DETECTION OF NUCLEIC ACIDS BY STRAND INVASION BASED AMPLIFICATION

Field of the Invention

The invention relates to a method for detection of a target nucleic acid sequence in a sample in the presence of at least one protein capable of binding to single-stranded DNA, wherein an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to the target nucleic acid sequence is used for detection. The sequence of the oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides. The invention also relates to kits suitable for use in this method, and their use for diagnosis of an infection by a pathogen.

Background to the Invention

Detection of target nucleic acid sequences has been performed using DNA probes comprising fluorophore/quencher pairs. Such probes exhibit a change in quenching activity on binding to a target nucleic acid, allowing for quantitative detection of target. Probes can be used to monitor DNA amplification in real time, and different targets may be detected in the same reaction through use of different fluorophore/quencher pairs on each probe for the targets to be detected, thus allowing multiplexing. Examples of previous probe systems for detection of DNA amplification include hybridisation probes showing conformational changes on target binding (US7241596), molecular beacons (US5925517), Taqman chemistry (US6214979), and endonuclease-cleavable probes (US7435561 and US20050214809). An isothermal DNA amplification process relying on an upstream primer, a downstream primer, and a strand invasion system is described in WO 2009/150467.

Summary

The present disclosure is based in part on the recognition of a problem associated with use of DNA probes in detection assays where proteins capable of binding to single-stranded DNA are present. The present inventors found that DNA probes were unable to provide for a specific, template-dependent signal in the presence of proteins capable of binding to single-stranded DNA, and indeed gave a signal even in absence of template. This problem led them to investigate a solution that could provide for reliable template-

dependent detection of DNA amplification in the context of proteins capable of binding to single-stranded DNA. The inventors surprisingly discovered that the incorporation of RNA nucleotides, modified RNA nucleotides and/or Peptide Nucleic acid (PNA) nucleotides into the sequence of an oligonucleotide probe labeled with a fluorophore and a quencher provided resistance to disruption of fluorescent signal by proteins capable of binding to single-stranded DNA as compared to a corresponding fully DNA probe.

In one aspect, there is provided a method for detection of a target nucleic acid sequence in a sample in the presence of at least one protein capable of binding to single-stranded DNA comprising contacting said sample with at least one oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to said target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides and wherein at least one said protein capable of binding to the single-stranded DNA is a recombinase.

The disclosure provides an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to a target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides. In another aspect, there is provided a kit, comprising an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to said target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides and in addition at least one protein capable of binding to single-stranded DNA wherein said at least one protein is a recombinase. In another aspect, there is provided a method for diagnosis of a disease in a subject, comprising carrying out a method for detection of a nucleic acid sequence of the disclosure in a sample from said subject to detect a target nucleic acid sequence associated with said disease.

Brief Description of the Figures

Figure 1 shows the effect of (A) a recombinase (UvsX), (B) a single strand DNA binding protein (gp32), (C) a reagent mixture for strand invasion based amplification , D) the *E.coli* recombinase RecA, and E) the single stranded DNA binding protein ET-SSB on signal from probes containing a fluorophore and quencher. X-axis for (A) and (B): left to right - four conditions for each tested probe (probe only; probe with complementary template; probe with protein(s); probe with protein(s) and complementary template). X axis for (C): left to right - two conditions for each tested probe (probe and reagent composition; probe, reagent composition and complementary template). X axis for (D) and (E)): left to right - two conditions for each tested probe (probe only, probe with protein).

SEQ ID NO:22 is the nucleotide sequence of a DNA primer.

SEQ ID NO:23 is the nucleotide sequence of a DNA strand invasion oligonucleotide.

SEQ ID NO:24 is the nucleotide sequence of a 2'-fluoro RNA probe.

SEQ ID NO:25 is the nucleotide sequence of a 2'-fluoro RNA probe.

SEQ ID NO:26 is a target DNA nucleotide sequence from *C. difficile* ATCC BAA 1382

SEQ ID NO:27 is the nucleotide sequence of a DNA primer.

SEQ ID NO:28 is the nucleotide sequence of a DNA primer.

SEQ ID NO:29 is the nucleotide sequence of a DNA strand invasion oligonucleotide.

SEQ ID NO:30 is the nucleotide sequence of a 2'-fluoro RNA probe.

SEQ ID NO:31 is a target DNA nucleotide sequence from *S. typhimurium* ATCC 14028

SEQ ID NO:32 is the nucleotide sequence of a 2'-O-methyl RNA probe.

SEQ ID NO:33 is the nucleotide sequence of a DNA primer.

SEQ ID NO:34 is a complementary target DNA nucleotide sequence for the probes of SEQ ID NOs 1 to 8.

SEQ ID NOs: 35 to 43 represent the nucleotide sequences of chimeric DNA/RNA, DNA/2'-fluoro RNA and DNA/2'-O-methyl RNA probes.

SEQ ID NO:44 is a complementary target DNA nucleotide sequence for the probes of SEQ ID NOs 9 and 10.

SEQ ID NOs :45 to 48 represent the nucleotide sequences of chimeric DNA/RNA probes.

Detailed Description of the Invention

It is to be understood that different applications of the disclosed methods may be tailored to the specific needs in the art. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting. In addition as used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "an oligonucleotide" includes two or more such oligonucleotides, and the like.

