Method for Detection of Enterovirus

TECHNICAL FIELD

The present invention relates to the detection of enteroviruses which cause hand, foot and mouth disease, and in particular the detection of the E71 virus serotype. More specifically, the present invention relates to primers and probes for use in polymerase chain reaction based tests, capable of differentiating E71 virus from other viruses which cause hand, foot and mouth disease. Kits of primers and probes are also provided, together with the use of the primers and probes in methods of diagnosis.

BACKGROUND

Hand, foot and mouth disease (HFMD) is a common viral illness of children and infants that causes fever and appearance of vesicular rashes on hand, feet and the buccal mucosa. The main etiological agents of HFMD are enteroviruses belonging to the Picornaviridae family, namely Enterovirus 71 (EV71), Coxsackievirus A16 (CA16), Coxsackievirus B2 (CB2) and Echovirus 7 (Echo 7). Picornaviridae family is a large and diverse group of small RNA viruses characterized by a single positive-strand genomic RNA.

EV71 has emerged as an important strain due to its exceptional virulence, which often results in neurological complications and rapid death. Neurological complications caused by EV71 include aseptic meningitis, brainstem encephalitis, pulmonary edema and poliomyelitis-like paralysis. In recent outbreaks of HFMD in Asia, more than 300 fatalities have been reported. In China, a recent epidemic reported more than 250,000 cases, of which 250 lead to fatalities. In Singapore, by mid 2010, more than 19,000 cases were reported and amongst which 12% were found to be caused by EV71.

Whilst the viral genomes of all of the known enteroviruses have been sequenced and are available in public, few diagnostic methods for enterovirus identification are available. Conventional diagnostic method for enterovirus identification requires special detection of viruses in infected tissues by cell culture, which is followed by neutralization tests with serotype-specific antisera. Whilst this method is currently deemed to be the "gold

standard", this method requires two weeks of growth and antigenic typing that could be hindered by non-neutralizable viruses due to unwanted aggregation, antigenic drifts, or the presence of multiple viruses in the specimens.

Serological methods such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) have been developed previously. These methods may be used to detect a rise in neutralizing antibody titer during the acute phase infection or convalescence. However, these methods have been shown to have limited sensitivity.

Polymerase chain reaction (PCR) techniques have also contributed significantly to laboratory diagnosis of virus infection. Whilst PCR techniques have provided for means of detecting enteroviruses that have improved speed and sensitivity, these methods still suffer lack of specificity.

Further attempts to improve PCR techniques include the use of reverse-transcriptase PCR (RT-PCR) strategy. RT-PCR is found to be more sensitive than normal conventional diagnosis methods. However, the two-step RT-PCR is time-consuming and cross-contamination may still occur. Conventional RT-PCR used to detect enteroviruses requires about six hours of assay time. Whilst this has provided for a relatively fast diagnosis tool, this still falls short of providing for a fast and sensitive assay, especially in view of the wide variation in clinical presentation associated with EV71 and its potential for severe neurological complications and rapid death. Furthermore, with the increasing number of cases reported each year, in anticipation of a severe outbreak, a rapid and sensitive assay is needed.

Accordingly, there is a need to provide a method of diagnosing EV71 that overcomes or at least ameliorates one or more of the disadvantages described above.

SUMMARY OF THE INVENTION

The present inventors have developed an assay to identify and differentiate hand, foot and mouth disease-causing enteroviruses. The present inventors have further developed an

assay that identifies and differentiates EV71 virus from other hand, foot and mouth-causing enteroviruses. Primers and probes, kits and methods of the present invention have been designed to ensure optimal sequence length for specificity and sensitivity and allow specific amplification of enteroviruses and allow the detection and differentiation of EV71 virus with high sensitivity. The present assay differentiates even newly identified mutated strains of EV71 virus that differ from other strains of EV71 virus previously identified.

Thus, in a first aspect there is provided a primer or probe having a target sequence in the region from 1bp to 746bp of 5' untranslated region (5' UTR) of an enterovirus genomic sequence. The primer or probe may comprise or consist of the nucleotide sequence of any of SEQ ID NO: 1 to 3, or complements thereof.

In a second aspect there is provided a forward primer as described herein for amplifying a nucleotide sequence of an enterovirus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 1, or complements thereof.

In a third aspect there is provided a reverse primer as described herein for amplifying a nucleotide sequence of an enterovirus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO: 2, or complements thereof.

In a fourth aspect there is provided a probe as described herein in which the probe sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO: 3 or complements thereof.

In a fifth aspect there is provided a primer or probe having a target sequence in the VP1 region from 2442bp to 3332bp of EV71, wherein said primer is not SEQ ID NO:9. In one embodiment, the primer or probe consists of the nucleotide sequence of any of SEQ ID NO: 4 to 6, or complements thereof.

In a sixth aspect there is provided a forward primer as described herein for amplifying a nucleotide sequence of an EV71 virus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO:4, 10, 11, 12, 13, 14, 15 or 16, or complements thereof.

In a seventh aspect there is provided a reverse primer as described herein for amplifying a nucleotide sequence of an EV71 virus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 5, 17, 18, 19, 20, 21, 22, 23 or 24 or complements thereof.

In an eighth aspect there is provided a probe as described herein in which the probe sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 6.

In a ninth aspect there is provided a set of primers for amplifying enterovirus in a test sample comprising a pair of forward and reverse primers, in which the forward primer comprises or consists of SEQ ID NO:1 or complements thereof; and the reverse primer comprises or consists of SEQ ID NO:2, or complements thereof.

In a tenth aspect there is provided a set of primers for amplifying EV71 virus in a test sample comprising a pair of forward and reverse primers, in which the primers comprise or consist of sequences selected from the group consisting of Set 2 to Set 65, or complements thereof.

In an eleventh aspect there is provided a set of primers and a probe for detecting enterovirus in a test sample comprising or consisting of a forward primer comprising or consisting of SEQ ID NO:1 or complements thereof; a reverse primer comprising or consisting of SEQ ID NO:2, or complements thereof; and a probe comprising or consisting of SEQ ID NO:3 or complements thereof.

In a twelfth aspect there is provided a set of primers and a probe for detecting EV71 virus in a test sample comprising or consisting of a set of primers as described herein and a probe comprising or consisting of SEQ ID NO: 6 or complements thereof.

In a thirteenth aspect there is provided a kit for detecting enterovirus and EV71 in a test sample comprising a set of primers and a probe for detecting enterovirus as described herein and a set of primers and a probe for detecting EV71 as described herein.

In a fourteenth aspect there is provided a set as described herein or a probe as described herein in which the nucleotide sequence of the probe is labelled with a detectable label, and in which the detectable label is directly or indirectly attached to the probe. In one embodiment, the detectable label comprises a fluorescent moiety attached at a 5' end of the probe. In a further embodiment, the nucleotide sequence of the probe further comprises a quencher moiety attached at a 3' end of the probe. In one embodiment of the set or probe as described herein, the detectable label of each of the probe sequences of SEQ ID NO:3 and SEQ ID NO:6, is such that the probe sequences are independently detectable.

In a fifteenth aspect there is provided a method for determining the presence or absence of an enterovirus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with at least one primer or probe or set as described herein.

In a sixteenth aspect there is provided a method for determining the presence or absence of an EV71 virus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with at least one primer or probe or set as described herein.

