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(71) Applicant: **MICROBIOME LIMITED** [NL/NL]; Troubadoursborch 59, 3992 BE Houten (NL).

(72) Inventor: **SAVELKOUL, Paul Hendrik Maria**; c/o van der Boechorststraat 7, 1081 BT Amsterdam (NL).

(74) Agents: **JANSEN, C.M.** et al.; V.O., Carnegieplein 5, 2517 KJ Den Haag (NL).

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(54) Title: NOVEL ISOLATION AND AMPLIFICATION PROCESS CONTROL

(57) Abstract: The invention relates to a positive control nucleic acid molecule comprising first and second primer hybridization sequences derived from a first microorganism which flank an internal nucleic acid sequence that is not naturally present in the first microorganism. The invention further relates to a positive process control microorganism comprising said positive control nucleic acid molecule, to a receptacle comprising said positive control microorganism, and to methods for determining whether a first microorganism is present or absent in a test sample, comprising contacting the test sample with the positive process control microorganism and generating amplification products using first and second primers that hybridize to the first and second primer hybridization sequences.

Title: **NOVEL ISOLATION AND AMPLIFICATION PROCESS CONTROL**

1. FIELD OF THE INVENTION

The present invention relates to a novel isolation and amplification control, to methods employing said control and to uses of said control.

2. BACKGROUND OF THE INVENTION

The detection of pathogenic microorganisms in patients and of microbial contaminants in, for example, food and drinking water, is an ongoing challenge. Amplification-based detection systems are often prone to inhibition by substances present in environmental samples, thereby potentially leading to inaccurate target quantification or false-negative results. For example, amplification inhibitors such as acidic polysaccharides in sputum and polymerase inhibitors in cerebrospinal fluid will negatively effect amplification-based detection reactions.

Controls have been developed that monitor the accuracy and precision of a part of the analytical process from sample preparation, storage, transportation, and extraction to amplification for the detection of a specific microorganism. For example, Internal amplification controls (IACs) have been developed to help controlling the amplification of nucleic acid in a test sample.

Therefore, at present most controls only serve as a control for the amplification reaction without controlling the steps of transportation, storage, sample preparation, and extraction that occur prior to amplification of the target sequence. Having accurate standards and controls for the whole process is key to any testing program, and is especially important for IVD-CE certification, in point of care systems where one often uses completely closed systems, and for in house protocols.

3. SUMMARY OF THE INVENTION

The present invention provides a novel isolation and amplification control (IAC) and methods for diagnosing of microbial infections in mammals, preferably humans, that employ this novel control. The IAC allows to control the complete procedure from obtaining a sample to detection of amplified fragments, or part of the procedure. The control provides substantial benefit as it allows the use of only one positive control for both isolation of nucleic acid and amplification of specific parts of the nucleic acid, for which routinely two different controls are used. In addition, the control can be added upon collection of a sample to serve as a control for the entire processing operations of the sample, including storage, transport, lysis, extraction and amplification; allows the reliable, quantitative determination of a lower limit of detection of a microorganism; and is easy to generate. In addition, the IAC provides detection of a control nucleic acid in the same genomic (chromosomal) environment as the actual target nucleic acid.

The invention therefore provides a method for determining whether a first, target microorganism is present or absent in a test sample, the method comprising contacting the test sample with a second, killed, intact positive process control microorganism, comprising a control nucleic acid, comprising first and second primer hybridization nucleic acid sequences from the first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence (X) that is not present between the first and second hybridization nucleic acid sequences in the first microorganism, and wherein an internal nucleic acid sequence (Y) that is present between the first and second hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control microorganism; whereby the first and second primer are able to amplify a target nucleic acid that is present in the first microorganism of between 50

and 500 bases, preparing a nucleic acid extract from the test sample, the extract comprising nucleic acid from at least the second microorganism and, if present in the sample, nucleic acid from the first microorganism, contacting the extract with the first and second primer to amplify at least the control nucleic acid and, if present, the target nucleic acid from the first microorganism; and detecting the presence or absence of the control nucleic acid and the target nucleic acid, whereby the presence of the target nucleic acid is indicative for the presence of the first microorganism.

If the presence or absence of more than one first microorganism is to be analyzed in a test sample, for example microorganisms A and B, the second, positive process control microorganism may comprise a first primer hybridization nucleic acid sequence from microorganism A and a second primer hybridization nucleic acid sequences from microorganism B, whereby the first and second primer hybridization nucleic acid sequences in the second, positive process control microorganism flank an internal nucleic acid sequence that is not present in microorganism A or microorganism B.

Therefore, the invention provides a method for determining whether at least two microorganisms A and B are present or absent in a test sample, comprising contacting the test sample with a second, killed, intact, positive process control microorganism comprising a control nucleic acid comprising first (A1) and second (B2) primer hybridization nucleic acid sequences, whereby said first primer hybridization nucleic acid sequence A1 is from microorganism A and is not present in microorganism B, said second primer hybridization nucleic acid sequence B2 is from microorganism B and is not present in microorganism A, wherein A1 and B2 flank an internal nucleic acid sequence that is not present in microorganisms A and B, preparing a nucleic acid extract from the test sample, the extract comprising nucleic acid from at least the second microorganism and, if present in the sample, nucleic acid from microorganism A and/or B, contacting the extract with primers A1 and A2, and with primers B1 and B2, wherein A1 and B2

amplify the control nucleic acid and, if present, A1 and A2 amplify target nucleic acid from microorganism A and B1 and B2 amplify target nucleic acid from microorganism B, and detecting the presence or absence of the control nucleic acid and the target nucleic acids from microorganisms A and B, whereby the presence of target nucleic acid from microorganisms A is indicative for the presence of microorganism A in the test sample, and whereby the presence of target nucleic acid from microorganisms B is indicative for the presence of microorganism B in the test sample.

The skilled person will understand that the second, killed, positive process control microorganism may further comprise a second control nucleic acid comprising first (C1) and second (D2) primer hybridization nucleic acid sequences, whereby said first primer hybridization nucleic acid sequence C1 is from microorganism C and is not present in microorganism D, said second primer hybridization nucleic acid sequences D2 is from microorganism D and is not present in microorganism C, wherein C1 and D2 flank an internal nucleic acid sequence that is not present in microorganisms C and D.

Said control nucleic acid is preferably integrated in the genome of the second microorganism.

Said test sample preferably is a bodily fluid or a biopsy sample.

It is preferred that a genomic nucleic acid extract is prepared from the test sample, whereby the second, positive process control microorganism comprises a control nucleic acid that is integrated into its genome.

It is further preferred in a method according to the invention that mammalian eukaryotic cells that are present in the test sample are lysed prior to lysis and preparation of a nucleic acid extract from the microorganisms. Prior lysis of eukaryotic cells results in increased sensitivity and specificity of the subsequent analysis of microorganisms.

It is further preferred that the length of the amplified part (e.g. internal nucleic acid sequence plus primer hybridization sequences) of the

control nucleic acid is larger than the length of the amplified part of the target nucleic acid or target nucleic acids. A greater length of the amplified region of the control nucleic acid will result in an optimal amplification of the target nucleic acid from the first microorganism, or from microorganisms A and B, as amplification in general is biased towards smaller fragments. Amplification of a larger fragment of the internal control will not significantly affect amplification of smaller fragments from the target nucleic acid.

In a preferred method according to the invention, the second positive process control microorganism is present in a receptacle prior to the addition of a test sample to the receptacle. Surprisingly, the second positive process control microorganism that is added to a receptacle, which is subsequently stored at varying temperatures including room temperature for varying periods of time, is left intact, meaning that genomic DNA can be isolated from said positive process control microorganism using isolation methods that will not allow isolation of genomic DNA of damaged cells, such as the Polaris method. The invention further provides a positive control nucleic acid molecule comprising first and second primer hybridization sequences derived from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not naturally present between the first and second hybridization sequences in the first microorganism, and wherein an internal nucleic acid sequence that is present between the first and second hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control nucleic acid; further comprising outside nucleic acid bordering sequences that allow integration of the positive control nucleic acid molecule into the genome of a second microorganism.

Further provided is a positive control nucleic acid molecule comprising first (A1) and second (B2) primer hybridization sequences

derived from microorganisms A and B, respectively, whereby A1 and B2 in the positive control nucleic acid molecule flank an internal nucleic acid sequence that is not naturally present in microorganisms A and B, further comprising outside nucleic acid bordering sequences that allow integration of the positive control nucleic acid molecule into the genome of a second microorganism.

The invention further provides a positive process control microorganism, comprising a positive control nucleic acid molecule comprising first and second primer hybridization sequences derived from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not naturally present between the first and second hybridization sequences in the first microorganism, and wherein an internal nucleic acid sequence that is present between the first and second hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control microorganism.

Also provided is a positive process control microorganism, comprising a positive control nucleic acid molecule comprising first (A1) and second (B2) primer hybridization sequences derived from microorganisms A and B, respectively, wherein A1 and B2 flank an internal nucleic acid sequence that is not naturally present microorganisms A and B.

Further provided is a receptacle comprising a positive process control microorganism according to the invention.

Said positive process control microorganism preferably is an intact microorganism, preferably an intact non-pathogenic microorganism. The term “intact” indicates that the positive process control microorganism may be killed, but that lysis is required for isolation of the positive control nucleic acid molecule, in contrast to currently used controls like viruses and isolated plasmids.

Said positive process control microorganism preferably is a bacterium. If the first microorganism is a bacterium, and/or if microorganisms A and B are bacteria, it is further preferred all microorganisms stain either positive or negative in Gram-staining.

Said positive process control microorganism preferably is fungus, such as a *Candida* species or *Aspergillus* species, especially in the case that the first microorganism is a fungus, such as a *Candida* species or *Aspergillus* species.

In one embodiment, the positive process control microorganism according to the invention is a microorganism that is resistant to lysis such as, for example, a spore-forming bacterium such as *Bacillus subtilis*.

Said positive process control microorganism preferably is killed prior to the addition of the positive process control microorganism to a test sample.

The invention further provides a use of a receptacle comprising a positive process control microorganism according to the invention to control the entire processing operations of the sample, including storage, transport, lysis, extraction and amplification.

In addition, the invention provides the use of a receptacle comprising a positive process control microorganism according to the invention to a quantitatively control the lower detection limit of the first microorganism.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Schematic overview of the construction of the internal control: A: target in a original micro organism; B: single internal control in a micro organism missing the target; C: Double process control for two targets. All primer sequences are specific for a target in the original microorganism, but

originally absent in the process control micro organism. Thin solid line: original target; Thick solid line: artificial DNA sequence.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1 Definitions

The term "test sample", as used herein, refers to a food, environmental, agricultural, biopharmaceutical, pharmaceutical, or water sample, including a clinical sample for example for diagnostic purposes. A preferred test sample is a sample from a subject that is suffering from, or suspected to be suffering from, an infection with a microorganism. Said sample is or comprises stool, a bodily fluid or a tissue sample such as, for example, a biopsy sample. Said bodily fluid preferably is cerebrospinal fluid, saliva, sweat, urine or blood, including blood plasma, which is prepared by removing red and white blood cells, for example by centrifugation, and blood serum, which is prepared by formation of a blood clot, and removal of the clot using, for example, a centrifuge.

The term "microorganism" includes bacteria, viruses and fungi.