Method for detection of a target nucleic acid sequence

Sample

Any sample may be used for detection of the target nucleic acid sequence, provided that nucleic acid can be obtained or derived from the sample. The sample may be for instance an environmental sample, a reference sample or a clinical sample. Where the methods of the invention are used for diagnosis of a disease by detection of a target nucleic acid sequence, the sample is commonly a clinical sample, for example a sample obtained from a patient suspected of having, or having the disease. Suitable types of clinical sample vary according to the particular type of disease or infection that is present, or suspected of being present in a subject. The sample may be a saliva, sputum, blood, plasma, serum, urine or stool sample. The sample may be a cell or tissue sample. In preferred embodiments, the samples are taken from animal subjects, such as mammalian subjects. The samples will commonly be taken from human subjects, but the present invention is also applicable in general to domestic animals, livestock, birds and fish. For example, the invention may be applied in a veterinary or agricultural setting. In embodiments where the invention detects infection of an infection by *Clostridium difficile (C. difficile)* or *Salmonella typhimurium* (*S. typhimurium*) the sample is preferably a stool sample. The stool sample may be taken from a subject having a gastrointestinal tract infection. The infection may be present in a patient having diarrhoea.

The sample comprises nucleic acid which may be DNA or RNA. If the nucleic acid is present in the sample in a suitable form allowing for detection according to the invention, the sample may be used directly. However, typically, nucleic acid is derived, obtained or extracted from the sample. Methods for processing samples containing nucleic acids, extracting nucleic acids and/or purifying nucleic acids for use in detection methods are well-known in the art. Total nucleic acid may be isolated or DNA and RNA may be isolated separately.

Typically, a sample is processed in an appropriate manner such that nucleic acid is provided in a convenient form for contacting with the oligonucleotide probe and single-stranded DNA binding protein and optional further nucleic acid components. Where the nucleic acid is DNA, the DNA is typically provided in double-stranded form. Where the

modified deoxyribonucleotides at its 5' end and/or 3'end. Alternatively, the deoxyribonucleotides or modified deoxyribonucleotides may be interspersed in a sequence of ribonucleotides.

The oligonucleotide probe is typically about 8 to about 25 nucleotides in length. The probe is typically at least 8 nucleotides in length or less than 30 nucleotides in length, more preferably less than 25 nucleotides in length. The probe may be at least 10, at least 12, or at least 15 nucleotides in length. The probe may be about 10 to about 20, about 12 to about 25, about 15 to about 25, or about 12 to about 22 nucleotides in length. The length of the probe is selected according to the requirement for specific or selective hybridisation with a region of the target sequence under the conditions used, and may be selected based on the length of primers used for amplification of the target sequence, as discussed below.

Specific or selective hybridisation refers to the binding of an oligonucleotide (for example a probe or primer) only to a particular nucleotide sequence under given conditions, when that sequence is present in a nucleic acid in a sample, such as a complex biological mixture including total cellular and foreign DNA or RNA. Appropriate hybridisation conditions are known in the art. See for example, Sambrook, Fritsche and Maniatis "Molecular Cloning: A Laboratory Manual", 2nd Ed. Cold Spring Harbor Press (1989). Appropriate hybridisation conditions are also provided in the Examples below. As is known to the skilled person, appropriate hybridisation conditions may vary depending on the length of a probe and its base composition. Hybridisation is typically performed at the same temperature as amplification, and thus also depends on the activity profile of the enzymes used for amplification, including the polymerase and the recombinase as applicable depending on the method of amplification.

The nucleotide sequence of the probe may be partly or full complementary to a region of the target nucleic acid sequence. An oligonucleotide probe of less than 25 nucleotides in length will typically comprise a region of at least 10, at least 15 or at least 20 nucleotides in length which is complementary to a region of the target nucleic acid sequence. The oligonucleotide probe may further comprise flanking regions of 1, 2, 3, 4, 5, 8 or 10 nucleotides in length 5' or 3' to the complementary region which are not complementary to the target nucleic acid sequence. The oligonucleotide probe may comprise a total of 1, 2, 3, 4, or 5, 8 or 10 nucleotides at the 5' and 3' ends flanking the complementary sequence, which are not complementary to the target nucleic acid sequence. Such flanking regions may be self-complementary, leading to a hairpin structure.

and a fluorophore at an internal position. The use of an oligonucleotide probe as a primer is described in more detail below.

The oligonucleotide probe may be labeled with any fluorophore and any quencher. The fluorophore and quencher will be selected such that the absorption spectrum of the quencher overlaps with the emission spectrum of the fluorophore. The fluorophore and quencher will further be selected and positioned in the probe such that, upon hybridization with a target template, the fluorophore produces an increase in signal due to reduced quenching effect.