In a seventeenth aspect there is provided a method for determining the presence or absence of an enterovirus and an EV71 virus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with

at least one primer or probe or set as described herein for detecting enterovirus and/or with at least one primer or probe or set as described herein for detecting EV71. In one embodiment of the method as described herein, the method further comprises the step of determining whether the nucleotide sequence hybridises to the at least one primer or probe under stringent conditions, thereby detecting whether the sample contains the virus. In one embodiment of a method as described herein, in situ hybridisation is used to detect whether the nucleotide sequence hybridises to the at least one primer or probe.

In one embodiment of a method as described herein, the method comprises the steps of: (a) contacting the nucleotide sequence obtained or derived from the biological sample with at least one forward primer suitable for detecting enterovirus as described herein and at least one reverse primer suitable for detecting enterovirus as described herein, or at least one set suitable for detecting enterovirus as described herein, under amplification conditions to generate an amplicon of a region of an enterovirus genomic sequence; and (b) detecting hybridization between the amplicon and at least one probe suitable for detecting enterovirus as described herein.

In one embodiment of a method as described herein, the method comprises the steps of:
(a) contacting a nucleotide sequence obtained or derived from a biological sample with at least one forward primer suitable for detecting EV71 as described herein and at least one reverse primer suitable for detecting EV71 as described herein, or at least one set suitable for detecting EV71 as described herein, under amplification conditions to generate an amplicon of a region of an EV71 genomic sequence; and
(b) detecting hybridization between the amplicon and at least one probe suitable for detecting EV71 as described herein.

In one embodiment of a method as described herein, the amplification conditions comprise an amplification reaction, and the amplification reaction is a polymerase chain reaction (PCR).

In one embodiment of a kit as described herein, the kit further comprises amplification reagents.

In an eighteenth aspect there is provided a method of treating a patient infected with an enterovirus or EV71 virus comprising: determining the presence of an enterovirus or EV71 virus in a biological sample derived from the patient using a method as described herein and then administering an anti-viral composition or medication or a immunotherapy to the patient.

In a nineteenth aspect there is provided a use of a composition comprising an anti-viral composition or medication or a immunotherapy in the manufacture of a medicament for the treatment of a patient infected with an enterovirus, wherein the presence of an enterovirus in a biological sample from the patient has been determined using a method as described herein.

In a twentieth aspect there is provided a composition comprising an anti-viral composition or medication or a immunotherapy for the treatment of a patient infected with an enterovirus, wherein the presence of an enterovirus serotype in a biological sample from the patient has been determined using a method as described herein.

BRIEF DESCRIPTION OF FIGURES, TABLES AND SEQUENCES

Figures

Figure 1. Schematic overview of TaqMan Probe Assay. In TaqMan probe-based approach, the probe contains one fluorophore at its 5'terminus and a quencher at its 3'terminus (a). When in close proximity, the two dyes formed a quenched system and no fluorescence was observed. Upon hybridization (b), the 5'exonuclease activity of the Taq polymerase would hydrolyse the probe during PCR amplification. Once the fluorophores are separated from the quencher (c), the emission of the fluorophore were no longer quenched and fluorescence detected (d) (Adapted from Applied Biosystems, USA).

Figure 2. Graph showing specific detection of enteroviruses at 520 nm and water was present as negative control. Other enteroviruses analysed include CA16, CB2, CB3, Echo 6, Echo 7.

Figure 3. Graph showing specific detection of EV71 at 556nm, other enteroviruses and water are present as negative control.

Figure 4. Conserved 5'UTR sequence (SEQ ID NO:7) of EV71 virus, showing region to which the forward primer (SEQ ID NO: 1) and reverse primer (SEQ ID NO: 2) bind and the probe sequence (SEQ ID NO:3).

Figure 5. Conserved VP1 sequence (SEQ ID NO:8) of EV71 virus, showing region to which the forward primer (SEQ ID NO: 4) and reverse primer (SEQ ID NO: 5) bind and the probe sequence (SEQ ID NO:6).

Figure 6A, B, C, D, E and F. Alignment report of EV71 showing a mutated strain of EV71 that differs from other strains of EV71 previously identified.

Figure 7 A and B. Amplification of HFMD-causing enteroviruses with specific serotyping of EV71 by multiplex RT-PCR in CFX96 real-time PCR detection system.

Figure 8 A and B. Amplification of HFMD-causing enteroviruses with specific serotyping of EV71 by multiplex RT-PCR in Rotorgene real-time PCR detection system.

Tables

Table 1. Overview of the primer and probe sequences for detection of HFMD-causing enteroviruses and specific detection of EV71.

Table 2. Nucleotide sequences of the EVFPnew.

Table 3. Nucleotide sequences of the EVFPnew.

Table 4. Components of the multiplex real-time RT-PCR setup.

Table 5. Components of the multiplex real-time RT-PCR setup, with re-adjusted volumes

Sequences

SEQ ID NO:1: Forward Primer for enterovirus, with a target nucleotide sequence in the 5'UTR region of enteroviruses of from position 414 to 434.

SEQ ID NO:2: Reverse Primer for enterovirus, with a target nucleotide sequence in the 5'UTR region of enterovirus of from position 600 to 577.

SEQ ID NO:3: Probe for enterovirus, with a target nucleotide sequence in the 5'UTR region of enterovirus of from position 443 to 467.

SEQ ID NO:4: Forward Primer for EV71, with a target nucleotide sequence in the VP1 of from position 2466 to 2489, wherein Y_1 / Y_2 respectively represent C/T.

SEQ ID NO:5: Reverse Primer for EV71, with a target nucleotide sequence in the VP1 of from position 2669 to 2647, wherein R_1 / R_2 respectively represent A/G

SEQ ID NO:6: Probe for EV71, with a target nucleotide sequence in the VP1 of from position 2498 to 2521.

SEQ ID NO:7: Conserved 5'UTR region for enterovirus (from 1 to 746 of enterovirus genome)

SEQ ID NO:8: Conserved VP1 region for EV71 (from 2442 to 3332 of enterovirus genome).

SEQ ID NO:9: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is T; R is G; and Y_2 is C, respectively.

SEQ ID NO:10: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is T; R is G; and Y_2 is T, respectively.

SEQ ID NO:11: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is T; R is A; and Y_2 is C, respectively.

SEQ ID NO:12: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is T; R is A; and Y_2 is T, respectively.

SEQ ID NO:13: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is C; R is G; and Y_2 is C, respectively.

SEQ ID NO:14: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is C; R is G; and Y_2 is T, respectively.

SEQ ID NO:15: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is C; R is A; and Y_2 is C, respectively.

SEQ ID NO:16: Forward Primer for EV71 according to SEQ ID NO:4, wherein Y_1 is C; R is A; and Y_2 is T, respectively.

SEQ ID NO:17: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is T; R_1 is G; and R_2 is G, respectively.

SEQ ID NO:18: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is T; R_1 is G; and R_2 is A, respectively.

SEQ ID NO:19: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is T; R_1 is A; and R_2 is G, respectively.

SEQ ID NO:20: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is T; R_1 is A; and R_2 is A, respectively.

SEQ ID NO:21: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is C; R_1 is G; and R_2 is G, respectively.

SEQ ID NO:22: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is C; R_1 is G; and R_2 is A, respectively.

SEQ ID NO:23: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is C; R_1 is A; and R_2 is G, respectively.

SEQ ID NO:24: Reverse Primer for EV71 according to SEQ ID NO:5, wherein Y is C; R_1 is A; and R_2 is A, respectively.

DETAILED DESCRIPTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of stated integers or steps but not to the exclusion of any other integer or step or group of integers or steps.