The term "first microorganism", as is used herein, refers to bacteria, especially pathogenic bacteria such as *Bacillus* spp., *Clostridium* spp., *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Chlamydia* spp., *Salmonella* spp., *Neisseria* spp., *Brucella* spp., *Mycobacterium* spp., *Listeria* spp., *Haemophilus* spp., *Helicobacter* spp., *Francisella* spp., *Legionella* spp., *Pseudomonas* spp., *Yersinia* spp., *Vibrio* spp., *Escherichia* spp. and *Klebsiella* spp., fungi, especially pathogenic fungi including *Aspergillus* spp., *Candida* spp., *Cryptococcus* spp., *Coccidioides* spp., *Corynebacterium* spp., *Histoplasma* spp. and *Pneumocystis* spp., and viruses, including DNA and RNA viruses such as hemorrhagic fever viruses, including ebola virus and Marburg virus, hepatitis virus, coronavirus, herpesvirus, West Nile virus and Japanese encephalitis virus.

The terms "second microorganism" and "second positive control microorganism", as are used herein, refer to a bacterium, virus or fungus, harboring a control nucleic acid comprising first and second primer hybridization nucleic acid sequences from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not present between the first and second hybridization nucleic acid sequences in the first microorganism. Said control nucleic acid is preferably integrated into the genome of the second microorganism. Preferably said second microorganism is of the same class as the first microorganism, meaning that the second microorganism preferably is a virus in case the first microorganism is a virus; the second microorganism preferably is a bacterium in case the first microorganism is a bacterium, the second microorganism preferably is a fungus in case the first microorganism is a fungus. If the first microorganism is a Gram-negative bacterium, the second microorganism preferably is a Gram-negative bacterium. If the first microorganism is a Gram-positive bacterium, the second microorganism preferably is a Gram-positive bacterium.

The term "flank" in the context of primer hybridization means that at least one primer hybridises to a target sequence adjacent one end (eg. the 5′ end) of the nucleotide sequence of interest and at least one primer hybridises to a target sequence at the other end (e.g. the 3′ end) of the nucleotide sequence of interest. Preferably, at least one forward primer hybridises to a target sequence adjacent one end (e.g. the 5′ end) of the nucleotide sequence of interest and at least one reverse primer hybridises to a target sequence at the other end (eg. the 3′ end) of the nucleotide sequence of interest.

The term "control sample" as used herein, refers to a negative control sample, for example a blood sample that does not comprise the indicated microorganism or microorganisms, or a sample of nucleic acids

isolated therefrom, or a buffer control, for example 10 mM Tris, pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA).

The term "killed", as used herein, refers to the inactivation of the microorganism such that it is not able to replicate its genomic nucleic acid and/or the divide. A killed microorganism remains intact, indicating that lysis is required for isolation of the positive control nucleic acid molecule. Methods for killing a microorganism are known in the art, including heat treatment, radiation such as ultra-violet radiation and ionizinq radiation such as X-ray radiation, and/or chemical treatment.

The term "nucleic acid extract", as is used herein, refers to a DNA and/or RNA extract that is prepared from a test sample using methods known in the art. It is preferred that the nucleic acid extract is enriched for nucleic acid sequences from microorganisms that are present in the test sample. For this, the presence of eukaryotic non-fungal cells from the subject is preferably reduced in the test sample, more preferably removed from the test sample, prior to the preparation of a nucleic acid extract.

The term "control nucleic acid", as is used herein, refers to a nucleic acid molecule that comprises first and second primer hybridization nucleic acid sequences from one microorganism, whereby the first and second primer hybridization nucleic acid sequences flank (eg. are at either side or end of) an internal nucleic acid sequence that is not present between the first and second hybridization nucleic acid sequences in said first, naturally occurring, microorganism. The control nucleic acid can be amplified using the same first and second primer that are used to amplify a target nucleic acid in a microorganism that is to be detected and serves as a control for the amplification said target nucleic acid. The length of the control nucleic acid preferably is larger than the length of the target nucleic acid. The length of the control nucleic acid preferably is between 60-600 basepairs (bp), preferably between 80-300 bp.

The term "positive control nucleic acid molecule", as is used herein, refers to a control nucleic acid that additionally comprises outside bordering nucleic acid sequences that are at least 90% identical over substantially their whole length to genomic sequences of the second microorganism, more preferred at least 95% identical, more preferred at least 99% identical, more preferred 100% identical to genomic sequences of the second microorganism. Said outside bordering nucleic acid sequences allow integration of the positive control nucleic acid molecule into the genome of the second microorganism, thereby generating a recombinant microorganism. The term "substantially the whole length" is used to indicate that the outside bordering nucleic acid sequences may comprise additional nucleotide sequences, for example at the 5' and/or 3' ends, that are not present in the genome of the second microorganism.

The term "target nucleic acid", as is used herein, refers to a nucleic acid molecule that is present in a microorganism that is to be detected, preferably in the genome of said microorganism, preferably a pathogenic microorganism. The target nucleic acid comprises first and second primer hybridization nucleic acid sequences that flank a nucleic acid sequence that can be amplified using said first and second primer that are able to hybridize to the first and second primer hybridization nucleic acid sequences. The length of the target nucleic acid preferably is between 50-500 base pairs (bp), preferably between 70-250 bp.

The term "first primer", as is used herein, refers to a single-stranded oligonucleotide or oligonucleotide mimic of 15–50 bases, preferably 16-30 bases, that is complementary to the first primer hybridization nucleic acid sequences that are located on one side of the region to be amplified.

The term "second primer", as is used herein, refers to a single-stranded oligonucleotide or oligonucleotide mimic of 15–50 bases, preferably 16-30 bases, that is complementary to the second primer hybridization

nucleic acid sequences that are located on another side of the region to be amplified, relative to the first primer hybridization nucleic acid sequences.

The term "oligonucleotide mimic", as is used herein, refers to a modified oligonucleotide comprising, for example, locked nucleic acid (LNA®), synthetic peptide nucleic acid, mimics comprising 2'O-Me modified nucleotides or phosphonoacetate modified oligonucleotides, and/or mimics comprising phosphodiester-, phosphonocarboxylate-, methylphosphonate- or phosphorothioate internucleotide bonds.

The sequence of the first primer and second primer determine the specificity of the amplification reaction. Preferred primers are preferably about 100% identical to a region on a nucleic acid template such that only the region between the two primers hybridization nucleic acid sequences in a target nucleic acid template is amplified. The distance between the two primer hybridization nucleic acid sequences on a nucleic acid template will determine the size of the amplified product.

The term "probe", as is used herein, refers to a single-stranded oligonucleotide or oligonucleotide mimic of 15–50 bases, preferably 16-30 bases, that is complementary to a nucleic acid sequence within the region that is amplified. A preferred probe is about 100% identical to a region on a target nucleic acid template.

The term "amplification" or "amplify", as is used herein, refers to the *in vitro* amplification of a specific nucleic acid sequence, such as to test for presence of a given fungus, virus or bacterium in a test sample. *In vitro* amplification methods include amplification of a target nucleic acid sequence using, for example, ligase chain reaction (LCR), isothermal ribonucleic acid amplification such as nucleic acid sequence-based amplification (NASBA) and cleavage-based signal amplification of RNA, transcription mediated amplification, strand displacement amplification and, preferably, polymerase chain reaction (PCR). An amplification is preferably specific, meaning that only a region between two hybridization

nucleic acid sequences is amplified. An amplification reaction preferably is performed in a reaction chamber such as a vial or a well of a microtiter plate, or a fluidic chamber of a cartridge system. Said reaction chamber preferably includes the general ingredients of an amplification reaction, such as first and second primers, buffer, dNTPs, DNA polymerase, preferably in lyophilized form.

The term "PCR reaction", as is used herein, refers to an amplification reaction that is characterized by repeated cycles of denaturation of target nucleic acid template, annealing of primers, and extension (synthesis) of new nucleic acid strand. The specificity of a PCR reaction is substantially determined by the % identity of the primers to the target nucleic acid template and the annealing temperature.

The term "real-time PCR reaction", as is used herein, refers to a PCR amplification reaction to which a labeled probe or a dye is added to generate a signal. The intensity of the signal is a measure for the amount of product that is generated. Detection of the signal in real-time allows quantification of the amount of starting material. A real-time PCR reaction is performed in specialized thermal cyclers with detection systems that detect the signal, for example a LightCycler 480II (Roche Diagnostics, Almere, The Netherlands), or a StepOne™ Plus (Thermo Fisher Scientific Inc., Waltham, MA USA). However, a separate probe does not need to be present. Some real-time PCR reactions incorporate a dye in the primer (e.g. Scorpion® primers; Premier Biosoft, Palo Alto, CA, USA) and are comprised in the scope of the present invention.

The term "Ct value", as used herein, refers to the cycle threshold in real time PCR, which is the number of cycles required for a fluorescent signal to cross a background level. Ct levels are inversely proportional to the amount of target nucleic acid in the sample, meaning that a lower Ct level indicates a higher amount of target nucleic acid template in the sample. In general, for a real time PCR assay undergoing 40 cycles of amplification, a

Ct value of < 29 is indicative of abundant target nucleic acid template in a sample, a Ct value of 30-37 is indicative of a moderate amount of target nucleic acid template, while a Ct value of 38-40 is indicative of a low amount of target nucleic acid template.

The term "detectable label", as is used herein, refers to a label that is detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radioactive labels, fluorescent labels, electron-dense reagents, enzymes (as commonly used in ELISAs), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available.

The term "colony forming unit-equivalent", as is used herein, refers to an amount of a micro-organism that is detected in a real-time PCR reaction according to the invention, when compared to a standard agar plate culture. A cell of a micro-organism will in principle give rise to a colony in an appropriate standard agar plate culture test, and detection of this amount of a micro-organism in a real-time PCR reaction according to the invention will result in detection of a colony forming unit-equivalent.

5.2 Sample preparation

The present inventors have developed a positive process control microorganism comprising a control nucleic acid comprising first and second primer hybridization nucleic acid sequences from a first microorganism that is to be detected, wherein the first and second primer hybridization nucleic acid sequences in the positive process control microorganism flank an internal nucleic acid sequence that is not present between the first and second hybridization nucleic acid sequences in the first microorganism. This allows to discriminate between the first microorganism that is to be detected and the positive process control microorganism when using first and second primer that hybridize to the first and second primer hybridization nucleic acid sequences for amplification of a region between the two primers.

As is indicated herein above, if the presence or absence of more than one first microorganism is to be analyzed in a test sample, for example microorganisms A and B, the second, positive process control microorganism may comprise first primer hybridization nucleic acid sequence (A1) from microorganism A and second primer hybridization nucleic acid sequence (B2) from microorganism B, whereby A1 and B1 in the second, positive process control microorganism flank an internal nucleic acid sequence that is neither present in microorganism A nor in microorganism B, and which differs in length from the region between the first and second primer hybridization nucleic acid sequences (A1 and A2) in microorganism A, and from the region between the first and second primer hybridization nucleic acid sequences (B1 and B2) in microorganism B.

The positive process control microorganism can be added to a test sample before, upon or shortly after collection of the test sample. Therefore the positive process control microorganism will serve as a control for the entire processing operations of the test sample, including storage, transport, lysis, extraction and amplification.

Said positive process control microorganism preferably is present in a receptacle to which a test sample is added. Said receptacle is, for example, a sample collection tube, including a blood tube such as a heparin tube and an EDTA tube. Said positive process control microorganism is preferably present in dried, preferably lyophilized, form in said receptacle.

As an alternative, said positive process control microorganism is present in an aqueous solution in said receptacle. Said aqueous solution preferably comprises a stabilizer, for example glycerol and/or dimethylsulphoxid.