The quencher may be non-fluorescent, for example a non-fluorescent chromophore. The quencher may be a dark quencher. Alternatively, the quencher may fluoresce with a different emission spectrum to the fluorophore, such that when specifically monitoring fluorescence of the fluorophore or the quencher, a change in either signal may report on hybridisation to the target template. The fluorophore and quencher are preferably positioned at the 5' and 3' termini of the probes, in particular in embodiments where polymerase-dependent extension of the probe is undesirable. The fluorophore may be located at the 5' terminus and the quencher at the 3' terminus of the probe. Alternatively, the quencher may be located at the 5' terminus and the fluorophore at the 3' terminus of the probe. The fluorophore and/or the quencher may also be located at internal positions within the probe, such as ten or less nucleotides away from the 5' or 3' terminus of the probe. For example, in a probe of less than 25 nucleotides in length the fluorophore or quencher may be located 1 to 3, 1 to 5, 1 to 8 or 1 to 10 nucleotides away from the 5' or 3' terminus of the probe. Preferably, only one of the fluorophore and the quencher is at an internal position, with the other member of the pair at the 5' or 3' terminus.

The fluorophore and quencher are typically positioned at least eight nucleotides apart in the sequence of the probe, more preferably at least ten, or at least twelve nucleotides apart, depending on the length of the probe. Where the probe is 15 to 25 nucleotides in length, the fluorophore and quencher may be at least eight, at least ten, at least twelve, at least fifteen or at least twenty nucleotides apart. The fluorophore and quencher may be located at the 5' and 3' termini, and thus the maximum distance apart that is possible in the probe. The distance between the fluorophore and quencher will be selected such that when the probe is hybridised to the target nucleic acid sequence (in an open or linear conformation) there will be reduced quenching of the fluorophore by the quencher, leading to a detectable signal for the presence of the target nucleic acid

The fluorophore and quencher are typically covalently attached to the probe. The fluorophore and quencher may be attached by any suitable linker to one or more nucleotides present in the sequence of the probe. The skilled person is able to select any appropriate linker based on their common general knowledge. Suitable linkers are discussed for example in Agrawal S (ed.): Protocols for Oligonucleotides and Analogs: Synthesis and Properties: Humana Press; 1993.

Particular oligonucleotide probes provided herein are complementary to nucleic acid target sequences in toxigenic *C .difficile* and *S. typhimurium.* Preferred examples of such probes are SEQ ID NOs 24 and 25 for toxigenic *C. difficile* and SEQ ID NO: 30 for *S. typhimurium.* The method of the invention may also use the probes and probe-primers of SEQ ID NOs 3 to 10, 17 to 19, 32, 35 to 43 and 48.

Variants of SEQ ID NOs 3 to 10, 17 to 19, 24, 25 , 30, 32, 35 to 43 and 48 may also be used in the method of the invention. Variants of 3 to 10, 17 to 19, 24, 25, 30, 32, 35 to 43 and 48 include probes which have a corresponding nucleotide sequence to that of the original probe but which comprise an alternative fluorophore and/or quencher. The above variants may comprise any appropriate fluorophore-quencher pair from the fluorophores and quenchers described herein.

Variants of SEQ ID NOs 3 to 10, 17 to 19, 24, 25, 30, 32, 35 to 43 and 48 may also have a corresponding nucleotide sequence to that of the original probe composed of a different pattern of natural ribonucleotides , modified ribonucleotides or PNA nucleotides. For example, the original probes of SEQ ID NOs 24, 25 and 30 are composed of 2'-fluoro ribonucleotides. The nucleotide sequence of variants thereof may comprise or be composed solely of natural ribonucleotides. Additional variants thereof may comprise alternative modified ribonucleotides in place of 2'-fluoro ribonucleotides, or a mixture of natural and modified ribonucleotides. Preferred alternative modified ribonucleotides include 2'-O-methyl ribonucleotides, and LNA (locked nucleic acid) nucleotides. Thus, for example variants of SEQ ID NOs 24, 25 and 30 may be composed of 2'-fluoro ribonucleotides and 1 to 8, 1 to 5 or 1 to 3 natural ribonucleotides, or 2'-fluoro ribonucleotides and 1 to 8, 1 to 5 or 1 to 3 2'-O-methyl ribonucleotides. Variants of SEQ ID NOs 24, 25 and 30 may comprise at least 20% of the corresponding LNA or PNA nucleotides, or be composed solely of corresponding LNA and PNA nucleotides.

Variant probes may also be chimeric probes which comprise 1 to 10, 1 to 5 or 1 to 3 deoxyribonucleotides or modified deoxyribonucleotides in place of corresponding ribonucleotides or PNA nucleotides in the sequence of SEQ ID NOs 3 to 10, 17 to 19, 24,

25, 30, 32 and 35 to 43. The method of the invention may also use a variant of the DNA probe of SEQ ID NO: 16 which comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides.

Variants of SEQ ID NOs 3 to 10, 17 to 19, 24, 25, 30, 32, 35 to 43 and 48 may also be oligonucleotides of less than 25 nucleotides in length comprising a region which is partly or fully complementary to at least eight contiguous nucleotides of the corresponding original probe sequence. Preferably, said variants will comprise a region which is partly or fully complementary to at least nine, at least ten, at least twelve or at least fourteen contiguous nucleotides of the corresponding original probe sequence. The above variants may comprise a region which has 1, 2, 3, 4 or 5 mismatches (substitutions) with respect to the corresponding region of the original probe sequence (and thus the target sequence) and thus is partly complementary thereto. Thus, for instance, the variants may comprise a region of at least twelve nucleotides in length which has 1, 2, or 3 mismatches to a corresponding region of at least twelve nucleotides of the original probe sequence. Any mismatches are preferably at least 4, at least 5, or at least 8 nucleotides apart.

