. Immobilisation or probes on microspheres and detection of sequences bound or hybridised thereto is also known in the art.

The chikungunya and dengue probes may be immobilised on coded microspheres, such that identification of the microsphere reveals the identity of a probe immobilised thereon. The coding may comprise colour coding. Thus, nucleic acid probes capable of binding to different nucleic acids, each characteristic of a particular viral strain, may be immobilised on different microspheres (which may be coded). For example a plurality of dengue nucleic acid probes capable of binding to different dengue nucleic acids, each characteristic of a dengue strain, may be used.

The method may comprise detecting detectable labels to detect amplified nucleic acids bound to microsphere-immobilised chikungunya and dengue probes. Furthermore, as the microspheres are coded, detection of the coding allows identification of the microsphere and reveals the identity of a probe immobilised thereon. The identity of an amplified nucleic acid sequence bound thereto is therefore obtained.

In the example provided below, a detectable label comprising a phycoerythin is incorporated into the amplified nucleic acid by biotin labelling the chikungunya primers and allowing binding of the amplified nucleic acid products to strepavidin-phycoerythin. The amplified nucleic acid is then allowed to hybridise and bind to a probe which is immobilised on a colour coded microsphere.

Different probes characteristic of different strains of dengue (and capable of binding to nucleic acids from these different strains) are immobilised on differently colour coded microspheres. The labelled amplified nucleic acids are allowed to hybridise to the immobilised probes.

The presence of an amplified nucleic acid is detected by laser excitation of the phycoerythrin and detection of an emitted signal. The coding of the microsphere is detected by laser excitation of a dye bound thereto (as part of the colour coding procedure) and the identity of the bead determined. The nature of the probe attached to the microsphere is determined by reference to the particular microsphere. Detection of both signals (which may be simultaneous or sequential) detects both the presence of an amplified nucleic acid as well

as determines its identity. For simultaneous detection, the two emitted signals may be of different wavelengths, and the two lasers accordingly may also be of different wavelengths.

DETECTION OF CHIKUNGUNYA AND DENGUE INFECTION

The methods described here may be used for detection of chikungunya and dengue infection in an individual. A sample is taken from the individual and tested for the presence of chikungunya and dengue nucleic acids, as described elsewhere. Presence of such nucleic acids establishes presence of the virus in the sample, and indicates chikungunya and dengue viral infection. Presence of a particular strain in the sample indicates infection by that strain.

It will be appreciated that all that it is not strictly necessary for intact chikungunya and dengue virus to be present in the sample. All that is necessary for detection is the presence of detectable (e.g., amplifiable) chikungunya and dengue nucleic acid in the sample.

The methods described here include therapeutic and prophylactic methods. Such methods include detection of chikungunya and dengue infection, optionally together with strain detection. They further include choosing a suitable therapy based on the presence of infection, or knowledge of infection by a particular strain, or both. Suitable therapies include antivirals, chikungunya and dengue vaccines, etc, as known in the art.

EXAMPLE ASSAY FOR DETECTING AND SUBTYPING CHIKUNGUNYA AND DENGUE USING RT-PCR

We set out an example of an assay to detect chikungunya and dengue in a sample. This example comprises a one-step Reverse Transcription-PCR assay to detect both Chikungunya virus and Dengue virus with Dengue serotype confirmation.

Our assay uses gene-specific confirmation probes, thus specific and is able to produce DEN serotype information. Therefore this assay will have potential commercial application in those countries where the detection of these etiological agents is crucial to patient management and disease control.

It should be made clear that this example is non-limiting and other methods of detecting chikungunya and dengue using the sequences disclosed here are available.

Duplex One-step RT-PCR

Duplex one-step RT-PCR is performed with the QIAGEN one-step RT-PCR kit (catalog no. 210210) in a 25-µl reaction volume containing 5 µl of RNA sample and all above seven primers on a thermal cycler, for example, Eppendorf Mastercycler-ep-gradient-S (Hamburg, Germany). Biotin-labeled PCR products are generated by 40 cycles of manufacture's recommended cycling condition with some modifications (see Examples).

Detection and Subtyping of Pathogen(s)

The biotin-labeled PCR product is then transferred to a hybridization suspension containing the above five target-specific capture probes, namely CHIKV and DENV (DEN1, 2, 3 and 4).

These probes are covalently linked to a specific set of color-coded microspheres.

DNA hybridization is carried out at stringent conditions and the biotin-labeled PCR products are captured by the bead-bound probes followed by stringent washing to remove unbound PCR products.

A streptavidin-phycoerythrin conjugate is used to add fluorescence to the biotin-labeled PCR products captured by the microspheres.

In the detection step each bead is identified by it's color-coding by one laser. A second laser detects the presence of the dye and when this is detected at the same time as a specific microsphere a signal is generated.

