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(54) Title: METHOD FOR DETERMINING THE PRESENCE OF DIARRHOEA CAUSING PATHOGENS

(57) Abstract: This invention relates to the field of detection of diarrhoea causing pathogens from patient, food or environmental samples. Particularly, the present invention provides a polymerase chain reaction (PCR) based assay method for detection of diarrhoea causing pathogens. The present invention further provides materials such as primers, primer pairs and probes for use in the method of the invention. Preferably, the method of the invention is a multiplex real-time PCR (RT-PCR) assay for rapid determination of clinically important pathogens related to traveller's diarrhoea.

## Method for determining the presence of diarrhoea causing pathogens

### FIELD OF THE INVENTION

This invention relates to the field of detection of diarrhoea causing pathogens from patient, food or environmental samples. Particularly, the present invention provides a polymerase chain reaction (PCR) based assay method for detection of diarrhoea causing pathogens, particularly ETEC and *Campylobacter* species. The present invention further provides materials such as primers, primer pairs and probes for use in the method of the invention. Preferably, the method of the invention is a multiplex real-time PCR (RT-PCR) assay for rapid determination of clinically important pathogens related to traveller's diarrhoea.

### BACKGROUND OF THE INVENTION

Diarrhoea is a major health problem worldwide causing morbidity, but also mortality especially of infants in the developing countries. Diarrhoea is the most reported problem for travellers and is commonly caused by contamination of food or water. In most cases traveller's diarrhoea is mild and short of duration, but severe infections with abdominal pain, bloody diarrhoea and septicaemia exist.

The causes of acute diarrhoea of travellers are many and varied. In addition to classical diarrhoeal bacteria, such as *Salmonella*, *Campylobacter*, *Shigella* and *Yersinia* also diarrhoeal *E. coli* strains are associated with traveller's diarrhoea. (enterohemorrhagic *E. coli*; EHEC, enterotoxigenic *E. coli*; ETEC, attaching and effacing *E. coli*; A/EEC or enteroaggregative *E. coli*; EAEC, enteropathogenic *E. coli*; EPEC, verocytotoxin producing *E. coli*; VTEC, enterohemorrhagic *E. coli*; EHEC, enteroinvasive *E. coli*; EIEC). *Salmonella* infection can cause a variable clinical disease starting from a mild, subclinical infection, or lead to severe systemic infection, typhoid fever. *Salmonella* sp. invades the host through the colonic epithelial cells, especially M cells using a type III secretion system. They are also able to survive within phagosomes of macrophages, and evade the host immune system by several ways (Coburn et al., 2007). *Campylobacter jejuni* and *coli* are among the large *Campylobacter* family predominant human stool pathogens causing watery diarrhoea, fever and typically hard abdominal pain. By

diagnostic means they must be dissected from the other *Campylobacter* species not associated with diarrhoea. They are able to invade the colonic epithelium lining and replicate intracellularly and cause apoptosis (Poly and Guerry, 2008; Allos, 2001). *Yersinia enterocolitica* and *pseudotuberculosis* harbour a virulence plasmid containing relevant  
5 adhesion and invasion proteins, such as YadA (Bottone, 1999, El Tahir et al., 2001). For *Y. enterocolitica* a virulence plasmid is required to cause a clinical disease, whereas *Y. pseudotuberculosis* has additional genomic virulence factors as well. *Yersinia pestis* is the plague pathogen, which harbours genomic virulence factors and three virulence plasmids which are all required to cause a clinical disease (Bottone, 1999). The traditional pathogens  
10 are also associated with late onset symptoms, such as reactive arthritis, sacroiliitis and acute anterior uveitis.

*Vibrio cholerae* is a highly virulent environmental pathogen living in free waters in some of the tropical countries, especially causing epidemics in catastrophe areas. It typically causes massive watery diarrhoea leading to patient death if not sufficiently resuscitated.  
15 The essential virulence factor is cholera toxin which consists of two subunits A and B. The cholera toxin is able to bind irreversibly to the G-proteins in the colonic epithelial cells responsible for liquid and electrolyte uptake causing non-voluntary continuous secretion into gut lumen (Nelson et al., 2009).