Variants of SEQ ID NOs 3 to 10, 17 to 19, 24, 25 , 30 and 32 may also be oligonucleotides of less than 25 nucleotides in length which have at least 70% sequence identity to the sequence of the corresponding original probe sequence, preferably at least 75%, at least 80%, more preferably at least 85%, at least 90%, or at least 95% sequence identity.

The above variant probes may comprise 5' and/or 3' flanking regions to the region which is partly or fully complementary to the original probe. The 5' and/or 3' flanking regions may comprise sequence which is non-complementary to the target nucleic acid sequence, or sequence complementary to the regions that flank the binding region of the original probe in the target nucleotide sequence. For example, in variants of SEQ ID NOs 24, 25 and 30, the 5' flanking region may comprise a sequence of 1-10, or 1-5 nucleotides in length which is complementary to the 1-10 or 1-5 nucleotides which are 5' to the binding region of the probe in the relevant target nucleic acid sequence of SEQ ID NO:26 or SEQ ID NO:31.

Detection of a signal

The detection of the signal from the probe may be performed by any suitable means for detection of fluorescence. The probe may be used to detect the target nucleic acid sequence without any prior DNA amplification. More typically, the probe is used to detect

The invention thus provides a method for detection of amplification of a target nucleic acid sequence in a sample in the presence of at least one protein capable of binding to single-stranded DNA comprising contacting said sample with an oligonucleotide probe as described above under conditions promoting amplification of said target nucleic acid sequence.

Such conditions typically comprise the presence of one or more primers and a DNA polymerase enzyme. The skilled person will be able to select suitable primers for a particular target sequence depending on the type of DNA polymerase to be used. Where the DNA polymerase enzyme is an RCA enzyme (such as phi29), random primers or a single species of primer amplifying the target nucleic acid sequence may be used. More typically, the amplification conditions will comprise presence of an upstream primer and a downstream primer for the target nucleic acid sequence.

As discussed above, the oligonucleotide probe may provide a primer function and thus the amplification conditions may comprise the presence of an oligonucleotide probe which acts as a downstream primer and a separate upstream primer (and optionally no other downstream primer(s)) or the presence of an oligonucleotide probe which acts as an upstream primer and a separate downstream primer (and optionally no other upstream primer(s)).

Where SIBA is used, the conditions typically further comprise the presence of a strand invasion oligonucleotide. The features of preferred primers and strand invasion oligonucleotides for SIBA amplification are described in more detail below.

Suitable conditions for amplification of the target nucleic acid sequence further comprise any conditions used to provide for activity of polymerase enzymes known in the art. The conditions typically include the presence of all four dNTPs, dATP, dTTP, dCTP and dGTP or analogues thereof, suitable buffering agents/pH and other factors which are required for enzyme performance or stability. The conditions may include the presence of detergents and stabilising agents. The temperature used is typically isothermal, i.e. constant throughout the amplification process. The temperature used typically depends on the nature of the polymerase enzyme and other enzyme components, and also reflects the hybridisation temperature required for the primers and strand invasion oligonucleotides.

The polymerase used typically has strand-displacement activity. The term "strand displacement" is used herein to describe the ability of a DNA polymerase, optionally in conjunction with accessory proteins, to displace complementary strands on encountering a region of double stranded DNA during DNA synthesis. Suitable DNA polymerases include

polI from *E. coli*, *B. subtilis*, or *B. stearothermophilus*, and functional fragments or variants thereof, and T4 and T7 DNA polymerases and functional fragments or variants thereof. A preferred polymerase is Bsu DNA polymerase or a functional fragment or variant thereof.

The amplification conditions comprise the presence of a protein capable of binding single-stranded DNA. The protein capable of binding single-stranded DNA may be any protein which causes a change in fluorescent signal from an oligonucleotide probe labeled with a fluorophore and quencher in the absence of a complementary template, and which can bind to single-stranded DNA. The protein may be any single-stranded binding protein (SSB) or any protein which is capable of binding single-stranded DNA and also has another functional activity. The protein capable of binding single-stranded DNA may be a recombinase or a recombinase accessory protein or cofactor. The protein capable of binding single-stranded protein may be mesophilic or thermophilic.

The amplification conditions comprise the presence of a recombinase. Any recombinase system may be used in the method of the invention. The recombinase system may be of prokaryotic or eukaryotic origin, and may be bacterial, yeast, phage, or mammalian. The recombinase may polymerise onto a single-stranded oligonucleotide in the 5'-3' or 3'-5; direction. The recombinase may be derived from a myoviridae phage, such as T4, T2, T6, Rb69, Aeh1, KVP40, Acinetobacter phage 133, Aeromonas phage 65, cyanophage P-SSM2, cyanophage PSSM4, cyanophage S-PM2, Rbl4, Rb32, Aeromonas phage 25, Vibrio phage nt-1, phi-1, Rbl6, Rb43, Phage 31, phage 44RR2.8t, Rb49, phage Rb3, or phage LZ2. In a preferred embodiment, the T4 recombinase UvsX (Accession number: P04529) or a functional variant or fragment thereof is used. The Rad systems of eukaryotes or the recA-Reco system of *E. coli* or other prokaryotic systems may also be used. The recombinase may be *E. coli* RecA.