For this, a positive process control microorganism, preferably a non-pathogenic microorganism or a killed, intact microorganism comprising the positive control nucleic acid molecule is added to a receptacle. The positive process control microorganism preferably is added to a receptacle in

an aqueous solution. The volume of the solution, preferably aqueous solution, is preferably less than 10 milliliter, preferably less than 1 milliliter, preferably less than 100 microliter (microl), preferably less than 50 microl, preferably less than 10 microl, preferably less than 5 microl, such as 1 microl, 2 microl, 3 microl, or 4 microl. It is preferred that the receptacle, after addition of the positive process control microorganism, is incubated such as to allow evaporation of the solution.

The invention therefore provides a receptacle, comprising a positive process control microorganism according to the invention. Said receptacle preferably comprises a positive process control microorganism, preferably a dried positive process control microorganism. The term receptacle, as is used herein, refers to a container that holds, or can hold, a test sample. Said receptacle preferably is a sample collection tube, such as a blood collection tube, including a heparin tube and an EDTA tube.

A preferred receptacle is a tube, preferably a sample collection tube, with a closure that is evacuated to create a vacuum inside the tube, facilitating the draw of a predetermined volume of liquid.

A further preferred receptacle comprising a positive process control microorganism according to the invention is a centrifuge tube, including a microcentrifuge tube, a test tube, or a multiwell plate, such as a 6 well plate, a 12 well plate, a 24 well plate, a 48 well plate, a 96 well plate, a 184 well plate, a 384 well plate, or a 1536 well plate.

Said positive process control microorganism may be present in at least one of the wells of a multiwell plate. It is preferred that all wells of a multiwell plate comprise the positive process control microorganism.

Said receptacle may be made of any material. Preferred material is glass, including soda-lime or borosilicate glass, and plastic such as polyolefins (including polyethylene and polypropylene), polyethylene terephthalate (PET), potassium oxalate/sodium fluoride, polyacrylic,

polytetrafluoroethylene, polysiloxane, polyvinyl chloride, polyacrylonnitrile, and polystyrene.

The length of the amplified part (e.g. internal nucleic acid sequence plus primer hybridization sequences) of the control nucleic acid that is present in the positive process control microorganism preferably is larger than the length of the amplified part of the target nucleic acid or target nucleic acids. A greater length of the amplified region of the control nucleic acid will result in an optimal amplification of the target nucleic acid from the first microorganism, or from microorganisms A and B, as amplification in general is biased towards smaller fragments. Amplification of a larger fragment of the internal control will not significantly affect amplification of smaller fragments from the target nucleic acid.

The positive process control microorganism can be added to a test sample from a subject that is suffering from, or suspected to be suffering from, an infection with a microorganism. Said sample is or comprises stool, a bodily fluid or a tissue sample such as, for example, a biopsy sample. However, said positive process control microorganism can be added to any food, environmental, agricultural, biopharmaceutical, pharmaceutical, clinical or water sample in which a specific microorganism, especially a pathogenic microorganism, is to be detected.

The microorganisms to be detected can be present in a food sample, such as meat, fish, fruit, vegetable, beer, wine, eggs, or milk, including processed forms of any of these food samples. Furthermore, a microorganism to be detected may be present in a pharmaceutical product, personal care product, dairy product or in samples of plant, animal, human or environmental origin, including clinical samples and clinical environments.

Early detection of a specific microorganism, especially of a pathogenic microorganism, and timely therapeutic intervention are crucial for improved outcome of patients with infections such as sepsis. The early detection of specific pathogenic microorganisms will allow the rapid

identification of patients with infections, and will allow the early start of a specific therapy that is targeted to the identified microorganism. Similarly, early detection of a specific microorganism in a pharmaceutical product, a personal care product, a dairy product or in samples of plant, animal, human or environmental origin, including drinking water, will allow timely cleaning and disinfection to eliminate the specific microorganism from the product or sample.

The test sample includes, but is not limited to, whole blood, a tissue biopsy, lymph, bone marrow, amniotic fluid, hair, skin, urine, sputum, semen, anal secretions, vaginal secretions, perspiration, saliva, buccal swabs, various environmental samples (for example, agricultural, water, and soil), a food sample, a clinical sample, and a pharmaceutical sample. Methods to retrieve a test sample from an individual or from any other source are known in the art.

Said positive process control microorganism is added to a test sample prior to the preparation of a nucleic acid extract from the test sample, preferably directly after the retrieval of a test sample from an individual or from any other source. In this way, the positive process control microorganism will serve as a control for the entire processing operations of the test sample, including storage, transport, lysis, extraction and amplification of nucleic acids. As an alternative, said positive process control microorganism is present in a receptacle to which a test sample is added.

Nucleic acids may be extracted by using generally known techniques for isolation of nucleic acids. Methods and compositions for isolation of genomic DNA from a test sample, for example blood, preferably employ aqueous solvents without use of organic solvents and chaotropic salts. The isolated genomic DNA is preferably free of polymerase inhibitors. However, the presence of any inhibiting substance will be indicated by the IAC.

The test sample such as, for example, a blood sample, is preferably pre-treated to induce selective lysis of mammalian cells and degradation of non-target DNA, which results in increased sensitivity and specificity of the subsequent analysis of microorganisms. Kits for the removal of human cells and the degradation of non-target human DNA are known in the art. Very suitable systems have been described, such as the Polaris system (WO2011/070507 and WO2012/168003) and/or are commercially available, such as the MolYsis pretreatment kit (Molzym GmbH & Co. KG, Bremen, Germany). These methods allow larger sample volumes to be processed. This will result in increased sensitivity, rendering the molecular detection of microorganisms applicable. The Polaris method is preferred for whole blood samples as it is more reproducible, less labour intensive, and faster compared to the MolYsis method.

It was surprisingly found that a positive process control microorganism that is added to a receptacle and is stored in said receptacle for several weeks at room temperature, still is intact after addition of a test sample to said receptacle, allowing the isolation of genomic DNA from said positive process control microorganism using, for example, the Polaris method.

Total genomic DNA may be purified from test samples using, for instance, a combination of physical and chemical methods. Very suitably commercially available systems for DNA isolation are used, such as the QIAamp blood mini kit columns (Qiagen, Venlo, The Netherlands), the NucliSENS® easyMAG® nucleic acid extraction system (bioMérieux, Marcy l'Etoile, France) or the MagNA Pure 96 System (Roche Diagnostics, Almere, The Netherlands).

As an alternative, ribonucleic acid (RNA) may be isolated from a test sample using, for example, the Ambion Ribopure™ kit or the Qiagen RNeasy kit. The RNA extract can be freshly prepared from the test sample at the moment of preparing the test sample, or it can be prepared from test

samples that are stored at -70°C until processed. Alternatively, tissues or biopsies can be stored under conditions that preserve the quality of the protein or RNA. Examples of these preservative conditions are fixation using e.g. formaline and paraffin embedding, RNase inhibitors such as RNAsin® (Pharmingen) or RNasecure® (Ambion), aquous solutions such as RNAlater® (Assuragen; US06204375), Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect (HOPE; DE10021390), and RCL2 (Alphelys; WO04083369), and non-aquous solutions such as Universal Molecular Fixative (Sakura Finetek USA Inc.; US7138226).

RNA is preferably converted into complementary DNA (cDNA) prior to amplification, using a RNA-dependent DNA polymerase or reverse transcriptase. Methods for the conversion of RNA into cDNA are known in the art and include recombinant M-MuLV reverse transcriptase or AMV reverse transcriptase. Suitable commercially available systems for cDNA synthesis include commercially available systems for DNA isolation are used, such as the qScript™ cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD) and the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA). It is preferred that random primers, for example hexamers or nonamers, or gene-specific primers are used for cDNA synthesis.

Because the positive process control microorganism is added to a test sample prior to the preparation of a nucleic acid extract from the test sample, the extracted nucleic acid will contain nucleic acid from the positive process control microorganism.

5.3 Amplification reaction

Different amplification methods, known to a skilled artisan, can be employed for amplification, including but not limited to Polymerase Chain Reaction (PCR), rolling circle amplification, nucleic acid sequence-based amplification, transcription mediated amplification, and linear RNA

amplification. A preferred amplification method is PCR, including end-point PCR and real-time PCR.

PCR is a technology that relies on thermal cycling, consisting of cycles of repeated heating and cooling of a reaction for DNA melting and enzymatic replication of the DNA. Primers containing sequences that specifically hybridize to the target region, and a DNA polymerase are key components to enable selective and repeated amplification. As PCR progresses, the amplified DNA product that is generated is itself used as a template for replication, resulting in a chain reaction in which the DNA template is exponentially amplified.

A preferred DNA polymerase is a thermo stable polymerase, preferably a thermo stable recombinant polymerase. Preferred commercially available DNA polymerases include AptaTaq Fast DNA Polymerase and LightCycler® FastStart Enzyme (Roche Diagnostics, Almere, The Netherlands).

End-point PCR measures presence or absence of an accumulated PCR product at the end of the PCR process. An amplified product of a specific size is monitored, for example by electrophoresis using a polyacrylamide or agarose gel. An approximate quantification of amplified product may be obtained, for example by ethidium bromide staining of the agarose gel.

Real-time PCR, also called quantitative PCR (qPCR), is a technique which is used to amplify and simultaneously quantify a template DNA molecule. The detection of the amplification products can in principle be accomplished by any suitable method known in the art. The amplified products may be directly stained or labelled with radioactive labels, antibodies, luminescent dyes, fluorescent dyes, or enzyme reagents. Direct DNA stains include for example intercalating dyes such as acridine orange, ethidium bromide, ethidium monoazide or Hoechst dyes.

Alternatively, the amplified product may be detected by incorporation of labelled dNTP bases into the synthesized DNA fragments. Detection labels which may be associated with nucleotide bases include, for example, fluorescein, cyanine dye and BrdUrd.

Methods for detection of an amplified fragment are known to a person skilled in the art and include the use of gel electrophoresis for determining presence and length of an amplified fragment. Said detection preferably is performed during or following the amplification, more preferably without any further handling of the amplification reaction mixture. Said method for detection preferably comprises labeling of the amplified fragments during the amplification reaction. Said label preferably is a fluorescent label.

When using for example Scorpion primers or a probe-based detection system, a primer or the probe is preferably labelled with a detectable label, preferably a fluorescent label. Preferred labels for use in this invention comprise fluorescent labels, preferably selected from Atto425 (ATTO-TEC GmbH, Siegen, Germany), Atto 647N (ATTO-TEC GmbH, Siegen, Germany), YakimaYellow (Epoch Biosciences Inc, Bothell, WA, USA), Cal610 (BioSearch Technologies, Petaluma, CA, USA), Cal635 (BioSearch Technologies, Petaluma, CA, USA), FAM (Thermo Fisher Scientific Inc., Waltham, MA USA), TET (Thermo Fisher Scientific Inc., Waltham, MA USA), HEX ((Thermo Fisher Scientific Inc., Waltham, MA USA), cyanine dyes such as Cy5, Cy5.5, Cy3, Cy3.5, Cy7 (Thermo Fisher Scientific Inc., Waltham, MA USA), Alexa dyes (Thermo Fisher Scientific Inc., Waltham, MA USA), Tamra (Thermo Fisher Scientific Inc., Waltham, MA USA), ROX (Thermo Fisher Scientific Inc., Waltham, MA USA), JOE (Thermo Fisher Scientific Inc., Waltham, MA USA), fluorescein isothiocyanate (FITC, Thermo Fisher Scientific Inc., Waltham, MA USA), and tetramethylrhodamine (TRITC, Thermo Fisher Scientific Inc.,

Waltham, MA USA). A probe is preferably labeled at the 5' end with a detectable label, preferably a fluorescent label.