VIRUSES

The following hand, foot and mouth disease viruses were analysed in this invention: Enterovirus 71 (EV71); Cocksackievirus A16 (CA16); Cocksackievirus B2 (CB2); Cocksackievirus B3 (CB3); Echovirus 6 (Echo 6); and Echovirus 7 (Echo 7). Accordingly, as used herein, the term “enterovirus” refers to these viruses. In one embodiment, the term enterovirus may also refer to other enteroviruses. The methods used herein are capable of distinguishing the enteroviruses listed above and may also and additionally distinguish further enteroviruses not listed above, provided that the primers and probes are capable of specific hybridisation to the sequences thereof.

PRIMERS AND PROBES

The nucleotide sequences presented herein are contiguous, 5’ to 3’ nucleotide sequences, unless otherwise described.

As used herein, the term "amplicon" refers to a product of an amplification reaction. An example of an amplicon is a DNA or an RNA product (usually a segment of a gene, DNA or RNA) produced as a result of PCR, real-time PCR, RT-PCR, competitive RT-PCR, ligase chain reaction (LCR), gap LCR, strand displacement amplification (SDA), nucleic acid sequence based amplification (NASBA), transcription-mediated amplification (TMA), or the like.

The term "primer" is used herein to mean any single- stranded oligonucleotide sequence capable of being used as a primer in, for example, PCR technology. Thus, a "primer" according to the invention refers to a single- stranded oligonucleotide sequence that is capable of acting as a point of initiation for synthesis of a primer extension product that is substantially identical to the nucleic acid strand to be copied (for a forward primer) or substantially the reverse complement of the nucleic acid strand to be copied (for a reverse primer). A primer or probe sequence may be suitable for use in, for example, PCR technology. As used herein, the primer and probe sequences do not include the full length nucleotide sequence of the EV71 virus genome. By single-stranded includes, for example, hairpin structures formed by single-stranded nucleotide sequences.

The design of a primer, for example its length and specific sequence, depends on the nature of the DNA and/or RNA targets and on the conditions at which the primer is used, for example, temperature and ionic strength.

The primers may consist of the nucleotide sequences described herein, or may be 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, 100 or more nucleotides which comprise or fall within the sequences described herein, provided they are suitable for specifically binding a target sequence, under stringent conditions. In one embodiment, the primer sequence is less than 35 nucleotides in length, for example the primer sequence is less than 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, or 17 nucleotides in length.

Slight modifications of the primers or probes, in length or in sequence, can be carried out to maintain the specificity and sensitivity required under the given circumstances. In one

embodiment of the present invention, probes and/ or primers described herein may be extended in length by 1, 2, 3, 4 or 5 nucleotides or reduced in length by 1, 2, 3, 4 or 5 nucleotides, for example, in either direction.

The term "probe" is well known in the art and is used herein to mean any single-stranded oligonucleotide sequence capable of binding nucleic acid and of being used as a probe in, for example, PCR technology.

Primer and probe sequences can be synthesised using any methods well known in the art. For example, nucleotide sequences can be obtained from First Base Pte Ltd, Singapore.

As used herein, unless otherwise described or amended, the term "comprising" in relation to the probe and/ or primer sequences described herein may be considered to include sequences that are extended in length by 1, 2, 3, 4 or 5 nucleotides, for example, in either direction.

As used herein, any "hybridisation" is performed under stringent conditions. The term "stringent conditions" means any hybridisation conditions which allow the primers to bind specifically to their defined target nucleotide sequence within the enterovirus or EV71 nucleotide sequence, but not to any other enterovirus or EV71 nucleotide sequence or to any other known sequences within the viral genome.

For example, specific hybridisation of a probe to its nucleic acid target region under "stringent" hybridisation conditions, include conditions such as 3X SSC, 0.1% SDS, at 50°C. The skilled person knows how to vary the parameters of temperature, probe length and salt concentration such that specific hybridisation can be achieved. Hybridisation and wash conditions are well known in the art.

"Specific binding" or "specific hybridisation" of a probe to its target region of the enterovirus or EV71 virus nucleotide sequence means that the primer or probe forms a duplex (double-stranded nucleotide sequence) with part of this region or with the entire

region under the experimental conditions used, for example under stringent hybridisation conditions, and that under those conditions the primer or probe does not form a duplex with other regions of the nucleotide sequence present in the sample to be analysed.

As used herein, the term "target sequence" is a region of the enterovirus or EV71 virus nucleic acid sequence (either DNA or RNA, e.g. genomic DNA, messenger RNA, or amplified versions thereof) to which the sequence of the probe or primer has partial (i.e. with some degree of mismatch) or total identity; although the reverse primer is the reverse complement (or, as above, has some degree of mismatch) of the sequence it recognises.

For a primer, the target sequence generally refers to a region of the enterovirus or EV71 virus sequence that differs by at least one nucleotide compared to another, or to all other, enterovirus or EV71 virus nucleotide sequences and is a sequence to which the primer is capable of binding under stringent conditions. For a probe, the target sequence is generally an "amplicon", that is, a sequence that is amplified by the primer sequences, and is a sequence to which the probe is capable of binding under stringent conditions. Provided, of course, that a reverse primer fulfils such conditions to a target sequence that is the reverse complement of the primer sequence.

The present invention provides methods for the detection of enterovirus and detection of EV71 virus. Specific regions within 5'UTR of enterovirus and/or VP1 of EV71 have been identified that facilitate the specific detection of enterovirus and/or EV71 virus. In one embodiment of the present invention, enterovirus virus may be identified by detection of a target sequence in the region defined by SEQ ID NO: 7. In one embodiment of the present invention, EV71 virus may be identified by detection of a target sequence in the region defined by SEQ ID NO: 8. The target sequence may be identified by any means known in the art. For example, the target sequence may be detected using primer(s) and/or probe(s) as described herein.

In one embodiment, a primer as described herein has a target sequence in a region identified in the column labelled "Position" in Table 1. Thus, in one embodiment, there is provided a primer or set of primers having a target sequence in the region from 1 to 746bp of the 5'UTR of enteroviruses. In a further embodiment, there is provided a primer or set of primers having a target sequence in the region from 2442 to 3332bp of the VP1 of EV71. More specifically, there is provided a forward primer and a reverse primer as described herein in which, under amplification conditions, for example in a method as described herein, the forward and reverse primer are capable of amplifying a sequence in these regions. Additionally, there is further provided a probe as described herein that is capable of hybridising to such an amplified sequence. In one embodiment of the present invention, the target sequences of the primers are in the regions identified in the column labelled "Position" in Table 1.

Suitably, the primer or probe may be at least 95% identical to its target sequence over the length of the primer or probe, suitably greater than 95% identical such as 96%, 97%, 98%, 99% and most preferably has 100% identity over its length to its target virus sequence. The primers or probes of the invention may be identical to the target sequence at all nucleotide positions of the primer or probe, or may have 1, 2, or more mismatches depending upon the length of probe, temperature, reaction conditions and requirements of the assay, for example. Provided, of course, that the reverse primer fulfils these conditions to a target sequence that is the reverse complement of the primer sequence.

Suitably each nucleotide of the primer or probe can form a hydrogen bond with its counterpart target nucleotide. Preferably the complementarity of primer or probe with the target sequence is assessed by the degree of A:T and C:G base pairing, such that an adenine (A) nucleotide pairs with a thymine (T), and such that a guanine (G) nucleotide pairs with a cytosine (C), or vice versa. In the RNA form, T may be replaced by U (uracil). In one example, inosine (I) may be included in a sequence considered complementary to another sequence, in view of its ability to indiscriminately pair with adenine, thymine, or cytosine. As used herein, the letters Y_1 or Y_2 when used within a nucleotide sequence indicate that indicated nucleotide is C or T; and the letters R_1 or R_2

when used within a nucleotide sequence indicate that indicated nucleotide is A or G. The use of the subscript is only present to differentiate the R or Y nucleotide and does not have any other meaning in this context.