The conditions may further comprise the presence of recombinase accessory proteins, such as single-stranded binding protein (e.g. T4 gp32, accession number P03695) and recombinase loading agent (e.g. UvsY, accession number NP_049799.2). In a preferred embodiment, the conditions comprise the presence of the T4 gp32, UvsX and UvsY proteins. The recombinase (such as UvsX), and where used the recombinase loading agent (such as UvsY) and single stranded DNA binding protein (such as gp32), can each be native, hybrid or mutant proteins from the same or different myoviridae phage sources. A native protein may be a wild type or natural variant of a protein.

The protein capable of binding single-stranded DNA may alternatively be any protein used to provide single-strand binding activity in a DNA amplification method. The amplification method may be PCR. The protein capable of binding single-stranded DNA may be Extreme Thermostable Single-Stranded DNA Binding Protein (ET-SSB), which may be obtained from New England Biolabs.

The conditions may further comprise other factors used to enhance the efficiency of the recombinase such as compounds used to control DNA interactions, for example proline, DMSO or crowding agents which are known to enhance loading of recombinases onto DNA (Lavery P. et al. J. Biol. Chem. 1992, 267, (13), 9307-9314).

The conditions may also comprise the presence of an ATP regeneration system. Various ATP regeneration systems are known to the person skilled in the art, and include glycolytic enzymes. Suitable components of an ATP regeneration system may include one or more of phosphocreatine, creatine kinase, myokinase, pyrophosphatase, sucrose and sucrose phosphorylase. The conditions may further comprise the presence of ATP.

Additional components such as magnesium ions, DTT or other reducing agents, salts, BSA/PEG or other crowding agents may also be included.

The various components described above may be provided in varying concentrations to provide for DNA amplification. The skilled person can select suitable working concentrations of the various components in practice. Where the oligonucleotide probe overlaps with the sequence of the upstream or downstream primer, any competition observed between binding of the primer and the probe may be minimized by either using a lower concentration of probe or a probe with a reduced region of overlap, such as a probe whose length is shorter than the reverse primer from the 3′-end.

Strand invasion based amplification (SIBA)

The features of the preferred method for amplification of the target nucleic acid sequence, SIBA, are discussed below. The invention provides a method for detection of amplification of a target nucleic acid sequence in a sample in the presence of at least one protein capable of binding to single-stranded DNA comprising contacting said sample with at least one oligonucleotide probe as described herein, at least one upstream primer, at least one downstream primer, at least one strand invasion oligonucleotide under conditions promoting amplification of said target nucleic acid sequence and at least one said protein capable of binding to single stranded DNA is a recombinase. As discussed above, the oligonucleotide probe may itself act as the upstream primer or downstream primer, or alternatively separate upstream and downstream primers are provided in combination with

region of the opposing strand of the duplex target nucleic acid sequence to the upstream primer, at a position proximal or overlapping with the 3' binding site of the strand invasion oligonucleotide. The 5' binding sites of the upstream and downstream primers are typically at least 45 nucleotides, more preferably at least 50, at least 55 or at least 60 nucleotides apart on the duplex target sequence.

The upstream and/or downstream primer may have a region of sequence overlap with the sequence of the strand invasion oligonucleotide. The region of sequence overlap is typically 1-8 nucleotides in length, and may be at least 5 or at least 6 nucleotides in length. The downstream primer may also have a region of sequence overlap of 1-8 nucleotides in length with the sequence of the strand invasion oligonucleotide. Alternatively, there may be no sequence overlap between the upstream and/or downstream primer and the strand invasion oligonucleotide, with the primer binding instead at a position that is proximal in the target sequence to the binding site of the strand invasion oligonucleotide.

Where a primer binds proximal to the strand invasion oligonucleotide, typically there is 25 nucleotides or less, more preferably 20 nucleotides or less, 15 nucleotides or less, or 10 nucleotides or less between the relevant binding site of the strand invasion oligonucleotide and the 5' end of the primer. This ensures that the primer is able to hybridise to the single-stranded region created by binding of the strand invasion oligonucleotide.

Preferably, each primer is designed to allow for specific detection of a particular target nucleic acid sequence, such as a particular genotype, or a nucleic acid sequence present in a particular target, such as a particular organism or a particular pathogen. Thus, each primer typically specifically or selectively hybridises to a complementary sequence found only in the target. However, each primer may also hybridise to other sequences, such as sequences found in other species, provided that when used in combination with the second primer, strand invasion oligonucleotide and oligonucleotide probe, specific detection of the target nucleic acid sequence is obtained.

Specific examples of suitable upstream and downstream primers for amplification of target nucleotide sequences in toxigenic *C. difficile* and *S. typhimurium* are provided herein. The method of the invention may use the primers of SEQ ID NOs 21 and 22 or variants thereof for amplification of a target nucleic acid sequence of toxigenic *C. difficile* (such as SEQ ID NO: 26) and the primers of SEQ ID NOs 27 and 28 or variants thereof for amplification of a target nucleic acid sequence of *S. typhimurium* (such as SEQ ID NO: 31).

recombinase enzyme. Thus, where a PNA oligonucleotide is used, the methods of the disclosure may be performed without presence of a recombinase enzyme.