A primer such as a Scorpion primer, or a probe preferably has a fluorescent label at one end and a quencher of fluorescence at the opposite end of the probe. The close proximity of the reporter to the quencher prevents detection of its fluorescence; breakdown of the probe by the 5' to 3' exonuclease activity of polymerase breaks the reporter-quencher proximity and thus allows unquenched emission of fluorescence, which can be detected after excitation with a laser. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence due to the breakdown of the probe and release of the reporter. Quenchers, for example tetramethylrhodamine TAMRA, dihydrocyclopyrroloindole tripeptide minor groove binder, are known in the art.

Preferred quenchers are Black Hole Quencher®-1 (BHQ1) and BHQ2, which are available from Biosearch Technologies, Petaluma, CA, USA). The BHQ1 dark quencher has strong absorption from 480 nm to 580 nm, which provides excellent quenching of fluorophores that fluoresce in this range, such as FAM, TET, CAL Fluor® Gold 540, JOE, HEX, CAL Fluor Orange 560, and Quasar® 570 dyes. The BHQ2 dark quencher has strong absorption from 599 nm to 670 nm, which provides excellent quenching of fluorophores that fluoresce in this range, such as Quasar® 570, TAMRA, CAL Fluor® Red 590, CAL Fluor Red 610, ROX, CAL Fluor Red 635, Pulsar® 650, Quasar 670 and Quasar 705 dyes. BHQ1 and BHQ2 may quench fluorescence by both FRET and static quenching mechanisms.

The term "specifically hybridizing" refers to a primer and/or a probe that is capable of hybridizing specifically under stringent hybridization conditions to a target nucleic acid template that is obtained or derived from at least the positive process control microorganism. Detection of the positive process control microorganism indicates that the processing

operations of the test sample, including storage, transport, lysis, extraction and amplification of nucleic acids, did not result in degradation of nucleic acid and/or the presence of amplification inhibitors. Degradation of nucleic acid and/or the presence of amplification inhibitors would have resulted in an indication that the first microorganism and second, positive process control microorganism were absent in the test sample.

The terms "stringency" and "stringent hybridization" refer to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The terms as used include reference to conditions under which a probe or primer will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g. at least 2-fold over background). Stringent conditions may be sequence dependent and will be different in different circumstances. Longer sequences hybridise specifically at higher temperatures. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridises to a perfectly matched probe or primer. Hybridization procedures are well known in the art and are described by e.g. Ausubel et al., 1998. Current Protocols in Molecular Biology, John Wiley, New York; and Sambrook et al., 2001. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

An oligonucleotide primer or probe, or an oligonucleotide mimic primer or probe, is able to hybridize to a target nucleic acid template when the length of the molecule is or resembles at least 15 bases. The length of the primer or probe is preferably less than 100 bases. A preferred length of a

primer or probe is between 15 and 50 bases, preferably between 16 and 30 bases.

In general, a primer or probe is able to hybridize to a target nucleic acid template when the percentage of sequence identity of the molecule is at least 90% over substantially the whole length, more preferred at least 91%, more preferred at least 92%, more preferred at least 93%, more preferred at least 94%, more preferred at least 95%, more preferred at least 96%, more preferred at least 97%, more preferred at least 98%, more preferred at least 99%, more preferred 100% identical to a nucleic acid that is obtained or derived from said target nucleic acid template over substantially the whole length of the primer or probe. The term "substantially the whole length" is used to indicate that the probe may comprise additional nucleotide sequences, for example at the 5' and/or 3' ends that are not present in the gene or region described herein above.

Efficient real-time PCR reactions are dependent upon high quality primer and probe design. Rules of thumbs for the design of primers include the selection of primers having a Tm between 58°C and 65°C while keeping the annealing temperatures of the primers as close as possible, having no more than two G's or C's in the last 5 bases at the 3' end, and the selection of primer pairs with minimal number of potential primer dimers and primer hairpins.

Rules of thumbs for the design of probes include the selection of probes that have a Tm between 68°C and 72°C, have no Gs on the 5' end, resemble a strand that has more C than G bases, and are as short as possible, without being shorter than 13 nucleotides.

Methods for the design of primers and probes are known in the art. For example, Premier Biosoft (Palo Alto, CA, USA) offers AlleleID® and Beacon Designer™ to design probes for real-time PCR assays that are free of dimers, repeats and runs and ensure signal fidelity. In addition, Primer3 (http://primer3.sourceforge.net) and Integrated DNA Technologies, Inc.

(www.idtdna.com/Scitools/Applications/Primerquest) provide online tools for the design of primers and probes for real-time PCR assays. Hence, the skilled person is able to design primers and probes for real-time PCR analyses of one or more target nucleic acid templates.

The primers and probes are preferably tested in single nucleic acid amplification reactions (monoplex) and combined nucleic acid amplification reactions (multiplex) to determine optimal combinations of specific nucleic acid amplification reactions.

The first and second primers act together to amplify an internal nucleic acid sequence of the second, positive process control microorganism and, if present, an internal nucleic acid sequence from the first microorganism.

Said first and second primers are preferably provided in a reaction chamber. Said reaction chamber preferably further comprises a buffer, dNTPs, and/or DNA polymerase, preferably in a lyophilized form.

The methods of the invention comprise contacting a test sample with a second, positive process control microorganism, comprising a control nucleic acid comprising first and second primer hybridization nucleic acid sequences from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not present between the first and second hybridization nucleic acid sequences in a first microorganism, amplifying at least the control nucleic acid and, if present, the target (internal) nucleic acid from the first microorganism, using first and second primers, providing a probe A that specifically hybridizes to the internal nucleic acid sequence from the first microorganism and a probe B that specifically hybridizes for the internal nucleic acid sequence of the second, positive process control microorganism, and detecting presence of a hybridization product comprising probe B and the amplified internal nucleic acid sequence of the second, positive process control microorganism and, if present, a hybridization product comprising

probe A and the amplified internal nucleic acid sequence of the first microorganism.

The methods of the invention also comprise contacting a test sample with a second, positive process control microorganism, comprising a control nucleic acid comprising first (A1) and second (B2) primer hybridization nucleic acid sequences, whereby said first primer hybridization nucleic acid sequence A1 is from microorganism A and said second primer hybridization nucleic acid sequence B2 is from microorganism B, wherein A1 and B2 in the control microorganism flank an internal nucleic acid sequence that is not present in microorganisms A and B, amplifying at least the control nucleic acid and, if present, the target (internal) nucleic acid from the microorganisms A and/or B, using primers A1, A2, B1 and B2, providing a probe A that specifically hybridizes to the internal nucleic acid sequence from microorganism A, a probe B that specifically hybridizes to the internal nucleic acid sequence of microorganism B, and a probe C that specifically hybridizes to the internal nucleic acid sequence of the positive process control microorganism, and detecting presence of a hybridization product comprising probe C and the amplified internal nucleic acid sequence of the second, positive process control microorganism and, if present, a hybridization product comprising probe A and the amplified internal nucleic acid sequence of microorganism A and/or a hybridization product comprising probe B and the amplified internal nucleic acid sequence of microorganism B.

The nucleic acid sequence of primer A1 is preferably from the same DNA strand as the nucleic acid sequence of probe A, and the nucleic acid sequence of primer B2 is preferably from the same DNA strand as the nucleic acid sequence of probe B.

5.4 Positive control nucleic acid molecule

A positive control nucleic acid molecule according to the invention comprises first and second primer hybridization sequences derived from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not naturally present between the first and second hybridization sequences in the first microorganism. Said positive control nucleic acid molecule preferably further comprises outside bordering nucleic acid sequences that allow integration of the positive control nucleic acid molecule into a plasmid or into the genome of a second microorganism.

An internal nucleic acid sequence that is present between the first and second hybridization nucleic acid sequences in a first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the positive control nucleic acid molecule. Said internal nucleic acid sequence that is absent in the positive control nucleic acid molecule preferably is between 1 and 100 nucleotides, more preferred between 5 and 50 nucleotides.

Examples of first and second primers are provided in Table 1. The skilled person will undoubtedly be able to deduce suitable first and second primer hybridization sequences that are able to hybridize to first and second primer sequences.

The skilled person will understand that a positive control nucleic acid molecule according to the invention may comprise additional primer hybridization sequences derived from a third and/or a fourth microorganism, such as third and fourth primer hybridization sequences derived from a third microorganism that flank the first and second primer hybridization sequences (see Figure 1), and fifth and sixth primer hybridization sequences derived from a fourth microorganism, that flank the third and fourth primer hybridization sequences.

The chromosomal integration of a positive control nucleic acid molecule in the genome of a second microorganism enables the engineering

of plasmid-free stable strains with single-copy insertions. Homologous recombination is conserved across all three domains of life as well as viruses, suggesting that it is a nearly universal biological mechanism. Homologous recombination is also used in gene targeting, a technique for introducing genetic changes into target organisms.

Suitable outside bordering nucleic acid sequences that allow integration of the positive control nucleic acid molecule into the genome of a second microorganism are known in the art. For example, a set of vectors which facilitates the sequential integration of exogenous sequences into the *Escherichia coli* chromosome by homologous recombination has been described (Martinez-Morales et al., 1999. J Bact 181: 7143-7148). A further vector that can be used is the suicide vector pBSL182. (Alexeyev and Shokolenko, 1995. Gene 160, 59-62). Similarly, integration vectors for Gram-positive microorganisms have been described, for example in Härtl et al., 2001. J Bact 183: 2696-2700), and a method of double crossover homologous recombination in Clostridia (WO2010084349) and Mycobacterium (Kendall and Frita, 2009. Methods Mol Biol. 465: 297-310) have been described.

In addition, homologous recombination in microorganisms such as yeast is well known. Homologous recombination will occur with as little as 30 bp of homology, but is more efficient with larger regions of homology (Sugawara et al., 2000. Mol Cell Biol 20: 5300-5309). Homologous recombination in *Aspergillus fumigatus* is known to be increased in certain mutants of this fungus (Krappmann et al., 2006. Eukaryot Cell 5: 212–215).

As an alternative, or in addition, a site specific recombinase such as, for example, Cre recombinase (minimal recognition sequence termed LoxP site 5'- ATAACTTCGTATANNNTANNNTATACGAAGTTAT) or the FLP recombinase (minimal recognition sequence termed FRT sequence 5'-GAAGTTCCTATTCtctagaaaGtATAGGAACTTC3') or a homing endonucleases such as, for example, PI-PspI (New England Biolabs;

recognition sequence 5'-TGGCAAACAGCTATTATGGGTATTATGGGT)) or PI-SceI (New England Biolabs; recognition sequence 5'-ATCTATGTCGGGTGCGGAGAAAGAGGTAAT) may be employed to - manipulate the genomic DNA of a microorganism to introduce a positive control nucleic acid molecule in the genome of a second microorganism.

Any of these methods and means can be used as outside bordering nucleic acid sequences by the skilled person to allow integration of the positive control nucleic acid molecule into the genome of a second microorganism.