By "biological sample" is meant a sample of tissue or cells from a patient that has been obtained from, removed or isolated from the patient.

The term "obtained or derived from" as used herein is meant to be used inclusively. That is, it is intended to encompass any nucleotide sequence directly isolated from a biological sample or any nucleotide sequence derived from the sample.

Table 1: Nucleotide sequence of the specific primer and probe for detection of HFMD-causing enteroviruses and specific detection of EV71.

Primer/Probe	Nucleotide Sequence (5' – 3')	Position	Seq ID No:
Enterovirus Detection			
5UTRFP3	ACATGGTGCGAAGAGCCTATT	414 to 434	1
5UTRRP3	GTCACCATAAGCAGCCAATATAAG	600 to 577	2
5UTR Probe	TAGTAGTCCTCCGGCCCCTGAATGC	443 to 467	3
EV71 Detection			
EVFPnew	GAGAGY_1TCTATAGGRGAY_2AGTGTG	2466–2489	4
EVRPnew	TGCYGTACTGTGTGAR_1TTAAGR_2A	2669–2647	5
EVprobe	ACTTACCCAGGCCCTGCCAGCTCC	2498–2521	6

Y_1 / Y_2 – C/T, R_1 / R_2 – A/G

Table 2: Nucleotide sequences of the EVFPnew forward primer for EV71 detection.

Nucleotide Sequence (5' – 3') GAGAG**Y_1**TCTATAGG**R**GA**Y_2**AGTGTG (SEQ ID NO:4)	Y_1	R	Y_2	SEQ ID NO:
GAGAG**T**TCTATAGG**G**GA**C**AGTGTG	T	G	C	9
GAGAG**T**TCTATAGG**G**GA**T**AGTGTG	T	G	T	10
GAGAG**T**TCTATAGG**A**GA**C**AGTGTG	T	A	C	11
GAGAG**T**TCTATAGG**A**GA**T**AGTGTG	T	A	T	12
GAGAG**C**TCTATAGG**G**GA**C**AGTGTG	C	G	C	13

GAGAGCTCTATAGGGGATAGTGTG	C	G	T	14
GAGAGCTCTATAGGAGACAGTGTG	C	A	C	15
GAGAGCTCTATAGGAGATAGTGTG	C	A	T	16

Table 3: Nucleotide sequences of the EVRPnew reverse primer for EV71 detection.

Nucleotide Sequence (5' – 3') TGCYGTACTGTGTGAR$_1$TTAAGR$_2$A (SEQ ID NO :5)	Y	R_1	R_2	SEQ ID NO:
TGCTGTACTGTGTGAGTTAAGGA	T	G	G	17
TGCTGTACTGTGTGAGTTAAGAA	T	G	A	18
TGCTGTACTGTGTGAATTAAGGA	T	A	G	19
TGCTGTACTGTGTGAATTAAGAA	T	A	A	20
TGCCGTACTGTGTGAGTTAAGGA	C	G	G	21
TGCCGTACTGTGTGAGTTAAGAA	C	G	A	22
TGCCGTACTGTGTGAATTAAGGA	C	A	G	23
TGCCGTACTGTGTGAATTAAGAA	C	A	A	24

Accordingly, the present invention provides a primer or probe comprising the nucleotide sequence of any of SEQ ID NO:1, 2, 3, 4, 5, 6, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, as shown in Tables 1-3.

The present invention further provides a set of primers for enterovirus detection comprising the following pairs of primers:
Set 1: SEQ ID NO:1 and 2;

The present invention further provides a set of primers for EV71 detection comprising one or more of the following pairs of primers:
Set 2: SEQ ID NO:10 and 5; Set 3: SEQ ID NO:11 and 5; Set 4: SEQ ID NO:12 and 5; Set 5: SEQ ID NO:13 and 5; Set 6: SEQ ID NO:14 and 5; Set 7: SEQ ID NO:15 and 5; Set 8: SEQ ID NO:16 and 5;
Set 9: SEQ ID NO:10 and 17; Set 10: SEQ ID NO:11 and 17; Set 11: SEQ ID NO:12 and 17; Set 12: SEQ ID NO:13 and 17; Set 13: SEQ ID NO:14 and 17; Set 14: SEQ ID NO:15 and 17; Set 15: SEQ ID NO:16 and 17;

Set 16: SEQ ID NO:10 and 18; Set 17: SEQ ID NO:11 and 18; Set 18: SEQ ID NO:12 and 18; Set 19: SEQ ID NO:13 and 18; Set 20: SEQ ID NO:14 and 18; Set 21: SEQ ID NO:15 and 18; Set 22: SEQ ID NO:16 and 18;

Set 23: SEQ ID NO:10 and 19; Set 24: SEQ ID NO:11 and 19; Set 25: SEQ ID NO:12 and 19; Set 26: SEQ ID NO:13 and 19; Set 27: SEQ ID NO:14 and 19; Set 28: SEQ ID NO:15 and 19; Set 29: SEQ ID NO:16 and 19;

Set 30: SEQ ID NO:10 and 20; Set 32: SEQ ID NO:11 and 20; Set 33: SEQ ID NO:12 and 20; Set 34: SEQ ID NO:13 and 20; Set 35: SEQ ID NO:14 and 20; Set 36: SEQ ID NO:15 and 20; Set 37: SEQ ID NO:16 and 20;

Set 38: SEQ ID NO:10 and 21; Set 39: SEQ ID NO:11 and 21; Set 40: SEQ ID NO:12 and 21; Set 41: SEQ ID NO:13 and 21; Set 42: SEQ ID NO:14 and 21; Set 43: SEQ ID NO:15 and 21; Set 44: SEQ ID NO:16 and 21;

Set 45: SEQ ID NO:10 and 22; Set 46: SEQ ID NO:11 and 22; Set 47: SEQ ID NO:12 and 22; Set 48: SEQ ID NO:13 and 22; Set 49: SEQ ID NO:14 and 22; Set 50: SEQ ID NO:15 and 22; Set 51: SEQ ID NO:16 and 22;

Set 52: SEQ ID NO:10 and 23; Set 53: SEQ ID NO:11 and 23; Set 54: SEQ ID NO:12 and 23; Set 55: SEQ ID NO:13 and 23; Set 56: SEQ ID NO:14 and 23; Set 57: SEQ ID NO:15 and 23; Set 58: SEQ ID NO:16 and 23;

Set 59: SEQ ID NO:10 and 24; Set 60: SEQ ID NO:11 and 24; Set 61: SEQ ID NO:12 and 24; Set 62: SEQ ID NO:13 and 24; Set 63: SEQ ID NO:14 and 24; Set 64: SEQ ID NO:15 and 24; and Set 65: SEQ ID NO:16 and 24.

The present invention further provides a probe for enterovirus detection comprising the nucleotide sequence of or comprising SEQ ID NO: 3. The probe may consist of the nucleotide sequences shown in SEQ ID NO: 3, or may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, 25, 30, 35, 40, 45, 50, 75, 100 or more nucleotides which comprise or fall within the sequence of SEQ ID NO: 3, provided they are suitable for specifically binding a target sequence within a 5'UTR of an enterovirus, as described herein.