This technology is called xMAP technology being developed by Luminex Corporation (TX, USA).

EXAMPLES

Examples 5 to 7 below describe a specific embodiment comprising a RT-PCR Assay Kit to Detect Chikungunya virus (CHIKV) & Dengue Virus (DENV) with DENV Serotype Confirmation by Luminex® Probe-Beads. This is a RT-PCR Based Detection Kit with Confirmation Probes: 100 Reactions / Kit, Lot CD-3 ~ 6

Example 1. Materials and Methods

Selection of Primers

In house Institute of Molecular and Cell Biology (IMCB) primers and probes for CHIK virus are selected by using the IND-63-WB1 strain (Gen Bank accession no. EF027140) sequence. The E1 coding region is chosen for primers and probes.

IMCB primers and probes for DEN viruses are selected by using the DEN virus type 1 strain D1/SG/06K2290DK1/2006 (EU081281), type 2 strain D2/SG/05K3295DK1/2005 (EU081177), type 3 strain Singapore (AY662691) and type 4 strain ThD4_0734_00 (AY618993) sequences respectively. The 5NS to 3'NTR coding and non-coding regions are chosen.

Viral RNA Extraction

All viral RNAs were extracted from the serum portion or virus culture supernatant by using a QIAGEN QIAamp viral RNA minikit (QIAGEN, Germany) according to the manufacturer's protocol. RNAs from some patient seras were automatically extracted with the EasyMag instrument (bioMerieux, France).

Viral DNA Standards

Serial 10-fold dilutions of CHIK and DEN RNAs were prepared from a culture of local clinical isolates, cultured in C6/36 and Vero cell lines respectively.

Example 2. Fluid Microbead-based DNA Hybridization Assay

Five µl of biotin-labeled PCR product are transferred to a 96-well plate containing 45µl of hybridization suspension, which made up of 40µl of hybridization solution (3M TMAC , 0.1% Sarkosyl, 50mM Tris HCl, pH 8.0, 4mM EDTA) and 5µl of probe-beads mix (2000 probe-coupled microbeads per each target).

The probe-beads mix holds the five target specific capture probes (CHIK, DEN 1, 2, 3 and 4) which are conjugated to the specific color-coded microbeads. The 96-well plate is then heated at 95°C for 10 min to allow DNA denaturation, followed by the hybridization process of target DNA to the specific probe at 60°C for 40 min. The reactions are pelleted by centrifugation before the post hybridization washing is carried out at 54°C for 10 min to remove the unbound PCR products.

A streptavidin-phycoerythrin (SAPE) conjugate is used to add fluorescence to the biotin-labeled PCR products captured by the microbeads. The signal measurement is then performed in the Qiagen LiquiChip 200 ® (Hilden, Germany). The laser excitation of microbeads and SAPE bound to the biotin-labeled PCR product generates fluorescence signals, which allows both classification and quantification of the fluid microbeads-based DNA hybridization assay.

A cutoff value of greater than twice the background fluorescence of samples containing all components except target is used to indicate a positive reaction.

Example 3 - Sensitivity of One-step RT-PCR and Fluid Microbead-based Assay for CHIK and DEN

The detection limits of this assays are determined according to PFU. This is performed with 10-fold dilutions of seed viruses that had been quantitated by a plaque forming assay.

The detection threshold of the assay for DEN virus is ranging from 0.001 to 0.1 PFU per reaction depending serotypes and 0.025 PFU per reaction for CHIK virus.

The result is indicated in Figure 1. The sensitivity of the DEN results are comparable with the result done by Lai YL et al. (2007). See Figure 1 (Continued).

Example 4 - Results of Clinical Samples

Clinical samples are assayed by this detection method and the results are compared with the results obtained with two individual single assays. CHIK-Taqman-based assay for CHIK virus is a modification of Pastorino (2005) and Pan DEN SYBR Green-based assay for all four serotypes of DEN virus are adopted from Barkham (2006).

Results from clinical samples obtained with this assay are coincided with that obtained with the single assays. They are shown in Figure 2.

Example 5. RT-PCR Chikungunya and Dengue Detection Kit - Product Description

This kit is made to detect the presence of Chikungunya virus and Dengue virus by one step RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) and to subtype Dengue virus using serotype specific probes for Dengue virus.

Four tubes of primer mixture are included in this kit:

1) CHIKV-U: Upper primer for Chikungunya virus

2) CHIKV-L: Lower primer for Chikungunya virus

3) DENV-U: Upper primer for Dengue virus, 1,2,3,4

4) DENV-L: Lower primer for Dengue virus, 1,2,3,4

This primer kit is optimized to detect Chikungunya virus and Dengue virus simultaneously in multiplex one-step RT-PCR assay using the 5 ul viral RNA sample in 25 ul reaction volume configuration.