Table 1. Selection of first and second primer sequences

	Species	PCR target	First primer 5′– 3′	Second primer 5′– 3′
1	*Escherichia coli*	16S rDNA	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA
		gadA + gadB	GGCTTCGAAATGGACTTTGCT	TGGGCAATACCCTGCAGTTT
2	*Pseudomonas aeruginosa*	phzE gene	GCCGAGGTCATGGAATTC	ATCCGCGCCATCATCTTC
		phzE gene	GCCGAGGTCATGGAATTC	ATCCGCGCCATCATCTTC
3	*Klebsiella* species	rhaA gene	AACCAGGCGTCGATAAT	GTTTACGGCGCAATCC
4	*Serratia marcescens*	gyrB gene	GACCGTGAAGACCACTTCCATTAC	ACGCCGATGTCGTCTTTCAC
5	*Staphylococcus* species	tuf gene	CCAACTCCAGAACGTGATTCTG	GTTGTCACCAGCTTCAGCGTAGT
			CCAACTCCAGAACGTGACTCTG	GTTATCACCAGCTTCAGCGTAAT
			CCAACACCAGAACGTGATTCTG–	GTTGTCACCAGCTTCAGCATAGT
6	*Staphylococcus aureus*	SA442	CATCGGAAACATTGTGTTCTGTATG	TTTGGCTGGAAAATATAACTCTCGTA
		hsdM gene	AAGGCGGAGGAATCACATGTC	TTCGCAATCGACCATAATTTTTT
7	*Enterococcus faecalis*	16S rDNA	CGCTTCTTTCCTCCCGAGT	GCCATGCGGCATAAACTG
		ncRNA	ATGCGTCTCGTCACAGTA	GGTACGATGATTTCATCTGT
8	*Streptococcus agalactiae*	cfb gene	TTCACCAGCTGTATTAGAAGTACATGC	CCCTGAACATTATCTTTGATATTTCTCA
9	*Acinetobacter baumannii*	23S rDNA	CGCTGTTGTTGGTGATGGAACT	AACAGTTGCAGCGGCCTG
10	*Enterococcus faecium*	hyp. protein	GCCAAAGGACCGCTTATTACG	GCTTTTCGCTGTTTTTTTAATGACT

		encoding ORF		
11	*Streptococcus pneumoniae*	comX gene	GGTCTCTGGCTAG ATGATTATTATCT CTT	ATAGTAAACTCCTTA AACACAATGCGTAA
12	*Staphylococcus* species	tuf gene	CCAACTCCAGAAC GTGATTCTG	GTTGTCACCAGCTTC AGCGTAGT
			CCAACTCCAGAAC GTGACTCTG	GTTATCACCAGCTTC AGCGTAAT
			CCAACACCAGAAC GTGATTCTG-	GTTGTCACCAGCTTC AGCATAGT
13	*Enterococcus* genus	23S rDNA	TGCGGGGATGAGG TGTG	CAAACAGTGCTCTAC CTCCATCAT
14	*Candida albicans*	ITS region	CATGCCTGTTTGA GCGTCRTTT	ATATGCTTAAGTTCA GCGGGT
15	*Candida glabrata*	ITS region	CATGCCTGTTTGA GCGTCRTTT	ATATGCTTAAGTTCA GCGGGT
16	*Candida krusei*	ITS region	CATGCCTGTTTGA GCGTCRTTT	ATATGCTTAAGTTCA GCGGGT
17	Pan-*Aspergillus*	ITS region	GCGTCATTGCTGC CCTCAAGC	ATATGCTTAAGTTCA GCGGGT
18	Gram-positive	16S rDNA	TGGAGCATGTGGT TTAATTCGA	TGCGGGACTTAACCC AACA
19	Gram -negative	16S rDNA	TGGAGCATGTGGT TTAATTCGA	TGCGGGACTTAACCC AACA
20	pan-*Candida*	ITS region	CATGCCTGTTTGA GCGTCRTTT	ATATGCTTAAGTTCA GCGGGT
21	*Tropheryma whipplei*	Repeat sequence	CCGCTAATTGAGA GATGCGATTCACA GATAAGAAGGCAG ATCCATCATC	CCAGACAGGTTGTGC CACAAGAGCTATGAG ATTGGTTGTTGTCTT TG

5.5 Positive process control microorganism

A recombinant, positive process control microorganism according to the invention, also termed second positive process control microorganism according to the invention, comprises a positive control nucleic acid molecule comprising first and second primer hybridization sequences derived from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not naturally present between the first and second hybridization sequences in the first microorganism, and wherein an internal nucleic acid sequence that is present between the first and second hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control microorganism.

It will be understood by a person skilled in the art that also the first and second primer hybridization sequences are not naturally present in a second positive process control microorganism, prior to the provision of a positive control nucleic acid molecule to a second microorganism.

Said second positive process control microorganism preferably is an intact microorganism, preferably a non-pathogenic microorganism or an attenuated microorganism. Said second positive process control microorganism preferably is killed prior contacting a test sample with said positive process control microorganism. The term “intact” indicates that the positive process control microorganism may be killed prior to adding the positive process control microorganism to the sample, but that lysis is required for isolation of the positive control nucleic acid molecule, in contrast to currently used controls like viruses and isolated plasmids.

Said positive control nucleic acid molecule may be present on an extra-chromosomal nucleic acid molecule, for example on a plasmid that comprises an appropriate origin of replication for the second microorganism.

The positive control nucleic acid molecule is preferably integrated, preferably as a single copy or as a defined number of copies, for example 2 or

more copies, 3 or more copies, 4 or more copies, 5 or more copies, 6 or more copies, 7 or more copies, 10 or more copies, into the genome of a second microorganism. For this, any of the methods and means indicated hereinabove can be used for the stable integration of a positive control nucleic acid molecule into the genome of a second microorganism, preferably of a single copy of the positive control nucleic acid molecule.

In the case that the first microorganism that is to be detected is a bacterium, the positive process control microorganism also is a bacterium. The positive process control microorganism preferably is of the same Gram staining as the first microorganism. It is preferred that the positive process control microorganism and the first microorganism both stain either positive or negative in Gram-staining. For example, it is preferred that the positive process control microorganism is a Gram+ staining microorganism if the first microorganism is Gram+, and the positive process control microorganism is a Gram$^-$ staining microorganism if the first microorganism is Gram$^-$.

In the case that the first microorganism that is to be detected is a fungus, the positive process control microorganism preferably is a fungus. For example, if the first microorganism that is to be detected is an *Aspergillus* spp., or *Candida* spp., the positive process control microorganism preferably is an *Aspergillus* or *Candida* species. Similarly, if the first microorganism that is to be detected is a *Cryptococcus* spp., *Coccidioides* spp., *Corynebacterium* spp., *Histoplasma* spp. or *Pneumocystis* spp., the positive process control microorganism preferably is a *Cryptococcus* spp., *Coccidioides* spp., *Corynebacterium* spp., *Histoplasma* spp. or *Pneumocystis* spp., respectively.

In the case that the first microorganism that is to be detected is a virus, the positive process control microorganism preferably is a virus. For example, if the first microorganism that is to be detected is a hemorrhagic fever viruses such as ebola virus and Marburg virus, a hepatitis virus, a

coronavirus, a herpesvirus, a West Nile virus, a human immunodeficiency virus, or a Japanese encephalitis virus, said positive process control microorganism preferably is an attenuated or inactivated hemorrhagic fever virus, hepatitis virus, coronavirus, herpesvirus, West Nile virus, human immunodeficiency virus, or Japanese encephalitis virus.

The positive process control microorganism preferably is a microorganism that requires stringent conditions for lysis, for example a spore-forming bacterium such as *Bacillus* spp., for example *Bacillus subtilis*.

A positive control nucleic acid molecule is preferably present as a single copy or as a defined number of copies, for example 2 or more copies, 3 or more copies, 4 or more copies, 5 or more copies, 6 or more copies, 7 or more copies, 10 or more copies, in the genome of a positive process control microorganism.

The invention in addition provides the use of a positive process control microorganism according to the invention as control for the entire processing operations of the sample, including storage, transport, lysis, extraction and amplification. Because the copy number, i.e. the number of integrated copies into the genome of the second positive process control microorganism is known, said use provides a quantitative control for the lower detection limit of the first microorganism.

For the purpose of clarity and a concise description, features are described herein as part of the same or separate aspects and preferred embodiments thereof, however, it will be appreciated that the scope of the invention may include embodiments having combinations of all or some of the features described.

The invention will now be illustrated by the following examples, which are provided by way of illustration and not of limitation and it will be understood that many variations in the methods described and the amounts indicated can be made without departing from the spirit of the invention and the scope of the appended claims.

6. EXAMPLES

Example 1

Materials and methods

Validation of *Tropheryma whipplei* real-time PCR

Primers and probes for real-time PCR

For optimal sensitivity and specificity, a *T. whipplei*-specific repetitive sequence, seven times present in the genomes of two completely sequenced strains, was chosen as target (Bentley et al., 2003. Lancet 361: 637-644; Raoult et al., 2003, Genome Res. 13: 1800-1809; Fenollar et al., 2004. J. Clin. Microbiol. 40: 1119-1120). A consensus sequence of these 14 sequences was used to design primers and a TaqMan probe with the Primer Express 2.0 software (Life Technologies Europe BV, Bleiswijk, The Netherlands). The BLAST software (http://blast.ncbi.nlm.nih.gov) was used to confirm the specificity of the obtained primers (Fwhi2 and Rwhi2) and probe (Pwhi2, Table 1). An internal control constructed to monitor DNA isolation and amplification contains an artificial DNA sequence with a probe sequence (PICcpctmp, Table 1) elongated with the Fwhi2 and Rwhi2 primer sequences. The primers and the FAM-labelled (Pwhi2) and VIC-labelled (PICcpctmp) TaqMan probes were synthesised by Biolegio BV (Nijmegen, The Netherlands).

Construction of a positive control for real-time PCR

A specimen from a patient with diagnosed WD, ascertained with a T. whipplei-specific16S rDNA PCR was used to amplify the 119 bp target with the primers Fwhi2 and Rwhi2. The PCR was performed in a 50 µl reaction mix containing 1 unit of AmpliTaq DNA polymerase (Life Technologies Europe BV, Bleiswijk, The Netherlands), dNTPs (200 µM of each), 3 mMMgCl2, PCR buffer II (Life Technologies Europe BV, Bleiswijk,

The Netherlands), 300 nM of each primer and 10 µl of template. Amplification conditions were 5 min at 95°C, followed by 45 cycles of 30 s at 95°C, 1 min at 59°C, and 1 min at 72°C. After the last cycle, the mix was left for 5 min at 72 °C. The PCR product was separated with electrophoresis on a 1% agarose gel, purified with the QIAquick gel extraction kit (Qiagen Benelux BV, Venlo, The Netherlands), and cloned into the pGEM-T Easy plasmid with the pGEM-T Easy Vector System 1 kit (Promega Benelux BV, Leiden, The Netherlands). Ligation was performed at 16 °C overnight. The ligation mixture was used to transform *E. coli* strain DH5α, selecting for ampicillin resistant bacteria. Bacterial colonies were screened with real-time PCR (see conditions below) to select for a plasmid with the correct insertion. The resulting plasmid, pWhi2,was isolated with the Qiagen plasmid mini kit and sequenced with the pUC/M13 Forward and reverse sequencing primers provided by the manufacturer (Promega Benelux BV, Leiden, The Netherlands), to confirm the presence of the whole real-time PCR target. Sequencing reactions were performed using the BigDye Terminator cycle sequencing kit (Life Technologies Europe BV, Bleiswijk, The Netherlands). The bands were separated on an ABI Prism 3100 Genetic Analyzer (Life Technologies Europe BV, Bleiswijk, The Netherlands).