*Shigella* and EIEC are genetically closely related. Both of these organisms invade the  
20 colonic epithelium mediated by the genes located in virulence plasmid pINV coding e.g. Ipa proteins and their transcription regulator invE (Lan and Reeves, 2002; Parsot, 2005). EAEC demonstrate characteristic adherence pattern to Hep-2 cells via specific fimbria encoded by genes which are located on plasmid under the regulation of AggR (Flores and Okhuysen, 2009). EPEC is characterized to possess pathogenicity island named the locus  
25 of enterocyte effacement (LEE). This island contains genes such as *eae* for intimate adherence of the EPEC strains to intestinal epithelial cells. EPEC is differentiated from EHEC by ability of EHEC strains to production shiga-like toxins I and II encoded by *stx1* and *stx2* genes. These cytotoxins cause acute inflammation in the intestine leading to abdominal pain and bloody diarrhoea. In addition, EHEC infection may lead to rare but  
30 severe, secondary complications such as haemolytic uremic syndrome (HUS) (Chen and Frankel, 2005; Karch et al., 2005). The challenge in multiplex PCR assays is to identify

EHEC variants so that there is no cross-reaction with *Shigella*/EIEC species, because the target genes expressing toxins in these bacteria are very similar.

Giardiasis is an infection of the small intestine caused by *Giardia lamblia* (also known as *G. intestinalis*), a flagellate protozoan. Giardiasis is the most commonly reported  
5 pathogenic protozoan disease worldwide. Travelers are the largest risk group for giardiasis infection, especially those who travel to the developing world. Giardiasis is spread via the fecal-oral route. Most people contract the disease by ingesting contaminated water or food, or by not washing their hands after touching something contaminated with *Giardia* cysts. Prevalence rates for giardiasis range from 2-7% in developed countries and 20-30% in  
10 most developing countries. The CDC estimates there are an upwards of 2.5 million cases of giardiasis annually. The most common symptoms of *Giardia* infection include diarrhea for a duration of more than 10 days, abdominal pain, flatulence, bloating, vomiting, and weight loss. Giardiasis is traditionally diagnosed by the detection of cysts or trophozoites in the feces, trophozoites in the small intestine, or by the detection of *Giardia* antigens in  
15 the feces.

Currently, the routine diagnostic of diarrhoea is mostly based on traditional cultivation methods and immunoassays, which are both laborious and time consuming. They are only available for *Salmonella*, *Campylobacter*, *Shigella* and *Yersinia* species as well as enterohaemorrhagic *Escherichia coli* (EHEC), whereas no cultivation method for other  
20 major diarrhoeagenic *E. coli* species, including ETEC, EPEC, EAEC, and EIEC exists. In recent years, DNA based methods for diagnosis of diarrhoeagenic *E. coli* has been published (Antikainen et al., 2009; Aranda et al., 2004; Brandal et al., 2007; Guion et al., 2008; Kimata et al., 2005; Müller et al., 2007; Vidal et al., 2005; Vidal et al., 2004).

ETEC causes watery diarrhoea by producing heat-labile (LT) and/or heat-stable (ST)  
25 enterotoxins [2-3]. ETEC is traditionally considered the most common cause in traveller's diarrhoea (Qadri et al., 2005). The present invention is particularly directed to improve the detection of ETEC in multiplex RT-PCR assays. The present invention provides two primer pairs and probes specific for the heat stable enterotoxin of ETEC encoded by the *est* gene and one primer pair and probe specific for the heat labile enterotoxin of ETEC  
30 encoded by the *elt* gene. These primers and probes are designed to amplify such target sequences in said genes that it renders possible efficient detection of global variants of

ETEC. Further problem was that the target gene *est* includes multiple repetitive elements and it was difficult to find conserved regions long enough for both the primers and the probe.