Results are obtained by agarose gel electrophoresis using ethidium bromide staining and/or by Luminex® detection platform for serotyping analysis.

Qiagen One Step RT-PCR Kit [Cat. no. 210210 or 210212] is recommended to use with this kit.

Example 6. RT-PCR Chikungunya and Dengue Detection Kit - Components

1. Primer tubes. CHIKV-U CHIKV-L DENV-U DENV-L See Figure 1. Storage Conditions (Short, Long) : 4°C, -20°C

2. Luminex Detection Kit

- Hybridization buffer, 8 ml

- Probe-beads mix, 500 ul (5ul/ rxn)

- Washing buffer, 30 ml

- Streptavidin, R-phycoerythrin conjugate solution (SA-PA)

Please store all reagents for Luminex detection at 4°C at all times.

Example 7. RT-PCR Chikungunya and Dengue Detection Kit - Protocol, One Step RT-PCR

1. Master Mix Preparation

In a RNase-Free thin-wall PCR tube (0.5 ml), add the following reagents per test/ per reaction. Qiagen One Step RT-PCR Kit [Cat. no. 210210, or 210212] is recommended to work with this kit.

Tubes:	Description	Vol (ul)/rxn	
CHIKV-U	U primer for CHIKV	0.38	Add to master mix.
CHIKV-L	L primer for CHIKV	0.40	
DENV-U	U primer for DENV all	0.35	
DENV-L	L primer for DENV all	0.35	
From Qiagen Kit	Qiagen 5x buffer	5.00	
	Qiagen dNTP Mix (10 mM ea.)	1.00	
	Qiagen Enzyme Mix (x25)	1.00	
	Qiagen RNase-free water	11.52	
	tRNA (2500 ng/ul)[1)]	0	
	RNA Internal control molecules[2)]	0	
	RNA Sample	5.0	
	Total rxn vol	25.0	

1) This tRNA is added here to avoid the non-specific PCR products if it is required. The final concentration of tRNA is 50 ng/ rxn in the reaction mixture.

2) This internal control (IC) RNA molecule is amplified by the primers included in this kit to produce 100-bp amplicon. The end-product is detectable by EtBr Agarose gel or gene specific probes conjugated to Luminex beads (included).

*It is essential to include a positive and negative control to validate the result.

2. Thermal Cycling Conditions

Step	Temp (°C)	RoboCycler® 96 by Stratagene Duration	Px2 Thermal Cycler by Thermo Electron (Tube mode) Duration	GeneAmp 9700 by ABI, Aluminium (Standard mode) Duration	Eppendorf Mastercycler by Eppendorf (100% ramp mode) Duration	Num of Cycles	Step
1	**56**	30 mins	30 mins	30 mins	30 mins	1	Reverse transcription
2	95	15 mins	15 mins	15 mins	15 mins	1	Initial denaturation
3	94	32 secs	13 secs	12 secs	27v	40	Denaturation
	55	88 secs	55 secs	61 secs	77 secs	40	Annealing
	72	34 secs	20 secs	19 secs	30 secs	40	Extension
4	72	3 mins	3 mins	3 mins	3 mins	1	Final Extension

3. Detection by Luminex®

Figure 2 shows a flow chart of a detection protocol.

Step 1: Sample Preparation

Mix 5 µl sample from PCR (3), 40 µl hybridisation buffer (1 x TMAC) (1) and 5µl beads mix (containing 2000 beads/each probe) (2) in a well of a multi-well titre plate. Seal with Parafilm. Do not spin.

Step 2: DNA Denataturation and Hybridisation

Incubate at 95°C for 10 min, then incubate at 60°C for 40 min using heating block such as ABI GeneAmp9700.

Centrifuge @ 1000G for 5 min at Room Temp. Discard supernatant by flicking twice and tapping twice over paper.

Step 3: Post Hybridization Wash (Repeat x2)

Wash with 100 µl Washing buffer-PBS, Tween 20 0.01% at T °C for 10 min (T = 54° for CHIKV & DENV Detection Kit). Centrifuge for 10 min. Discard supernatant.

Step 4: Signal Production

Add 70µl of 1/500 SA-PA in Washing buffer-PBS, 0.01% Tween 20; incubate for 5 mi at 52°C

Step 5: Signal Measurement

Reading by Luminex

Following bead numbers are used for each confirmation probe.

CHIKV: 29 DEN1: 88, DEN2: 89, DEN3: 90, DEN4: 72

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Each of the applications and patents mentioned in this document, and each document cited or referenced in each of the above applications and patents, including during the prosecution of each of the applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims.