The present invention further provides a probe for EV71 detection comprising the nucleotide sequence of any of SEQ ID NO: 6. The probes may consist of the nucleotide sequences shown in SEQ ID NO: 6, or may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, 25, 30, 35, 40, 45, 50, 75, 100 or more nucleotides which comprise or fall within the sequence of SEQ ID NO: 6, provided they are suitable for specifically binding a target sequence within a VP1 region of an EV71 virus nucleotide sequence, as described herein.

In one embodiment of the present invention, the probe sequence is less than 35 nucleotides in length, for example the probe sequence is less than 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17 or 16 nucleotides in length.

In one embodiment of the invention, in which a probe is to be used in a method in combination with a pair of primers, the pair of primers should allow for the amplification of part or all of 5'UTR of an enterovirus or VP1 region of an EV71 virus polynucleotide fragment to which probes are able to bind or to which the probes are immobilised on a solid support.

The primer and/or probe may additionally comprise a detectable label, enabling the probe to be detected. Examples of labels that may be used include: fluorescent markers or reporter dyes, for example, 6- carboxyfluorescein (6FAM™), NED™ (Applera Corporation), HEX™ or VIC™ (Applied Biosystems); TAMRA™ markers (Applied Biosystems, CA, USA); chemiluminescent markers, for example Ruthenium probes; and radioactive labels, for example tritium in the form of tritiated thymidine. ^{32}-Phosphorus may also be used as a radiolabel.

In one embodiment, the detectable label is directly or indirectly attached to the probe. The detectable label may comprise a fluorescent moiety attached at a 5' end of the probe. The nucleotide sequence of the probe may further comprise a quencher moiety attached at a 3' end of the probe. In one embodiment, the detectable label for each of the probe

sequences described herein, for example sequences comprising or consisting of SEQ ID NO:3 and SEQ ID NO:6, is such that the probe sequences are independently detectable.

In one embodiment of the present invention, the probe may comprise a fluorescent reporter dye as a detectable label, as described herein, at its 5'-end and a quencher moiety at its 3'-end. The quencher may comprise a non- fluorescent quencher (NFQ). Optionally, a Minor Groove Binder protein (MGB; Applied Biosystems) may be added to the probe, for example the 3' end of the probe.

In one embodiment, the quencher moiety may be a Black Hole Quencher (BHQ). In another embodiment, the probe may have an Eclipse(TM) Dark Quencher and an MGB(TM) moiety positioned at the 5 '-end of the probe.

KITS

In an embodiment in which a probe is to be used in multiplex PCR techniques, the probe is labelled with a means of detection that differs from any other probe used in the same reaction. By this means, each of the probe sequences are independently detectable, in the same reaction procedure. That is, in one embodiment, different labels, for example fluorescent dyes or markers, may be selected for detection of enterovirus and/or EV71 virus. The labels are attached to the 5' end of the probes. A quencher, for example, Black Hole Quencher (BHQ), is attached at the 3' end.

In one embodiment, the fluorescent dyes are selected from 6-FAM, HEX, Texas Red, and Cy5.

In a specific embodiment, the probe for enterovirus is labelled with 6-FAM and the probe for EV71 is labelled with HEX.

The sequence for the enterovirus and EV71 primers and probes according to the present invention are shown in Table 1. The sequences for the various embodiment of VP1 primers are shown in Tables 2 and 3. The TaqMan probes may be independently

detectable through independent labelling with different fluorescent dyes which emit fluorescence at different wavelengths. This allows the differentiation of EV71 from other enteroviruses by viewing the fluorescence in different channels of wavelength.

In one embodiment, the primer and probe sequences of the present invention may contain or comprise naturally occurring nucleotide structures or bases, for example adenine (A), cytosine (C), guanine (G), thymine (T) and uracil (U). Inosine (I), may be included in a sequence as required.

In a further embodiment, modified or synthetic analogues of nucleotide structures or bases may be included in the sequence of the probe. By "modified" or "synthetic" is meant a non-naturally occurring nucleotide structure or base. Such synthetic or modified bases may replace 1, 2, 3, 4, 5, 6, 7, 8, 9 or all of the bases in the probe sequence. In one embodiment, Cytosine may be replaced by 5 -Methyl dC and Thymine may be replaced by 5-Propynyl dU. A quencher such as BHQ may also be included within the sequence.

The present invention additionally provides a kit for detection of an enterovirus comprising the following components: (i) at least one primer or set of primers having a target sequence within the 5'UTR of enterovirus as described herein; and (ii) at least one probe having a target sequence within the 5'UTR of enterovirus amplified by the primers of (i), as described herein.

The present invention additionally provides a kit for detection of EV71 comprising the following components: (i) at least one primer or set of primers having a target sequence within the VP1 of EV71 as described herein; and (ii) at least one probe having a target sequence within the VP1 of EV71 amplified by the primers of (i), as described herein.

The present invention further provides a kit for detection and differentiation of both enterovirus and EV71 comprising the following components: (i) at least one primer or set of primers for enterovirus as described herein and at least one primer or set of primers for

EV71 as described herein; and (ii) at least one probe for enterovirus as described herein and at least one probe for EV71 as described herein.

In one embodiment, the kit comprises one forward primer, one reverse primer and a probe sequence which has a target sequence within the region amplified by the forward and reverse primers. In one embodiment the set of primers is capable of amplifying a portion (amplicon) of the sequence of the 5'UTR of an enterovirus and the probe is capable of hybridising under stringent conditions to the amplicon. In a further embodiment, the set of primers are capable of amplifying a portion (amplicon) of the sequence of the VP1 of an EV71 virus and the probe is capable of hybridising under stringent conditions to the amplicon.

In one embodiment, the kit may comprise:
(a) a pair of primers of or comprising SEQ ID NO: 1 and 2 and the probe of or comprising SEQ ID NO: 3; and/or
(b) a pair of primers of or comprising SEQ ID NO:4 and 5 or the pair of primers of any of Set 2 to Set 65 as described herein and the probe of or comprising SEQ ID NO: 6, and in which the primer of SEQ ID NO:4 is not SEQ ID NO:9.

In one embodiment of the present disclosure, the forward primer for use in the present invention is not SEQ ID NO: 9. For example, in one embodiment, the forward primer of SEQ ID NO:4 is not SEQ ID NO:9.

The kits as described herein may further comprise amplification reagents, as required. For example, amplification reagents may include enzymes having polymerase activity, enzyme co-factors, for example magnesium or manganese; salts; nicotinamide adenine dinucleotide (NAD); and deoxynucleotide triphosphates (dNTPs), (dATP, dGTP, dCTP and dTTP).

DETECTION METHODS

In a further embodiment of the present invention there is provided a method for determining the presence or absence of an enterovirus in a biological sample or test sample, comprising the step of contacting a nucleotide sequence obtained or derived from a biological sample with at least one primer or probe or set according as described herein.

In a further embodiment of the present invention there is provided a method for determining the presence or absence of EV71 virus, in a biological sample or test sample, comprising the step of contacting a nucleotide sequence obtained or derived from a biological sample with at least one primer or probe or set according as described herein.

In a further embodiment there is provided a method for determining the presence or absence of an enterovirus and also the presence or absence of an EV71 virus, comprising the step of contacting a nucleotide sequence obtained or derived from a biological sample with at least one primer or probe or set according as described herein.

In one embodiment, the nucleotide sequence is or has been isolated from a biological sample, for example a test sample.

A method of the present invention may further comprise amplifying the nucleotide sequence and detecting in the sample the amplified nucleotide sequence. Alternatively or additionally, the method of the present invention may further comprises contacting the nucleotide sequence or amplified nucleotide sequence with one or more probes as described herein.