Specific examples of suitable strand invasion oligonucleotides for target nucleotide sequences in toxigenic *C. difficile* and *S. typhimurium* are provided herein. The method of the invention may use the strand invasion oligonucleotide of SEQ ID NO: 23 or a modified derivative or variant thereof for amplification of a target nucleic acid sequence of toxigenic *C. difficile* (such as SEQ ID NO: 26) and the strand invasion oligonucleotide of SEQ ID NO 29 or a modified derivative or variant thereof for amplification of a target nucleic acid sequence of *S. typhimurium* (such as SEQ ID NO: 31).

As discussed above, it is preferred that a strand invasion oligonucleotide used in the invention comprises one or more modified oligonucleotides in its 3' region to block its use as a polymerase substrate. Thus, a modified derivative of SEQ ID NO: 23 or 29 may comprise one, two, three, four, five, six, seven, eight or more modified nucleotides in its 3' region, typically in the 10-15 or 10-20 nucleotides from the 3' terminus. The modifications may be selected from any of those discussed above. The modified derivative may be a PNA oligomer of corresponding sequence to SEQ ID NO: 23 or 29.

Variants of SEQ ID NOs 23 and 29 are typically oligonucleotides of greater than 30 nucleotides, more preferably at least 35, at least 40, or at least 45 nucleotides in length, comprising a region which is partly or fully complementary to at least 30 contiguous nucleotides of the corresponding original target-complementary sequence present in SEQ ID NO: 23 or 29. Preferably, said variants will comprise a region which is partly or fully complementary to at least 32, 35, 37, 40, 42 or 45 contiguous nucleotides of the target-complementary sequence present in SEQ ID NO: 23 or 29.

The above variants may comprise a region which has 1, 2, 3, 4, 5, 6, 7 or 8 mismatches (substitutions) with respect to the corresponding target-complementary region of the original strand invasion oligonucleotide of SEQ ID NO: 23 or 29 (and thus the target sequence) and thus is partly complementary thereto. Thus, for instance, the variants may comprise a region of at least 30 nucleotides in length which has 1, 2, 3, or 4, such as 1-4 or 1-3 mismatches to a corresponding region of at least 40 contiguous nucleotides of the corresponding original strand invasion oligonucleotide. The variants may comprise a region of at least 35, 40, 42, or 45 nucleotides in length which has 1, 2, 3, 4, 5 or 6, such as 1-5, or 1-3 mismatches to a corresponding region of an equivalent length in the corresponding original strand invasion oligonucleotide. Any mismatches in the variant

strand invasion oligonucleotide sequence may be at least 2, at least 4, at least 5, or at least 10 nucleotides apart.

Alternatively, the variants may comprise a region of at least 32, 35, 37, 40, 42 or 45 nucleotides in length which is in full complementarity with the target-complementary region of the original strand invasion oligonucleotide.

Variants of SEQ ID NOs 23 and 29 may also be oligonucleotides of greater than 30 nucleotides in length comprising a target-complementary region which has at least 70% sequence identity to the target-complementary sequence of the corresponding original strand invasion oligonucleotide, preferably at least 75%, at least 80%, more preferably at least 85%, at least 90%, at least 95% sequence identity.

The variant strand invasion oligonucleotides may comprise 5' and/or 3' flanking nucleotide sequence(s) to the region which is partly or fully complementary to the original strand invasion oligonucleotide sequence. The 5' and/or 3' flanking sequence(s) may be non-complementary to the target nucleic acid sequence, or may be complementary in sequence to the regions that flank the binding region of the original strand invasion oligonucleotide in the target nucleic acid sequence, such as the 5-10 or 5-15 nucleotides 5' and/or 3' to the binding region of the original strand invasion oligonucleotide in the target nucleic acid sequence.

The remaining sequence of the variant strand invasion oligonucleotides is typically unrelated to the target sequence, and also typically unrelated to the original strand invasion oligonucleotide.

The variant strand invasion oligonucleotides further comprise one or more modified oligonucleotides in their 3' region such as, two, three, four, five, six, seven, eight or more modified nucleotides, which may be in the 10-15 or 10-20 nucleotides from the 3'terminus The modifications may be selected from any of those discussed above.

Detection of target nucleotide sequences

Described herein are particular combinations of upstream and downstream primers, oligonucleotide probes and strand invasion oligonucleotides for detection of target nucleotide sequences. Thus, the invention provides a method for detection of a target nucleic acid sequence of toxigenic *C. difficile* in a sample in the presence of at least one single-stranded DNA binding protein which is a recombinase comprising contacting said sample with an oligonucleotide probe of SEQ ID NO: 24 or 25 or a variant of either thereof as described above, an upstream primer of SEQ ID NO: 21 or a variant thereof as described above, a

downstream primer of SEQ ID NO: 22 or a variant thereof as described above, and a strand invasion oligonucleotide of SEQ ID NO: 23 or a modified derivative or variant thereof as described above, under conditions promoting amplification of said target nucleic acid sequence. Such a method may comprise detection of the target nucleic acid sequence of SEQ ID NO: 26.