The present invention is further directed to improve the detection of diarrhea causing  
5 *Campylobacter* species in multiplex RT-PCR assays. The invention provides primer pairs and probes for *rimM* gene of *C. jejuni* and *gyrB* gene of *C. coli*. With these primers and probes the diarrhoea causing *Campylobacter* can be distinctively identified from other non-pathogenic *Campylobacter* and other diarrhea causing pathogens. A combination of two different genomic targets was required to solve the problem.

10 In WO2005/005659, it is disclosed a method for simultaneous screening diarrhoea causing bacteria such as *E. coli* groups: ETEC (enterotoxigenic *E. coli*), A/EEC (attaching and effacing *E. coli*) EPEC (enteropathogenic *E. coli*), VTEC (verocytotoxin producing *E. coli*) and EIEC (enteroinvasive *E. coli*); and *Shigella* spp. The method is a real-time multiplex PCR assay and the template DNA is isolated directly from a stool sample. Similarly as the  
15 present invention, WO2005/005659 is also directed to the problem of screening for human pathogenic *E. coli* in order to provide distinction between the pathogenic *E. coli* groups and other diarrhoea causing pathogens. However, the target sequences in *est* and *elt* genes of ETEC are different in the present invention from the targets disclosed by WO2005/005659. Moreover, the present invention is providing coverage of global ETEC  
20 variants by the use of three specific primer pairs and probes while WO2005/005659 in Table 3 discloses four primer pairs for the same purpose. Table 8 of the present specification show that ETEC variants can be detected by using the three primer pairs of the present invention.

In WO2005/083122, it is disclosed a method for detection and quantification of  
25 enteropathogenic bacteria in a fecal specimen, including *Shigella* species, *Salmonella* species, *Campylobacter* species, enterohemorrhagic *Escherichia coli* or Verocytotoxin-producing *Escherichia coli*, *Vibrio cholerae*, and *Clostridium perfringens*. The method is a real-time PCR assay based on TaqMan® probes.

In WO2007/056463, it is disclosed a method comprising amplification of a sample with a  
30 plurality of pathogen-specific primer pairs to generate amplicons of distinct sizes from each of the pathogen specific primer pairs. The method utilizes real-time and multiplex

PCR techniques. The method can be used for the detection of *Salmonella* species, *Campylobacter* species, diarrhoeagenic *Escherichia coli*, *Vibrio cholerae*, *Yersinia* species such as *Yersinia pestis*, and *Giardia lamblia*.

- In WO2005/090596, it is disclosed an assay for detecting micro-organisms, and in particular bacteria, based on multigenotypic testing of bacterial DNA from human, animal or environmental samples. The method may also be utilized as a real-time multiplex PCR technique using TaqMan® probes. The method can be used for the detection of *Salmonella* species, *Campylobacter* species, diarrhoeagenic *Escherichia coli*, *Vibrio cholerae*, *Yersinia* species such as *Yersinia pestis*.
- 10 In US2004/0248148, it is disclosed a 5' nuclease real-time polymerase chain reaction approach for the quantification of total coliforms, *E. coli*, toxigenic *E. coli* O157:H7, toxigenic *M. aeruginosa* (microcystin hepatotoxins), *Giardia lamblia*, and *Cryptosporidium parvum*. Multiplex PCR assay can also be used for simultaneous detection of two or more pathogens..
- 15 In WO02/070728, it is disclosed an assay that relies on a 'multiprobe' design in which a single set of highly conserved sequences encoded by the 16S rRNA gene serves as the primer pair, and it is used in combination with both an internal highly conserved sequence, the universal probe, and an internal variable region, the species-specific probe. The real-time system reliably identifies 14 common bacterial species.
- 20 CN101113471, CN101245384 and CN101235410 disclose PCR methods for rapid detection of diarrhoea causing pathogens from food samples.
- Fukushima et al., 2003, disclose a real-time PCR assay for detection of 17 species of food- or waterborne pathogens directly from stool sample. The detection levels were approximately  $10^5$  pathogenic bacteria per gram of stool, therefore the protocol for stool specimens for less than  $10^4$  pathogenic bacteria per gram of stool requires an overnight enrichment step to achieve adequate sensitivity.
- 25 Hidaka et al., 2009, disclose multiplex real-time PCR for exhaustive detection of diarrhoeagenic *E. coli*. This method is especially for the detection of pathogenic bacteria from a food sample, such as meat sample.