Construction of an internal control for real-time PCR

To monitor DNA isolation and amplification, an internal control (IC) was constructed consisting of a recombinant *E. coli*-strain containing the primer sequences of the *T. whipplei* real-time PCR with an artificial probe sequence. These sequences were inserted in the chromosome of *E. coli*. To facilitate preferential amplification of the *T. whipplei* target before the IC target, the sequence on the IC between the primers was made longer than the sequence at the *T. whipplei*-chromosome. Four primers were used to amplify fragments of pWhi2 in three PCRs. Two fragments were amplified with the primer pairs ICWhi2left/Rextpgem, and ICWhi2right

/Fextpgem, respectively (Table 1, Fig. 1). The PCR was performed in a 50 µl reaction mix containing 1 unit of AmpliTaq DNA polymerase (Life Technologies Europe BV, Bleiswijk, The Netherlands), dNTPs (200 µM of each), 3 mM MgCl2, PCR buffer II (Life Technologies Europe BV, Bleiswijk, The Netherlands), 300 nM of each primer and 10 µl of template. Amplification conditions were 5 min at 95°C, followed by 40 cycles of 30 s at 95°C, 60 s at 60°C, and 60 s at 72°C. The resulting PCR products, which partially overlap, were purified as above, mixed together, denatured for 3 min at 95°C and thereafter, full-length products were synthesized by lowering the temperature to 65 °C for 60 s and 72 °C for 3 min in the presence of 1 U AmpliTaq DNA polymerase, 3 mM MgCl2 and PCR amplification with primers FWhi2para and Rwhi2para was followed. Cycling conditions was as above for the two fragments, but 30 cycles were performed. The resulting fragment containing the IC sequences, was purified and cloned into pGEM-t Easy in *E. coli* as described above. Bacterial colonies were screened with real-time PCR (see conditions beneath) to select for a plasmid with the right insertion. The resulting plasmid, pICWhi2, was isolated with the Qiagen plasmid mini kit and sequenced with the pUC/M13 Forward and Reverse sequencing primers (Promega Benelux BV, Leiden, The Netherlands) to confirm the presence of the correct fragment. For insertion in the chromosome of *E. coli*, the insert was subcloned in the suicide vector pBSL182 (Alexeyev and Shokolenko, 1995. Gene 160: 59-62). Digestion of pICWhi2 was performed with NotI and SpeI. The resulting fragments were separated on an agarose gel, purified as described above and the insert, containing the IC target, was ligated into pBSL182, which had been digested with NotI and SpeI. The resulting ligation mix was used to transform *E. coli* DH5α, selecting for gentamycine-resistant colonies containing the IC sequence inserted in the chromosome. Presence of the right insert in strain ICwhi2 was confirmed with the real-time PCR mentioned above.

Table 1 Primers and probe sequences

Oligonucleotide	Sequence 5'-3'
Fwhi2	CACAGATAAGAAAGCAGATCCATCATC
Rwhi2	GAGCTATGAGATTGGTTGTTGTCTTTG
Pwhi2	FAM-AACCTTCCCTCATACAGACATAGCAACCCCA-TAMRA
PICcpctmp	VIC-AATGCACATCCGCCAAATCTTTCGCCAGA-TAMRA
ICWhi2left	TCTGGCGAAAGATTTGGCGGATGTGCATTGAGGCATAGGAAAG ATGATGGATCTGCTTTCT
Fextpgem	TAGGGCGCTGGCAAGTGTAG
ICWhi2right	TCCGCCAAATCTTTCGCCAGAATGCCTCTACTACGGAGATGAGA AAGTARTCTCCATAAC
Rextpgem	CCGATTCATTAATGCAGCTGG
FWhi2para	CCGCTAATTGAGAGATGCGATTCACAGATAAGAAGGCAGATCC ATCATC
RWhi2para	CCAGACAGGTTGTGCCACAAGAGCTATGAGATTGGTTGTTGTCT TTG
F16Swhi	GAAAGGCGTAGAGATACGCCC
R16Swhi	CGTGAAGCCCAAGACCGA
P16Swhi	FAM-CCTGTGTTGCCAGCGCGTAATGG-TAMRA

Nucleic acid extraction

200 µl of whole blood, plasma or CSF specimens was used to extract DNA.200 µl of phosphate buffered saline (PBS) was added to the biopsies. The specimens were all spiked with 30 CFU of internal control ICWhi2 and incubated with 6 units of proteinase K for 1h at 56°C.DNA extraction was performed using the NucliSensEasyMAG platform with the specific A stool protocol, as described by the manufacturer (BioMérieux, Benelux BV, Zaltbommel, The Netherlands). Purified nucleic acids were eluted in 110 µl of elution buffer and stored at -20 °C until further analysis.

T. whipplei real-time PCR

A 119 bp fragment of T. whipplei was amplified with the real-time primer and probe set described above. The internal control was amplified with the same primer set, but detected with a different probe. A control PCR amplifying a part of the 16S rDNA of T. whipplei was performed on a patient sample with the primers F16Swhi en R16Swhi and the probe P16Swhi. All primers and probes are described in Table 1. The PCR was performed in a 30 µl reaction mix containing TaqMan Universal PCR Master Mix (Life Technologies Europe BV, Bleiswijk, The Netherlands), 300 nM each of primers Fwhi2 and Rwhi2, 150 nM each of probes Pwhi2 and PICcpctmp, and 10 µl of DNA template. Cycling conditions were 2 min at 50°C, followed by 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C. Amplification, detection and data analysis was performed with an ABI 7500 real-time PCR and sequence detection system (Life Technologies Europe BV, Bleiswijk, The Netherlands).

Sensitivity and specificity of the test

The internal control strain ICwhi2 was cultured in Luria Bertani (LB) broth medium shaking at 37 °C until the culture reached the logarithmic phase (bacterial stock solution). Tenfold serial dilutions were plated on LB agar plates to determine the bacterial concentration in the original culture. Analytical sensitivity of the PCR was determined by using tenfold serial dilutions of DNA extracted from a bacterial solution of 107 CFU/ml.

Specificity for *T. whipplei* was verified using DNA extracted from cultures of the following bacterial species: *Bacteroides vulgatus* (ATCC 8482), *Bifidobacterium adolescentis* (clinical isolate), *Enterococcus faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Lactobacillus acidophilus* (ATCC 4356), *Propionibacterium acnes* (ATCC 6919), *Streptococcus milleri* (clinical isolate), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus*

epidermidis (ATCC 12228), and *Listeria monocytogenes* (clinical isolate). The bacteria were cultured overnight on blood agar plates. Bacterial DNA was extracted with the QIAamp DNA mini kit from colonies, following the instructions of the manufacturer (Qiagen Benelux BV, Venlo, The Netherlands).

The presence of *T. whipplei* in a clinical specimen was confirmed with real-time PCR detecting the 16S rRNA gene, followed by sequencing of the obtained fragment. A 223bp fragment was amplified with the primers F16Swhi and R16Swhi. Sequencing reactions were performed with the same primers. The obtained consensus sequence was compared with sequences in the GenBank using the BLASTn tool (http://www.ncbi.nlm.nih.gov/).

PART II. Prevalence of *T. whipplei* in gastrointestinal samples

Subjects

All individuals were recruited from the Outpatient Clinic of the Department of Gastroenterology and Hepatology of VU University medical centre, Amsterdam, the Netherlands, being an academic, third-line referral centre for gastrointestinal care.

Consecutive patients scheduled for routine gastroduodenoscopy or colonoscopy were asked to participate. Written informed consent was obtained prior to enrolment. Regular exclusion criteria for performing endoscopy were applied as exclusion criteria for these series, thus including high risk of perforation during endoscopy, recent myocardial infarction or uncontrolled haemorrhagic diathesis. In case of colonoscopy additional exclusion criteria were pregnancy and other bowel cleansing than with polyethylene glycol (Klean-Prep©, Norgine B.V., Amsterdam Z-O, The Netherlands).

Prior to the endoscopic procedure, a questionnaire was conducted including questions regarding height, weight, weight loss and altered bowel habits. These symptoms might be of importance in WD. Antibiotic and

probiotic use four weeks prior to endoscopy were documented because these variables could influence the prevalence of *T. whipplei*.

Bowel cleansing

In case of colonoscopy, individuals underwent bowel cleansing with 4 litres of polyethylene glycol (Klean-Prep©, Norgine bv, Amsterdam Z-O, The Netherlands) one day prior to the endoscopic procedure.

Sample collection

Colonoscopy

A full colonoscopy with or without intubation of the terminal ileum was performed. During withdrawal two mucosal biopsy specimens were harvested from the distal sigmoid colon, approximately 20 cm from the anal verge, with sterile reusable biopsy forceps (Cobra Medical BV, Groningen, The Netherlands). Specimens were transferred with a sterile needle in a test tube containing 500 µl phosphate buffered saline (PBS, pH 7) and firmly shaken for five seconds. The specimens were then transferred, with another sterile needle, in an empty test tube. Both test tubes were snap frozen in liquid nitrogen and subsequently stored at -20 °C until *T whipplei* analysis. In addition, two additional mucosal biopsy specimens were harvested from adjacent regions and subjected to regular histopathological examination.

Gastroduodenoscopy

In case of a gastroduodenoscopy, mucosal biopsy specimens were harvested from the second part of the duodenum (D2) for *T. whipplei* analysis, as described above, and histopathological examination.

The study was approved by the Medical Ethical Board of VU University Medical Centre, Amsterdam, The Netherlands.

Statistical analysis

Continuous variables were summarised with standard descriptive statistics including means, standard deviations, medians and ranges. Categorical variables were summarised with frequencies and percentages. Ninety-five percentage confidence intervals were provided for descriptive statistics. Comparisons between categorical variables were performed with $\chi 2$ test or with Fischer's exact test. P values less than 0.05 were considered statistically significant.

Results

PART I

Sensitivity and specificity of *T. whipplei* real-time PCR

To detect *T. whipplei* in clinical specimens, an internally controlled real-time PCR was set up, detecting a repeating sequence in the *T. whipplei* genome. The detection limit of the internal control was shown to be 1 CFU *E. coli* per PCR, corresponding to 1 copy of DNA (data not shown). No quantified strain of *T. whipplei* was available, but the detection limit of *T. whipplei* was assumed to be similar; 1 DNA copy per PCR, which theoretically corresponds to 0.14 CFU, presuming the target is being 7 times present on the chromosome.

Seventeen clinical specimens (whole blood, plasma, CSF and biopsies from skin, jejunum and duodenum) from patients with clinically certified WD, which had been shown to contain *T. whipplei* DNA (with 16SDNA PCR) in the past (data not shown), were retested. All samples were positive, indicating that the test detects known positive samples.

A BLAST search (NCBI) of the target sequence indicated no cross reactivity with any other bacterial species, indicative for high specificity. This was corroborated by the fact that ten PCRs on DNA from ten different bacterial isolates originating from gut and skin flora other than *T. whipplei* were negative.

Pilot study to investigate the specificity of the test

To investigate the clinical specificity of the novel test in patient specimens, a pilot study was performed with 21 colonic biopsy specimens. Indication for colonoscopy was different from suspicion of WD. Unexpectedly, three of the specimens were positive in the real-time PCR with Ct-values being 39.8, 34.5 and 18.5 respectively. The presence of *T. whipplei* in the first two positive specimens could not be confirmed with conventional 16S rDNA PCR, putatively due to low bacterial load, because, in contrast, the presence of *T. whipplei* in the highly positive sample was confirmed, followed by sequencing. A BLAST search revealed highest homology (100%) to the 16S rDNA gene of the completely sequenced *T. whipplei* strains Twist and Tw08/27. Analysis of stored mucosal biopsy specimens from colon and duodenum from the same patient, collected with an interval of 3 years, revealed that these biopsy specimens were PAS-positive.

PART II

Baseline characteristics

Patient characteristics are summarised in Table 2. In total, 243 individuals were included in this study. Mean age (±SD) was 55 ± 16 years old, of which 59% was female. Of the included individuals, 84 underwent a gastroduodenoscopy, 143 a colonoscopy, and 16 individuals underwent both procedures, successively on the same day. Indications for endoscopy are summarised in Table 2.