In one embodiment, the nucleotide sequence is isolated or purified from the biological sample. In RT- PCR, genomic DNA contamination, for example additional material from a patient's sample, may lead to false positive results. In one embodiment, the genomic DNA is removed or substantially removed from the sample to be tested or included in the methods of the present invention.

IN SITU HYBRIDISATION

In one embodiment of the present invention, an enterovirus may be detected using in situ hybridisation. In one embodiment of the present invention an EV71 virus may be detected using in situ hybridisation.

By "in situ hybridisation" is meant is a hybridisation reaction performed using a primer or probe according to the present invention on intact chromosomes, cells or tissues isolated from a patient for direct visualization of morphologic sites of specific DNA or RNA sequences.

Hybridisation of the polynucleotides may be carried out using any suitable hybridisation method and detection system. Examples of hybridisation systems include conventional dot blot, Southern blots, and sandwich methods. The enterovirus and/or EV71 virus, specific nucleic acid sequences, for example a probe or primer as described herein, can be labelled with biotin and the hybrid can be detected via a biotin- streptavidin coupling with a non-radioactive colour developing system. However, other reverse hybridisation systems may also be employed.

TISSUE SAMPLES

The methods as described herein are suitable for use in a sample of fresh tissue, frozen tissue, paraffin- preserved tissue and/or ethanol preserved tissue. The sample may be a biological sample. Non-limiting examples of biological samples include whole blood or a component thereof (e.g. plasma, serum), urine, saliva, cerebrospinal fluid, bronchioalveolar lavage fluid, breast milk, throat swabs, rectal swabs and blister fluid.

In one embodiment, the sample is a whole blood sample.

A biological sample as contemplated herein includes cultured biological materials, including a sample derived from cultured cells, such as culture medium collected from cultured cells or a cell pellet. Accordingly, a biological sample may refer to a lysate, homogenate or extract prepared from a whole organism or a subset of its tissues, cells or

component parts, or a fraction or portion thereof. A biological sample may also be modified prior to use, for example, by purification of one or more components, dilution, and/or centrifugation.

Well-known extraction and purification procedures are available for the isolation of nucleic acid from a sample. The nucleic acid may be used directly following extraction from the sample or, more preferably, after a polynucleotide amplification step (e.g. PCR) step. In specific instances, such as for reverse hybridisation assays, it may be necessary to reverse transcribe RNA into cDNA before amplification. In both latter cases the amplified polynucleotide is 'derived' from the sample.

The present invention additionally provides a method of treating a patient comprising: determining whether a patient-derived biological sample tissue contains a nucleotide sequence of an enterovirus and/or an EV71 virus, using a method as described herein, and treating the patient with a medicament or immunotherapy suitable for treatment of hand foot and mouth infection or the symptoms associated therewith.

Thus the present invention provides a method for screening, in clinical applications, biological samples from a human patient for the presence or absence of infection by an enterovirus and/or an EV71 virus. In one embodiment, there is provided a method of diagnosis of a patient, comprising a method or method step as described herein, in which a patient is diagnosed as suffering from or having had an enterovirus and/or an EV71 virus.

In yet a further embodiment of the invention, there is provided a method of treating a patient, the method comprising determining, through use of a method of the present invention, whether the patient is infected by an enterovirus and/or an EV71 virus, and subsequently administering a composition, medicament or immunotherapy to clear the infection or to lessen secondary symptoms or to prevent or ameliorate recurrence of disease.

Primers and probes of the invention may be used to detect an enterovirus and/or an EV71, using any method known in the art, non-limiting examples of which include the Polymerase Chain Reaction (PCR), for example, Real-Time Reverse Transcription (RT) PCR.

POLYMERASE CHAIN REACTION (PCR)

PCR is an enzyme-mediated reaction use to amplify a specific target DNA sequence. By amplifying the target DNA sequence in the DNA template, it is then able to produce millions more copies of the targeted DNA sequence. This is useful when a biological sample contains only small amounts of DNA. PCR is carried out in a mixture containing DNA polymerase, a pair of primers (forward and reverse) and four deoxynucleotide triphosphates (dNTPs) with the aid of thermal cycler. Although the PCR process is used to amplify DNA, RNA can also be amplified by carrying out an extra step of reverse transcription before the start of the assay to convert RNA into cDNA.

As used in methods described herein, primers are selected for their ability to amplify a region of the 5'UTR of an enterovirus and/or VP1 of an EV71 virus nucleotide sequence. For example, a forward primer and a reverse primer are selected for their ability to amplify a region of, and thus generate an amplicon of, 5'UTR of an enterovirus nucleotide sequence and/or a VP1 of an EV71 nucleotide sequence. A probe is then selected for its ability to hybridise to the sequence of the amplicon thus generated, for example, a probe is selected for its ability to hybridise to the amplicon of the 5'UTR of an enterovirus nucleotide sequence or a VP1 of an EV71 nucleotide sequence.

REAL-TIME REVERSE TRANSCRIPTION – POLYMERASE CHAIN REACTION

Thus, primers and probes as described herein may be used in the detection of a nucleic sequence by nucleic acid amplification technique is now the most commonly used technique in laboratories. The detection is done using the real-time reverse transcription Polymerase Chain Reaction (RT-PCR), nested PCR or nucleic acid sequence-based amplification (NASBA). In one embodiment of the present invention, the amplification reaction is RT-PCR. The RT-PCR may be a 3-step PCR method or a 2-step PCR method.

In one embodiment of the present invention as described herein, the RT-PCR reaction conditions comprise a 3-step PCR method, comprising the step of thermal cycling for 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds, for example in a CFX96 Real-Time PCR detection system or in a Rotorgene Real-time PCR detection system. The reaction mixture may be as shown in Table 4 or 5. For example, in one embodiment, the reaction mixture may be as shown in Table 5.

Optionally, the methods described herein may comprise the step of synthesising cDNA from RNA at 50C for 10 minutes, followed by a reverse transcriptase deactivation at 95C for 5 minutes.

Real time PCR is an improved form of conventional PCR. As in conventional PCR, the amplicons are detected by end-point analysis, by running the DNA on agarose gel after the reaction is complete. Whereas, real-time PCR is able to accumulate the amplified product to be detected as the reaction is going on. This is made possible by adding to the real-time PCR reaction, a fluorescent molecule such as a DNA binding dye and probes that report the increase in the amount of DNA with a proportion increase in the fluorescent signal. The probes that are commonly used are TaqMan probes.

In the end-point PCR graph, the x-axis represents the PCR cycle time. The amount of the PCR product is doubled in each cycle until it is enough to produce the fluorescence signal. The cycle number in which this occurs is called the threshold cycle (Ct). A Standard Curve is plotted to perform an absolute quantification by doing a serial dilution of the control template with known concentration. The amplified standard dilution series graph would be plotted by the machine as Ct against the log of the initial copy number or concentration. Therefore the initial copy number or concentration of the unknown sample can be quantified by comparing the Ct with the help of the standard curve.

TAQMAN PROBE CHEMISTRY

Figure 1a shows an illustrated picture of a TaqMan® probe. In one embodiment, the probe consists of two types of fluorophores: the fluorescent parts of reporter (R) dye and the quencher (Q) which is attached or unattached from the template DNA and before the polymerase acts. Such a probe uses fluorescence resonance energy transfer (FRET) theory. The reporter dye is found on the 5' end of the probe and the quencher at the 3' end. The TaqMan Probe is attached to a specific piece of template DNA after the denaturation steps. After the reaction mixture cools down, the primers anneal to the DNA. After all this is in position, shown in Figure 1b, *Taq* polymerase starts to add nucleotides and starts to cleave the TaqMan probe from the DNA template. The reporter is cleaved off first. Separating from the quencher, the reporter is free to emit its energy. This is shown in Figure 1c-d. The fluorescence can be quantified.