The invention further provides a method for detection of a target nucleic acid sequence of *S. typhimurium* in a sample in the presence of at least one single-stranded DNA binding protein which is a recombinase comprising contacting said sample with an oligonucleotide probe of SEQ ID NO: 30 or a variant thereof as described above, an upstream primer of SEQ ID NO: 27 or a variant thereof as described above, a downstream primer of SEQ ID NO: 28 or a variant thereof as described above, and at least one strand invasion oligonucleotide of SEQ ID NO: 29 or a modified derivative or variant thereof as described above, under conditions promoting amplification of said target nucleic acid sequence. Such a method may comprise detection of the target nucleic acid sequence of SEQ ID NO: 31.

The invention further provides a method for detection of the target nucleic acid sequence of SEQ ID NO: 20 in a sample in the presence of at least one single-stranded DNA binding protein which is a recombinase comprising contacting said sample with an oligonucleotide probe of SEQ ID NO: 13 or 14 or a variant thereof as described above, an upstream primer of SEQ ID NO: 11 or a variant thereof, a downstream primer of SEQ ID NO: 12 or a variant thereof, and at least one strand invasion oligonucleotide of SEQ ID NO: 15 a modified derivative or variant thereof, under conditions promoting amplification of said target nucleic acid sequence. Variants of SEQ ID NOs 11 and 12 may be selected according to the same criteria described above for variants of SEQ ID NOs 21, 22, 27 and 28. Variants and modified derivatives of SEQ ID NO 15 may be selected according to the same criteria described above for variants of SEQ ID NOs 23 and 29.

The invention also provides a method for detection of the target nucleic acid sequence of SEQ ID No: 34 in a sample in the presence of at least one single-stranded DNA binding protein which is a recombinase comprising contacting said sample with an oligonucleotide probe of any one of SEQ ID NOs 3 to 10 or a variant thereof.

Products of the invention

Nucleic acids

The disclosure further provides an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to a target nucleic acid sequence as a product *per*

se. The sequence of the oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides. The oligonucleotide probe of the disclosure may be any oligonucleotide probe described above for use in the methods of the invention. The disclosure also specifically provides an oligonucleotide probe of SEQ ID NOs: 3 to 10, 13, 14, 24, 25 or 30 or a variant of any thereof as described above.

Compositions

The disclosure also provides a composition and formulation comprising an oligonucleotide probe. The composition may be for example a solution, lyophilisate, suspension, or an emulsion in an oily or aqueous vehicle. The composition may further comprise one or more oligonucleotide components selected from an upstream primer, a downstream primer and a strand invasion oligonucleotide. The composition may further comprise one or more proteins selected from a DNA polymerase, protein capable of binding to single-stranded DNA, a recombinase and a recombinase accessory protein. The composition preferably comprises the oligonucleotide probe and in addition (i) a strand invasion oligonucleotide comprising a region complementary to the target nucleic acid sequence and/or (ii) at least one protein capable of binding to single-stranded DNA. Probe, primer and strand invasion oligonucleotide components of the composition are each designed for detection of the target nucleic acid sequence.

The composition may comprise an oligonucleotide probe of SEQ ID NO: 24 or 25 or a variant thereof in combination with (i) an upstream primer of SEQ ID NO: 21 or a variant thereof, (ii) a downstream primer of SEQ ID NO: 22 or a variant thereof and/or (iii) a strand invasion oligonucleotide of SEQ ID NO: 23 or variant thereof. Additionally, or alternatively the composition may comprise an oligonucleotide probe of SEQ ID NO: 30 or a variant thereof in combination with (i) an upstream primer of SEQ ID NO: 27 or a variant thereof, (ii) a downstream primer of SEQ ID NO: 28 or a variant thereof and/or (iii) a strand invasion oligonucleotide of SEQ ID NO: 29 or variant thereof. Additionally, or alternatively, the composition may comprise an oligonucleotide probe of any one of SEQ ID NOs 17 to 19 and 32 or a variant thereof in combination with (i) an upstream primer of SEQ ID NO: 11 or a variant thereof, (ii) a downstream primer of SEQ ID NO: 12 or a variant thereof and/or (iii) a strand invasion oligonucleotide of SEQ ID NO: 15 or variant thereof.

Kits

The invention further provides a kit comprising an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to a target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides, and in addition at least one protein capable of binding to single stranded DNA wherein said at least one protein comprises a recombinase. The kit may optionally further comprise instructions for use in a method of the invention. The kit may comprise a means for detection of amplified DNA. The kit may comprise an upstream primer and/or a downstream primer. The kit may comprise a strand invasion oligonucleotide. Each of the above oligonucleotides may be provided in the kit as a mixture, or in separate containers.

The kit or composition optionally comprises one or more of a DNA polymerase, a recombinase, and recombinase accessory proteins. Preferably, the DNA polymerase is Bsu polymerase. Preferably, the recombinase is bacteriophage T4 UvsX, optionally in combination with the recombinase accessory proteins UvsY and gp32. The kit or composition may further comprise dNTPs, suitable buffers and other factors which are required for DNA amplification in the method of the invention as described above.

The kit may comprise any combination of oligonucleotide probe, upstream primer, downstream primer and/or strand invasion oligonucleotide described above in connection with the methods and compositions of the invention.

Applications for detection of target nucleic acid sequences

The methods of the invention may be used in any application where a target nucleic acid sequence is desired to be detected.