Presence of *T. whipplei* in intestinal samples

Colonic mucosa (Table 3+4)

Of the 159 colonic biopsy specimens tested, 6 samples (3.8%; 95% C.I. 0.81-6.74) showed the presence of *T. whipplei* DNA. One of these patients

underwent an additional gastroduodenoscopy of which mucosal biopsy specimens tested negative.

Matching washing fluids of the positive samples were tested, of which three were positive with higher, equal and lower Ct-values.

Antibiotic and probiotic use did not influence the prevalence of *T. whipplei*. (2-sided Fischer's exact test, resp. p=0.476; p=0.348)

Table 2 Baseline characteristics

Characteristics	**Total (n=243*)**	**GDS (n=100)**	**Colonoscopy (n=159)**
Male/female (%/%)	99/144 (40.7/59.3)	41/59 (41.0/59.0)	67/92 (42.1/57.9)
Age mean, (SD)	54.85 (15.973)	52.06 (16.778)	56.90 (14.918)
BMI median (range)	24.190 (15.6-49.4)	24.062 (16.2-37.4)	24.857 (15.6-49.4)
Antibiotic use <1mth (%)	10.3	12.0	10.1
Probiotic use <1mth (%)	18.1	14.0	20.1
Indication endoscopy **			
- dyspepsia/pyrosis	21.3	51.0	0.0
- coeliac disease-related	9.9	24.0	0.0
- IBD-related	6.6	4.0	8.9
- cancer screening	64.9	22.0	87.3
- intervention	2.5	0.0	3.8
- (suspicion of) Whipple's disease	0.8	2.0	0.6
- miscellaneous	1.2	3.0	0.0
Unintentional weight loss (>10% IBW) (%)	12.2	14.1	9.8
Altered bowel habits (%)	32.8	37.0	28.9
Fatty diarrhoea (%)	14.7	20.0	12.3

GDS=gastroduodenoscopy, BMI=body mass index, IBD= inflammatory bowel disease, IBW=initial body weight
*sixteen individuals underwent both GDS and colonoscopy
** in some individuals more than one indication for endoscopy was present

Weight loss and self-reported fatty diarrhoea did not have an effect on *T. whipplei* prevalence (2-sided Fischer's exact test, resp. p=0.650; p=0.160), whereas a statistical trend was observed for an increased prevalence in patients with self-reported altered bowel habits (2-sided Fischer's exact test p=0.066). However, one of four patients with altered bowel habits and a positive test suffered from constipation, a symptom not expected to be related to the presence of *T.whipplei.*

Duodenal mucosa (Table 3+4)

One hundred duodenal biopsy specimens were tested, two of which were positive (2.0%; 95% C.I. -0.74-4.74). Matching washing fluids of the positive samples were negative. Prevalence of *T. whipplei* DNA was not statistically significant associated with antibiotic and probiotic use. (2-sided Fischer's exact test, resp p=1.000; p=1.000). Weight loss, altered bowel habits and self-reported fatty diarrhea were not statistically significant correlated with prevalence of *T. whipplei* DNA (2-sided Fischer's exact test, p=1.000; p=0.135; p=1.000, respectively).

Colonic mucosa versus duodenal mucosa

No statistical significant difference was reached between the prevalence of *T. whipplei* in colonic and duodenal mucosa. (2-sided Fischer's exact test p=0.715) The same holds true for sixteen individuals who underwent both procedures of which only one colonic sample was positive. (2-sided Fischer's exact test p=1.000)

Table 3 Results

	Colon			**Duodenum**		
	Tw -	**Tw +**	**p-value**	**Tw -**	**Tw +**	**p-value**
N **95% C.I.**	153 (96,2%)	6 (3,8%) 0.81-6.74		98 (98%)	2 (2%) -0.74-4.74	
AB	15	1	0.476	12	0	1.000
PB	30	2	0.348	14	0	1.000
WL	38	2	0.650	37	1	1.000
ABH	42	4	**0.066**	35	2	0.135
FD	17	2	0.160	20	0	1.000

Tw=*T. whipplei* PCR, C.I.= confidence interval, AB= antibiotic use, PB=probiotic use, WL= weight loss, ABH= altered bowel habits, FD= fatty diarrhoea

Table 4 Tw DNA + individuals

Pt.	M/F	Age	Presence Tw DNA				Indication	Clinical signs of intestinal WD			Endoscopy	Histology	Additional information
			C	WF	F	D		BMI	WL	FD			
1	M	79	+	+			Diarrhoea, weight loss (14kg/6mnths)	20.0	+ (14/6)	-	Multiple polyps, pandiverticulosis		Suspicion of self-limiting infectious colitis
2	M	28	+	+			Frequent, loose stools, for yrs	22.8	-	+	N.A.	Signs of resolved inflammation	
3	M	54	+	-	-		CRC-screening, occult blood loss in stools	30.4	-	-	Angiodysplasia coecum, small polyps in rectum	Adenoma with low-grade dysplasia	
4	F	47	+	-	-		Altered defaecation pattern, Fe-anaemia, abdominal complaints for years	25.4	-	+	Haemorrhoids		Arthralgias for years
5	M	73	+	-		-	Fe-anaemia, suspicion of diverticulitis and CRC	29.6	-	-	Duodenal lipoma, multiple colonic polyps, diverticulosis,	Tubulovillous adenoma's with mild dysplasia	Received trimethoprim-sulfamethoxazole twice daily for ten days

											no signs of diverticulitis		duo to suspicion of diverticulitis
6	F	42	+	+	+		Abdominal complaints	22.0	+ (14/12)	-	Multiple polyps sigmoid	Hyperplastic polyps	Arthralgias, rheuma serology -, ocular irritation or inflammation
7	M	58	-	-	+	-	Dyspepsia, abdominal complaints	29.1	-	-	Oesophagitis, gastritis, colonic polyp, diverticulosis	Hyperplastic polyp	
8	F	50		-		+	Dyspepsia, pyrosis	37.4	-	-	Gastritis	Reactive gastropathy with metaplasia	SLE (polyarthritis, vasculitis, ostemomyelitis, pericarditis)
9	F	28		-		+	Family history coeliac disease, weight loss, diarrhoea	17.3	+/- (2/2)	-	Gastritis, bulbitis, and duodenitis with whitish villi and reticular pattern	Marsh 1: >IEL	No coeliac disease, based on serology and genotyping
M	5/4	51.0						26.0	22.2	22.2			

Tw DNA + = *T. whipplei DNA positive*

Discussion

A novel real-time PCR targeting a repetitive sequence seven times present exclusively in the *T. whipplei* genome had an optimal detection limit of 1 DNA-copy per PCR. Different samples and sample types from patients with previously diagnosed WD tested all positive.

No cross-reaction was found with *in silico* testing using the BLAST-tool. Moreover, testing of several monocultures of different species revealed no cross reaction with the *T. whipplei* PCR.

In a pilot study of 21 individuals undergoing a colonoscopy for other reasons than (suspicion of) WD, three samples tested positive, one of which was confirmed by a 16S-based PCR and subsequent sequencing.

Therefore we tested 159 colonic samples of which six tested positive which resulted in a prevalence of 3,8%.

Presence of *T. whipplei* without evident symptomatology seems to indicate the bacterium to be a conditional pathogenic in the human gut, similar to *Clostridium difficile*. It is also conceivable that differences in pathogenicity of *T. whipplei* strains are essential in development of WD, although this could not be demonstrated in a recent study (Li et al., 2008. Microbiology 154: 521-527). The difference between carriage of *T. whipplei* and WD is yet unexplained. Hence, cultivation and subsequent whole-genome sequencing of *T. whipplei* strains, both from patients as well as from asymptomatic carriers, is of pivotal importance in order to elucidate the exact role of *T. whipplei* strains in the pathogenesis of (subclinical) Whipple's disease.

The prevalence of *T. whipplei* in duodenal biopsy samples was 2.0% (95% C.I. -0.74-4.74), consistent with reported numbers (0%-4.8%) (Ehrbar et al., 1999. Lancet 353: 2214; Maibach et al., 2002. J. Clin. Microbiol. 40: 2466-2471; Maiwald et al., 2001. Ann. Intern. Med. 134: 115-119; Fenollar et al., 2002. J. Clin. Microbiol. 42: 401-403.). Others (Edouard et.al. 2012. J Clin Microbiol. 50: 3917–3920) reported a higher percentage over a 12-year

period (7,24%). The fact that these samples were analysed in a tertiary center for WD is a likely explanation for this difference.

No statistically significant difference between prevalence of *T. whipplei* in colonic versus duodenal samples was observed.

Since an indication for endoscopy was present in all individuals in our study, the results may not be an accurate reflection of *T. whipplei* prevalence in the general population. As such, individuals undergoing endoscopy would have a higher change of pathology, amongst which WD (although with a very low incidence). However, in case of colonoscopy the prevalence was equal to faecal samples in which an indication for endoscopy was absent.

Example 2

Materials and methods

A process control specifically for application in blood samples taken for diagnosis of blood stream infections was constructed by genetically modifiying *Mycobacterium smegmatis*. This organism was chosen because it is considered to be non-pathogenic and it has a sturdy cell wall.

Using *Chlamydia pneumoniae* DNA as template, a first PCR product was generated using primers Extern 1 and CP links and a second PCR product using CP rechts and Extern 2. The PCR products were combined and used as template in a third PCR containing CtCp forward and CtCp reverse primers. The resulting PCR product was used as template in a fourth PCR containing primers MPCtCP forward and MPCtCP reverse. This PCR product was cloned into pGEM-T-easy (Promega Corporation; Woods Hollow Road, Madison, WI 53711 USA) following the manufacturers instructions, yielding plasmid pIAC-MpCtCp. A fifth PCR was set up, using pIAC-MpCtCp as template and primers Abau-IC-for plus Eco-IC-rev. The resulting PCR product was cloned into pGEM-T-easy, yielding plasmid

pIAC-Ec-Abau. A PCR product was generated using pIAC-Ec-Abau as template and IAC-myco-for and IAC-myco-rev as primers. The resulting PCR product was cut with HindIII and NsiI and ligated into the Mycobacterial integrative plasmid pMV361 (Kumar et al., (1998) Vaccine 16:1212-1215) which was transformed into *E. coli* DH5α. Selection of transformants was done on LB-agar plates containing 100 µg/ml streptomycin. Plasmids isolated from these transformants were checked by restriction analysis and electroporated into *M. smegmatis* strain MC2 155.