The invention will be further described by reference to the following, non-limiting, examples, in which RT-PCR refers to reverse-transcription polymerase chain reaction:

EXAMPLES

EXAMPLE 1

Design of enteroviruses virus primers and TaqMan Probes

The 5'UTR region of the enteroviruses encodes for RNA synthesis and translation. VP1 region possesses high degree of antigenic and genetic diversity that can be used to distinguish enterovirus serotypes. The 5'UTR and VP1 regions were analyzed from GenBank database, maintained by the National Institutes of Health, USA. *(http://www.ncbi.nlm.nih.gov).*

The primers and probes were designed to ensure optimal sequence length for specificity and sensitivity. The conserved region is from 1bp to 746bp for 5'UTR and 2442bp to 3332bp for VP1.

Primers and probes were designed using Lasergene 7 software. The specificity of the primers and probes were analysed by BLAST analysis in the GenBank of the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). A pair of primers (designated 5UTRFP3/5UTRRP3 (SEQ ID NO:1 and 2) and EVFPnew/EVRPnew for VP1 (SEQ ID NO:4 and 5). TaqMan probes designated 5UTR Probe (SEQ ID NO:3) and EVprobe (SEQ ID NO:6); were designed from the conserved 5'UTR and VP1 regions of EV71 Virus respectively, for specific amplification of enterovirus and EV71.

Different fluorescent dyes were selected for differentiation between 5'UTR region and VP1 region. The dyes were attached to the 5' end of the probes. A quencher, Black Hole Quencher (BHQ), was attached at the 3' end. The fluorescent dyes selected were 6-FAM for 5'UTR and HEX for VP1.

The sequence for 5'UTR and VP1 primers and probes are shown in Table 1. The TaqMan probes are each labelled with different fluorescent dyes that emit fluorescence at different wavelengths. This allows the detection and differentiation of enteroviruses and differentiation of EV71 in different channels. This system eliminates spectral crosstalk amongst the detection channels and enables fluorescence to be read from each sample well with high sensitivity.

- 6-FAM (6-carboxy-fluorescein) – 5'UTR (520nm)
- HEX (hexachloro-fluorescein) – VP1 (556nm)

The labels for each probe are as follows:

- 6-FAM (6-carboxy-fluroscein) was labelled at the 5' end of 5UTR probe
- HEX (hexachloro-fluorescein) was labelled at the 5' end of EV probe
- BHQ 1 was labelled at the 3' end of 5UTR and EV probe.

Details of the primer and probe sequences, together with the regions of the 5'UTR and VP1 gene to which they bind, are given in Figures 4 and 5.

EXAMPLE 2

Extraction of Viral RNA

RNA was extracted from the hand foot and mouth patient samples supplied using the QIAamp® viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at -20°C. All extractions were done in a biosafety level 2 cabinet to prevent any accidental infections from the live hand foot and mouth enterovirus RNA viruses.

EXAMPLE 3

Multiplex Real-time RT-PCR analysis

The multiplex real-time RT-PCR analysis was carried out using the CFX96 Real-Time PCR Detection system from Bio-Rad Laboratories, United States, with the components listed in Table 2.

Table 4: Components of the multiplex real-time RT-PCR setup

Reagent	Volume
One-Step RT-PCR reaction mix	12.5µL
Reverse Transcriptase	1.0µL
5UTRFP3 (10µM)	1.25µL
5UTRRP3 (10µM)	1.25µL
EVFP (10µM)	1.0µL
EVRP (10µM)	1.0µL
5UTR3 Probe (10µM)	1.0µL
EVProbe (10µM)	1.0µL
Nuclease-free H2O	2.5µL
RNA Template	2.5µL
Final Reaction Volume	25.0µL

The following conditions were set up for the Real-Time PCR reactions:
cDNA was synthesized from RNA at 50°C for 10 minutes, followed by a reverse transcriptase deactivation at 95°C for 5 minutes. Thermal cycling was performed for 40

cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds in CFX96 Real-Time PCR Detection system.

EXAMPLE 4

Real-time RT-PCR using CFX96 Real-Time PCR Detection System

Real-time RT-PCR analysis was carried out using the CFX96 Real-Time PCR Detection system from Bio-Rad Laboratories, United States. It uses optical technology that builds on the C1000™ thermal cycler and with its six-channel platform combines advance optical technology, creating a precise thermal control to deliver sensitive, reliable detection that detects excitation/emission wavelengths of fluorophores ranging from 450-730nm. Performing a real-time PCR experiment can be done with the help of the CFX Manager software. The CFX96 system's solid-state optical technology consisting of six filtered LEDs, each with a corresponding filtered photodiode, maximizes fluorescence detection for specific dyes in specific channels, providing sensitive detection for precise quantification and target discrimination. Scanning just above the sample plate, the optics shuttle individually illuminates and reads fluorescence from each well with high sensitivity and no cross talk. At every position and with every scan, the optics shuttle is reproducibly centred above each well, so the light path is always optimal and there is no need to sacrifice data collection in one of the channels to normalize to a passive reference.

EXAMPLE 5

Specificity of the designed primers and probes by real-time multiplex RT-PCR

The specific detection and differentiation of the hand foot and mouth-causing enteroviruses and specific stereotyping of EV71, obtained according to the methods described in Example 1 to 4, using the sequences shown in Table 1, are shown in Figures 2 and 3. In Figure 2, amplification of all the enteroviruses under analysis was observed in the fluorescence channel at 520 nm. When the fluorescence channel was switched to 556nm, only fluorescence which was emitted as a result of amplification of EV71 was observed (Figure 3). All other enteroviruses were shown to be negative.

The TaqMan probes were labelled with different fluorescent dye which emits fluorescence at different wavelengths. This allows the detection of enteroviruses in a channel, and simultaneous serotyping of EV71 in another channel. This system eliminates spectral crosstalk amongst the detection channels and it reads fluorescent from each sample well with high sensitivity.

EXAMPLE 6

Alignment report for EV71 sequences

An alignment report was prepared for designing specific primers for EV71 (EVFP and EVRP) and a probe (EVprobe), as shown in Figure 6. The alignment was based on VP1 sequences of 17 EV71 strains (S41, S10, MS, 1585 JVP1, 2933 JVP1, 75JVP1, 962JVP1, NUH0012-08, NUH0013-08, NUH0037-08, NUH0043-08, NUH0047-08, NUH0049-08, NUH0075-08, NUH0083-08, NUH0085-08, NUH0086-08) and 2 CA16 strains (CA16, CA16-2). All the sequences were obtained from GenBank.

Based on the alignment report, those in dots are represented by consensus nucleotides (A,T,G or C) across all 19 strains. The top row (indicated as "Majority") represent the consensus nucleotide.

In the alignment report, the following positions correspond to the primers: EVFP, EVRP; and the probe: EVprobe.

Position 25 - 48 - EVFP

Position 57 - 80 - EVprobe

Position 206 to 228 - EVRP

EXAMPLE 7

Re-optimization of the PCR assay

The PCR assay was re-optimised using a new PCR master mix (BioRad Laboratories). Volumes of the primers and probes were also readjusted, as shown in Table 5.

Table 5: Components of the multiplex real-time RT-PCR setup with readjusted volumes.