CLAIMS

1. A method of detecting chikungunya and dengue nucleic acids in a biological sample, the method comprising the steps of:

(a) an amplification step in which chikungunya and dengue nucleic acids are amplified by chikungunya primers and dengue primers in a nucleic acid amplification reaction comprising for example a polymerase chain reaction (PCR) such as reverse transcription polymerase chain reaction (RT-PCR) or duplex RT-PCR; and

(b) a detection step in which amplified nucleic acids from (a) are hybridised to a chikungunya nucleic acid probe and a dengue nucleic acid probe in a nucleic acid hybridisation reaction, preferably in which the amplified nucleic acids are hybridised to a plurality of dengue nucleic acid probes capable of binding to different dengue nucleic acids, each characteristic of a dengue strain, to enable identification of a nucleic acid from a particular dengue strain in a biological sample.

2. A method according to Claim 1, in which the chikungunya and dengue probes are immobilised on coded microspheres, such that identification of the microsphere reveals the identity of a probe immobilised thereon, preferably in which the coded microspheres comprise colour coded microspheres.

3. A method according to Claim 1 or 2, in which the amplified nucleic acids are labelled with a detectable label, preferably by labelling chikungunya nucleic acid primers and dengue nucleic acid primers with a detectable label such that amplification step (a) generates labelled amplified nucleic acids, the detectable label preferably comprising biotin or a biotin strepavidin-phycoerythin conjugate, or in which

4. A method according to any of Claims 1, 2 or 3, in which the method comprises detecting detectable labels to detect amplified nucleic acids bound to microsphere-immobilised chikungunya and dengue probes, the method preferably further comprising identifying a microsphere to identify a probe immobilised thereon and hence the identity of a bound amplified nucleic acid.

5. A method according to any preceding claim, in which a detectable label comprising a biotin strepavidin-phycoerythin conjugate is detected by means of a first laser and the identity

of a colour coded microsphere is determined by means of a second laser of a different wavelength, preferably in which the detectable label is detected simultaneously with the identification of the colour coded microsphere.

6. A method according to any preceding claim, in which:

(a) the chikungunya nucleic acid primers consist of the nucleic acid sequences shown in Table D1.1, or in which the dengue nucleic acid primers consist of the nucleic acid sequences shown in Table D1.2, or both; or

(b) the chikungunya nucleic acid probes consist of the nucleic acid sequences shown in Table D2.1, or in which the dengue nucleic acid probes consist of the nucleic acid sequences shown in Table D2.2, or both.

7. A method according to any preceding claim for simultaneously detecting the presence of chikungunya and determining the strain of dengue in a biological sample.

8. A nucleic acid sequence as shown in Table D1.1, Table D1.2, Table D2.1 or Table D2.2, or a variant, homologue, derivative or fragment thereof.

9. A combination of two or more sequences according to Claim 15, preferably comprising two or more sequences set out in Table D1.1 and Table D1.2, or two or more sequences set out in Table D2.1 and Table D2.2, or both.

10. A combination according to Claim 9, in which the combination consists of:

(a) each of the nucleic acid sequences shown in Table D1.1 and Table D1.2, or a variant, homologue, derivative or fragment thereof;

(b) each of the nucleic acid sequences shown in Table D2.1 and Table D2.2, or a variant, homologue, derivative or fragment thereof; or

(c) a combination consisting of (a) and (b) above.

11. A nucleic acid or combination according to Claim 8, 9 or 10, comprising a labelled nucleic acid, in which the label comprises for example biotin or a biotin strepavidin-phycoerythrin conjugate.

12. A nucleic acid or combination according to any of Claims 8 to 11, comprising an immobilised nucleic acid, preferably in which the nucleic acid is immobilised on a microsphere or an array such as a microarray.

13. A combination consisting of: (a) each of the nucleic acid sequences shown in Table D1.1 and Table D1.2, or a variant, homologue, derivative or fragment thereof; and (b) each of the nucleic acid sequences shown in Table D2.1 and Table D2.2, or a variant, homologue, derivative or fragment thereof; in which the nucleic acids of (a) are in solution and the nucleic acids of (b) are immobilised on microspheres.

14. A kit for the detection of chikungunya or dengue in a patient, the kit comprising a nucleic acid or combination according to any of Claims 8 to 13, together with instructions for use.

15. Use of a nucleic acid sequence or a combination according to any of Claims 8 to 14 as a primer for nucleic acid amplification of a chikungunya or dengue gene or as a probe for nucleic acid hybridisation of a chikungunya or dengue gene.

16. A method of diagnosis of chikungunya or dengue in an individual, the method comprising obtaining a biological sample from the individual and detecting chikungunya and dengue nucleic acids therein by a method according to any of Claims 1 to 7.

17. A method of treatment or prevention of chikungunya or dengue in an individual, the method comprising detecting chikungunya or dengue in an individual according to Claim 16 and administering a suitable treatment or prophylactic to the individual.