Table 5: primer sequences used in construction PCR fragments

Primer	Sequence
Extern 1	AAAGCGATCCCAAATGTTTAAGG
CP links	TGGCGAATTTGGCGGATGTGCATTCTATGTATCGGCTTAGCTGGATAC
CP rechts	GCA CAT CCG AAT CTT TCG CCA GAA TCG CAA TAG CGG ATC TCC TGC AGC ACC TTC
Extern 2	GAC GTC TGT TCG CCA AAA ATA T
CtCP forward	AAT GAT ACT GGA TTG ACT CCG ACA ACG TAT TCC CGT AAA ATCCAG CAC GTA AGC
CtCP reverse	CAT TAT GTT ATC ATT GCC ATT AGA AAG GGC AGA AGG TGC AGG AGA TCC
MPCtCP forward	CCT GGG TAG TAC ATT CGC AAG AAT GAT ACT GGA TTG ACT CCG ACA AC
MPCtCP reverse	CAC CTG TCA CTC GGT TAA CCT CCA TTA TGT TAT CAT TGC CAT TAG AAA GGG C
Abau-IC-for	ATCGCTGTTGTTGGTGATGGAACTGATACTGGATTGACTCCG
Eco_IC-rev	TGGGCAATACCCTGCAGTTTGTTATCATTGCCATTAG
IAC-myco-for	CGACATCGATAAGCTTACTCAAGCTATGCATCCAACGC
IAC-myco-rev	GAACCACTTAATTAATGCATTTGGGCCCGACGTCGCATGC

The modified *M. smegmatis* strain was inactivated by incubation for 2 hours at 85˚C after which no colonies grew on agar plates. The process control (10E6 cfu in 50 µl saline) was injected into EDTA blood collection tubes. This procedure did not disturb the vacuum in the tube. The EDTA blood collection tubes with process control were left at room temperature for 0, 7, 42 and 56 days after which 5 ml blood spiked with 1.5x10E6 cfu each of *E. coli* and *S. agalactiae*, as representative clinically relevant organisms, was added. The whole 5 blood sample was processed by the Polaris procedure (see above). Reaction conditions for PCR were: per reaction 12,5 µl Sensimix II (mastermix containing buffer, polymerase, dNTPs, obtained from Bioline, London, UK), 300 nM of each primer and 200 nM of probe added in a 2,5 µl

volume plus 10 µl of DNA. Cycling conditions were: one step of 10 minutes at 95˚C; then 45 cycles of 15 seconds at 95 ˚C plus 60 seconds at 60˚C. Fluorescence was monitored after each cycle in a LightCycler 480 realtime PCR machine (Roche Woods Hollow Road, Madison, USA).

Results

The presence of all spiked organisms was tested by specific real time PCR using primers and probes as indicated in Table 6).

Table 6. Primer and probe sequences. All sequences are indicated from 5'-end to 3'-end.

Target	Primer 1	Primer 2	Probe	Amplicon length (bp)
E. coli	GGCTTCGAAATGGACTTTGCT	TGGGCAATACCCTGCAGTTT	FAM-CTGTTGCTGGAAGACTACAAAGCCTCCCTG-BHQ1	94
S. agalactiae	CCCTGAACATTATCTTTGATATTTCTCA	TTCACCAGCTGTATTAGAAGTACATGC'	FAM- CAAGCCCAGCAAATGGCTCAAAAGCT-BHQ1	149
Process control	CGCTGTTGTTGGTGATGGAACT	TGGGCAATACCCTGCAGTTT	FAM-TCTGGCGAAAGATTTGGCGGATGTGCATT-BHQ1	187

The results are presented in Table 7.

Table 7. Real time PCR of spiked organisms

timepoint	Ct *E. coli*	Ct *S. agalactiae*	Ct Processing control
T= 0	20.1	22.8	25.0
T= 7 days	21.5	21.9	26.3
T= 42 days	22.1	22.7	28.8
T= 56 days	24.6	22.5	30.1

The data show that the processing control remains intact, at least partly, for at least 2 months. Stability may be improved by adding a stabilizing component, such as glycerol to the processing control.

5

Claims

1. A method for determining whether a first microorganism is present or absent in a test sample, comprising,

- contacting the test sample with a second, killed, positive process control microorganism, comprising a control nucleic acid, comprising first and second primer hybridization nucleic acid sequences from the first microorganism,

wherein the first and second primer hybridization nucleic acid sequences in the control nucleic acid comprised in the control microorganism flank an internal nucleic acid sequence that is not present between the first and second primer hybridization nucleic acid sequences in the first microorganism; and

wherein an internal nucleic acid sequence that is present between the first and second primer hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control microorganism;

whereby the first and second primer are able to amplify a target nucleic acid that is present in the first microorganism of between 50 and 500 bases;

- preparing a nucleic acid extract from the test sample, the extract comprising nucleic acid from at least the second microorganism and, if present in the sample, nucleic acid from the first microorganism;

- contacting the extract with the first and second primer to amplify at least the control nucleic acid and, if present, the target nucleic acid from the first microorganism; and

- detecting the presence or absence of the control nucleic acid and the target nucleic acid, whereby the presence of the target nucleic acid is indicative for the presence of the first microorganism.

2. A method for determining whether at least one of two microorganisms A and B are present or absent in a test sample, comprising

- contacting the test sample with a second, killed, positive process control microorganism, comprising a control nucleic acid comprising first (A1) and second (B2) primer hybridization nucleic acid sequences, whereby said first primer hybridization nucleic acid sequence A1 is from microorganism A and said second primer hybridization nucleic acid sequence B2 is from microorganism B, wherein A1 and B2 flank an internal nucleic acid sequence that is not present in microorganisms A and B;

- preparing a nucleic acid extract from the test sample, the extract comprising nucleic acid from at least the second microorganism and, if present in the sample, nucleic acid from microorganism A and/or B;

- contacting the extract with primers A1 and A2, and with primers B1 and B2, wherein A1 and B2 amplify the control nucleic acid and, if present, A1 and A2 amplify target nucleic acid from microorganism A and B1 and B2 amplify target nucleic acid from microorganism B;

- detecting the presence or absence of the control nucleic acid and the target nucleic acids from microorganisms A and B, whereby the presence of target nucleic acid from microorganisms A is indicative for the presence of microorganism A in the test sample, and whereby the presence of target nucleic acid from microorganisms B is indicative for the presence of microorganism B in the test sample.

3. The method according to claim 1 or claim 2, wherein the control nucleic acid is integrated in the genome of the second microorganism.

4. The method according to any one of claims 1-3, wherein the test sample is a bodily fluid or a biopsy sample.

5. The method according to any one of claims 1-4, wherein a genomic nucleic acid extract is prepared from the test sample.

6. The method according to any one of claims 1-5, wherein mammalian eukaryotic cells that are present in the test sample are lysed prior to lysis and preparation of a nucleic acid extract from the microorganisms.

7. The method according to any one of claims 1-6, wherein the length of the control nucleic acid is larger than the length of the target nucleic acid or target nucleic acids.

8. The method according to any one of claims 1-7, wherein the second, killed, positive process control microorganism is present in a receptacle prior to the addition of a test sample to the receptacle.

9. A receptacle comprising a positive process control microorganism, comprising a positive control nucleic acid molecule comprising first and second primer hybridization sequences derived from a first microorganism, wherein the first and second primer hybridization nucleic acid sequences flank an internal nucleic acid sequence that is not naturally present between the first and second primer hybridization sequences in the first microorganism, and wherein an internal nucleic acid sequence that is present between the first and second primer hybridization nucleic acid sequences in the first microorganism is absent between the first and second primer hybridization nucleic acid sequences in the control microorganism.

10. A receptacle comprising a positive process control microorganism, comprising a positive control nucleic acid molecule comprising first (A1) and second (B2) primer hybridization sequences derived from microorganisms A

and B, respectively, wherein A1 and B2 flank an internal nucleic acid sequence that is not naturally present microorganisms A and B.

11. The receptacle according to claim 9 or 10, comprising a killed, intact microorganism.

12. The receptacle according to any one of claims 9-11, comprising a non-pathogenic microorganism.

13. The receptacle according to any one of claims 9-12, wherein the positive process control microorganism is a bacterium.

14. The receptacle according to any one of claims 9-12, wherein the positive process control microorganism is a *Candida* or *Aspergillus* species.

15. The receptacle according to any one of claims 9-12, wherein the positive process control microorganism is a spore-forming bacterium such as *Bacillus subtilis*.

16. Use of a receptacle comprising a positive process control microorganism according to any one of claims 9-15 to control the entire processing operations of the sample, including storage, transport, lysis, extraction and amplification.

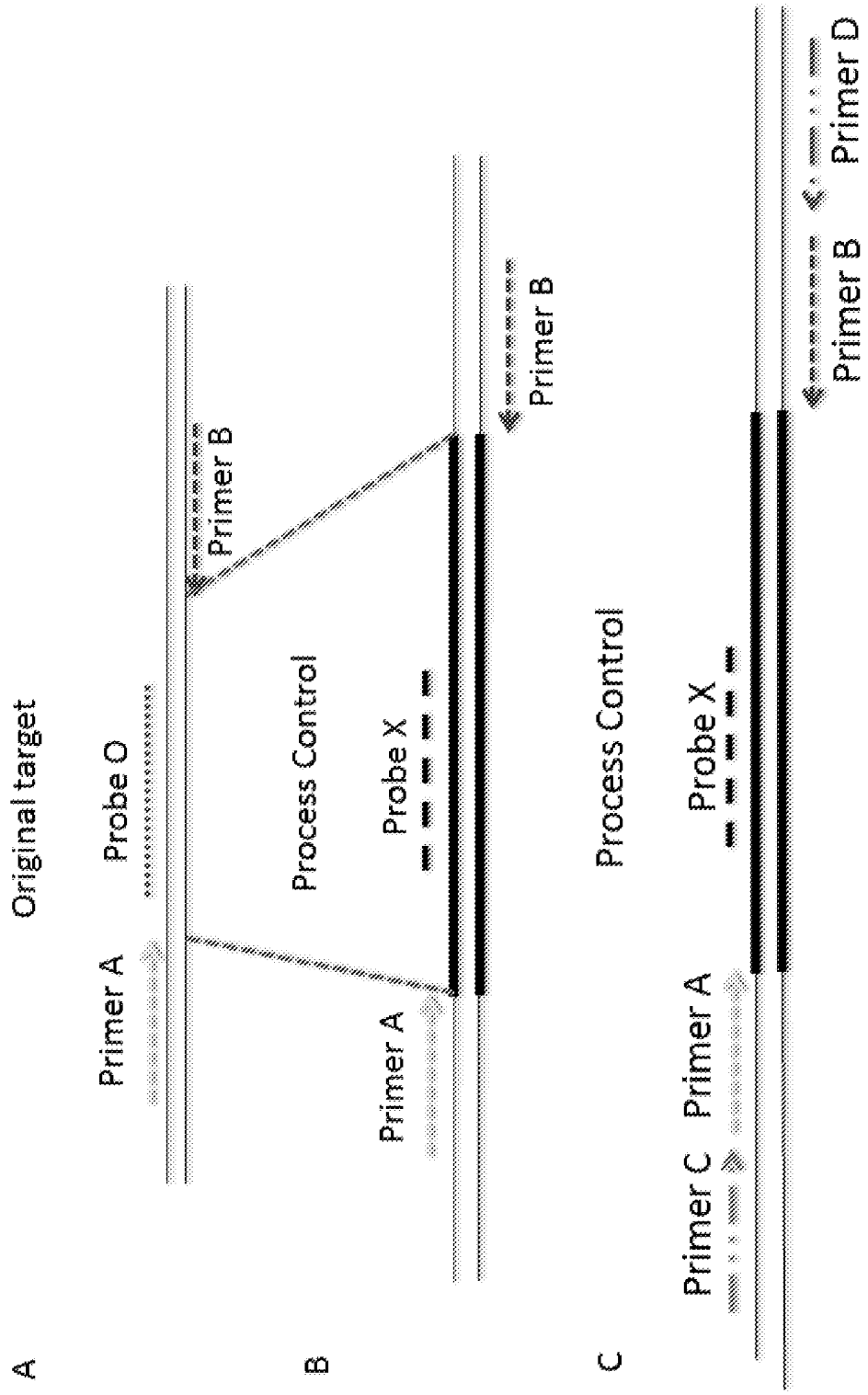
A
Original target
Probe O
Primer A
Primer B
B
Process Control
Probe X
Primer A
Primer B
C
Process Control
Probe X
Primer C
Primer A
Primer B
Primer D

Figure 1