Methods for diagnosis

The present invention is particularly advantageous in the medical setting. The detection methods of the invention provide a highly specific test to allow for determination of whether a clinical sample contains a target nucleic acid sequence. The method may be applied to a range of disease settings. The invention provides a method for diagnosis of a disease in a subject, comprising carrying out a method of detection of a target nucleic acid sequence of the invention in a sample from said subject to detect a target nucleic acid sequence associated with said disease.

Such a method may be for diagnosis of an infection by a pathogen in a subject, comprising detection of a target nucleic acid sequence from said pathogen. The determination of whether or not the pathogen is present may be in the context of any disease or illness present or suspected of being present in a patient. Such diseases may include those caused by, linked to, or exacerbated by the presence of the pathogen. Thus, a patient may display symptoms indicating the presence of the pathogen, and a sample may

gastrointestinal illness is due to an infection by toxigenic *C.difficile or S. typhimurium*. The diagnostic method may for instance provide an indication as to the toxinotype and virulence of *C. difficile* and whether the *C. difficile* is resistant to any antibiotics. Depending on the outcome of the test the medical treatment can then be optimised, for example by use of antibiotics.

Detection of Salmonella typhimurium

The invention further provides a method for detecting *S. typhimurium* in a sample, said method comprising contacting said sample in the presence of at least one protein capable of binding to a single stranded DNA wherein said at least one protein is a recombinase, with at least one upstream primer, at least one downstream primer and at least one strand invasion oligonucleotide under conditions promoting amplification of a target nucleic acid sequence comprising SEQ ID NO: 31, wherein each said primer and said strand invasion oligonucleotide comprises a region complementary to said target nucleic acid sequence; and wherein said strand invasion oligonucleotide renders at least a portion of the target nucleic acid sequence single-stranded to allow the binding of said upstream primer and a downstream primer, and wherein the at least one oligonucleotide probe comprises a fluorophore, a quencher and a region complementary to said target nucleic acid sequence. The oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides. Typically, said method for detecting *Salmonella typhimurium* is carried out in the presence of at least one protein capable of binding to single-stranded DNA. Typically, said protein capable of binding to single-stranded DNA is a recombinase.

Preferably, the upstream primer comprises the sequence of SEQ ID NO: 27 or a variant thereof, the downstream primer comprises the sequence of SEQ ID NO: 28 or a variant thereof, and the strand invasion oligonucleotide comprises the sequence of SEQ ID NO: 29 or a variant thereof.

The following Examples illustrate the invention.

Examples

Example 1 – Affinity of different oligonucleotides for single-stranded DNA binding proteins

CLAIMS

1. A method for detection of a target nucleic acid sequence in a sample in the presence of at least one protein capable of binding to single-stranded DNA comprising contacting said sample with at least one oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to said target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides and wherein at least one said protein capable of binding to single-stranded DNA is a recombinase.

2. A method according to claim 1, which comprises amplification of said target nucleic acid sequence, optionally under isothermal conditions promoting amplification of said target nucleic acid sequence.

3. A method according to claim 1 or 2, which comprises contacting said sample with at least one upstream primer and at least one downstream primer, each comprising a region complementary to said target nucleic acid sequence.

4. A method according to any one of the preceding claims, which comprises contacting said sample with a strand invasion oligonucleotide comprising a region complementary to said target nucleic acid sequence.

5. A method according to claim 3 or 4, where said oligonucleotide probe is capable of priming amplification of said target nucleic acid sequence and functions as said at least one upstream or downstream primer.

6. A method according to any one of the preceding claims, further comprising the presence of a protein capable of binding single-stranded DNA which is a recombinase accessory or cofactor protein, optionally wherein said recombinase accessory protein is selected from UvsY or gp32.

7. A method according to claim 1, wherein said recombinase is selected from UvsX or RecA.

8. A method according to any one of the preceding claims, wherein the modified RNA nucleotides present in the oligonucleotide probe comprise at least one 2'-fluoro ribonucleotide, 2'-O-methyl ribonucleotide or LNA ribonucleotide.

9. A method according to any one of the preceding claims, which comprises contacting said sample with RNase H.

10. A method according to any one of the preceding claims, wherein said oligonucleotide probe comprises the sequence of SEQ ID NO: 24, 25 or 30 or a variant of any thereof, comprising a corresponding nucleotide sequence which comprises ribonucleotides selected from natural ribonucleotides, 2'O- methylribonucleotides, PNA nucleotides or LNA nucleotides.

11. A kit comprising an oligonucleotide probe comprising a fluorophore, a quencher and a region complementary to a target nucleic acid sequence, wherein the sequence of said oligonucleotide probe comprises at least 20% RNA nucleotides, modified RNA nucleotides and/or PNA nucleotides, and in addition at least one protein capable of binding to single-stranded DNA wherein said at least one protein is a recombinase.

12. A kit according to claim 11, wherein (i) said kit further comprises an upstream and/or downstream primer comprising a region complementary to said target nucleic acid sequence; and a strand invasion oligonucleotide comprising a region complementary to said target nucleic acid sequence optionally wherein or (ii) said oligonucleotide probe is capable of priming amplification of said target nucleic acid sequence and functions as a said upstream primer or a said downstream primer.

13. A method for diagnosis of a disease in a subject, comprising carrying out a method as defined in any one of claims 1 to 12 in a sample from said subject to detect a target nucleic acid sequence associated with said disease.