Reagent	Volume
iQ Multiplex Powermix	12.5µL
iScript™ Reverse Transcriptase	0.5µL
5UTRFP3 (10µM)	0.75µL
5UTRRP3 (10µM)	0.75µL
EVFP (10µM)	0.75µL
EVRP (10µM)	0.75µL
5UTR3 Probe (10µM)	0.5µL
EVProbe (10µM)	0.5µL
Nuclease-free H_2O	4.0µL
RNA Template	2.5µL
Final Reaction Volume	25.0µL

The PCR conditions used were the same as described in Example 3: cDNA was synthesized from RNA at 50°C for 10 minutes, followed by a reverse transcriptase deactivation at 95°C for 5 minutes. Thermal cycling was performed for 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds in CFX96 Real-Time PCR Detection system. The same conditions were also evaluated on the Rotorgene real-time PCR platform from Qiagen.

The specificity and efficacy of the assays were analysed using EV71 and CA16 RNA. The results are shown in Figures 7A, 7B and 8A, 8B. As shown in the amplification plots, the fluorescence signals in both real-time PCR platforms were shown to be more stable and specific in their respective channels.

Gathering all the results, it is shown that this re-optimised invention is able to be applied across 2 different real-time PCR platforms which are commonly used in laboratories.

CLAIMS

1. A primer or probe having a target sequence in the region from 1bp to 746bp of 5'UTR of an enterovirus genomic sequence.

2. A primer or probe according to claim 1, comprising or consisting of the nucleotide sequence of any of SEQ ID NO: 1 to 3, or complements thereof.

3. A forward primer according to claim 1 or 2 for amplifying a nucleotide sequence of an enterovirus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 1, or complements thereof.

4. A reverse primer according to claim 1 or 2 for amplifying a nucleotide sequence of an enterovirus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO: 2, or complements thereof.

5. A probe according to claim 1 or 2 in which the probe sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO: 3 or complements thereof.

6. A primer or probe having a target sequence in the VP1 region from 2442bp to 3332bp of EV71, wherein said primer is not SEQ ID NO:9.

7. A primer or probe according to claim 6, consisting of the nucleotide sequence of any of SEQ ID NO: 4 to 6, or complements thereof.

8. A forward primer according to claim 6 or 7 for amplifying a nucleotide sequence of an EV71 virus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of any of SEQ ID NO:4, 10, 11, 12, 13, 14, 15 or 16, or complements thereof.

9. A reverse primer according to claim 6 or 7 for amplifying a nucleotide sequence of an EV71 virus in a test sample, in which the primer sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 5, 17, 18, 19, 20, 21, 22, 23 or 24 or complements thereof.

10. A probe according to claim 6 or 7 in which the probe sequence comprises or consists of the nucleotide sequence of SEQ ID NO: 6.

11. A set of primers for amplifying enterovirus in a test sample comprising a pair of forward and reverse primers, in which the forward primer comprises or consists of SEQ ID NO:1 or complements thereof; and the reverse primer comprises or consists of SEQ ID NO:2, or complements thereof.

12. A set of primers for amplifying EV71 virus in a test sample comprising a pair of forward and reverse primers, in which the primers comprise or consist of sequences selected from the group consisting of Set 2 to Set 65, or complements thereof.

13. A set of primers and a probe for detecting enterovirus in a test sample comprising or consisting of a forward primer comprising or consisting of SEQ ID NO:1 or complements thereof; a reverse primer comprising or consisting of SEQ ID NO:2, or complements thereof; and a probe comprising or consisting of SEQ ID NO:3 or complements thereof.

14. A set of primers and a probe for detecting EV71 virus in a test sample comprising or consisting of a set of primers of claim 12 and a probe comprising or consisting of SEQ ID NO: 6 or complements thereof.

15. A kit for detecting enterovirus and EV71 in a test sample comprising (a) a set of primers and a probe according to claim 13 and (b) a set of primers and a probe according to claim 14.

16. The set according to any of claims 11 to 14 or the probe according to any of claims 1, 2, 5, 6, 7 or 10 in which the nucleotide sequence of the probe is labelled with a detectable label, and in which the detectable label is directly or indirectly attached to the probe.

17. The set or probe of claim 16, in which the detectable label comprises a fluorescent moiety attached at a 5' end of the probe.

18. The set or probe of claim 17, in which the nucleotide sequence of the probe further comprises a quencher moiety attached at a 3' end of the probe.

19. The set or probe of claim 16 to 18, in which the detectable label of each of the probe sequences of SEQ ID NO:3 and SEQ ID NO:6, is such that the probe sequences are independently detectable.

20. A method for determining the presence or absence of an enterovirus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with at least one primer or probe or set according to any of claims 1 to 5, 11 or 13.

21. A method for determining the presence or absence of an EV71 virus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with at least one primer or probe or set according to any of claims 6 to 10, 12 or 14.

22. A method for determining the presence or absence of an enterovirus and an EV71 virus in a biological sample, comprising the step of contacting a nucleotide sequence obtained or derived from the biological sample with at least one primer or probe or set according to any of claims 1 to 5, 11 or 13 and/or with at least one primer or probe or set according to any of claims 6 to 10, 12 or 14.

23. A method according to claim 20, 21 or 22 further comprising the step of determining whether the nucleotide sequence hybridises to the at least one primer or probe under stringent conditions, thereby detecting whether the sample contains the virus.

24. A method according to any of claims 20 to 23, in which in situ hybridisation is used to detect whether the nucleotide sequence hybridises to the at least one primer or probe.

25. A method according to claim 20 or 22, comprising the steps of:
(a) contacting the nucleotide sequence obtained or derived from the biological sample with at least one forward primer according to claim 3 and at least one reverse primer according to claim 4, or at least one set according to claim 11, under amplification conditions to generate an amplicon of a region of an enterovirus genomic sequence; and
(b) detecting hybridization between the amplicon and at least one probe according to claim 5.

26. A method according to claim 21 or 22, comprising the steps of:
(a) contacting a nucleotide sequence obtained or derived from a biological sample with at least one forward primer according to claim 8 and at least one reverse primer according to claim 9, or at least one set according to claim 12, under amplification conditions to generate an amplicon of a region of an EV71 genomic sequence; and
(b) detecting hybridization between the amplicon and at least one probe according to claim 10.

27. A method according to claim 25 or 26 in which the amplification conditions comprise an amplification reaction, and in which the amplification reaction is a polymerase chain reaction (PCR).

28. The method of claim 25, 26 or 27, wherein the amplification conditions comprise a 3-step PCR reaction.

29. The method of claim 25, 26, 27 or 28, wherein the method comprises the step of providing a reaction mixture, and wherein the reaction mixture is as shown in Table 4 or 5.

30. A kit of claim 15, in which the kit further comprises (c) amplification reagents.

31. The kit of claim 14 or 15, wherein components (a), (b) and (c) are provided in the amounts shown in Table 4 or 5.

32. A method of treating a patient infected with an enterovirus or EV71 virus comprising: determining the presence of an enterovirus or EV71 virus in a biological sample derived from the patient using a method according to any of claims 20 to 29 and then administering an anti-viral composition or medication or a immunotherapy to the patient.

33. Use of a composition comprising an anti-viral composition or medication or a immunotherapy in the manufacture of a medicament for the treatment of a patient infected with an enterovirus, wherein the presence of an enterovirus in a biological sample from the patient has been determined using a method according to any of claims 20 to 29.

34. A composition comprising an anti-viral composition or medication or a immunotherapy for the treatment of a patient infected with an enterovirus, wherein the presence of an enterovirus serotype in a biological sample from the patient has been determined using a method according to any of claims 20 to 29.

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<120> Method for Detection of Enterovirus

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