Wang et al., 1997, disclose a protocol for PCR detection of 13 species of foodborne pathogens in foods.

There are some commercial multiplex PCR-based diarrheal pathogen detection kits available, for example xTAP GPP from Luminex and Diarrhea ACE Detection from  
5 Seegene. Both systems use multiplex PCR as a means for amplifying certain organism-specific nucleotide sequences but the final detection relies on analysis in another separate instrument. The means used for detection has an impact on the amplicon, primer and probe design because of different requirements of the detection formats. Further, gene or  
10 amplicon sequences used in the present invention for the detection of ETEC or *Campylobacter* have not been disclosed.

The number of pathogens causing diarrhoea is large and a diarrhoea test method should optimally identify all of them. Having one PCR reaction per species can be cumbersome, since the number of samples tested is typically large. It would be optimal to detect multiple species within one reaction. In a PCR setting the most obvious alternative is ‘multiplex’  
15 PCR amplification. In multiplex PCR, several oligonucleotide sets, each designed to amplify one species/species group, are included in the same reaction vessel and each oligonucleotide set is used to amplify its respective pathogen DNA during the same PCR reaction. In this invention, we describe a PCR based method for rapid detection of  
20 clinically important pathogens related to traveller’s diarrhea, particularly ETEC and/or *Campylobacter*. The present invention discloses primers and probes designed for target sequences conserved in global variants of ETEC and *Campylobacter*. These primers and probes are compatible for use in any multiplex RT-PCR determining the presence of multiple diarrhoea causing pathogens, since the target sites are unique for ETEC and  
*Campylobacter*.

25 Multiplex PCR presents a challenge for quantitation of the pathogen DNA (qPCR): the different amplicons compete for the same PCR reaction components (eg. DNA polymerase and MgCl<sub>2</sub>) and this can compromise the quantitative nature of the reaction between and, especially, quantitative comparisons between samples. It is commonly known in the art that there is bias in the amplification efficiencies between different template amounts or  
30 lengths so that e.g. short amplicons are favoured in the expense of longer ones.

At the same time, undesired cross-reactions of multiplex set oligo combinations must be avoided. One must also remember to check mis-priming to any other sequences present in the sample.

- 5 Finding suitable primer and probe sequences for the detection of a diverse group of pathogenic microbes can be far from trivial especially when designing multiplex set ups where all amplicons and templates should be amplified with equal efficiency (e.g., Giardia). Many of the species are relatively closely related, making it challenging to locate sequences that are unique for each species. Also, as there are a significant number of
- 10 global variants, it is difficult to identify globally conserved regions or a combination of minimal set of regions to detect all known variants (e.g., for EHEC, ETEC, pathogenic Yersinia, pathogenic Campylobacter and Shigella/EIEC). Some genes possess complex repetitive closely related elements which is challenging from the amplicon design point of view, especially when designing amplicons for multiplex PCR. For example, due to
- 15 repetitive elements and minor variants ETEC cannot be detected using only one amplicon.

The sample matrix, which in diarrhoea diagnostics is commonly a stool or food sample, is likely to contain a host of PCR inhibitors. This reduces amplification efficiency of the PCR reaction and thus even more careful optimization is expected from the amplicon design

20 step to verify that all templates and copy numbers are amplified equally but also efficiently enough. Hence, oligonucleotide design enabling high PCR efficiency (optimally as close to 100% as possible) is required. The detection method used may also affect amplification efficiency and/or bias.

- 25 The present inventors have now located DNA sequence regions that are well suited for specific and sensitive amplification and quantification of diarrhoea causing pathogens, particularly ETEC and Campylobacter. Accordingly, optimal primers and quantitative PCR probes have been designed in the present invention and validated for identification and quantification of diarrhoea causing pathogens. The amplicons have been designed to be so
- 30 specific that they can be combined into any multiplex sets with each other. Naturally a prerequisite to this is that all the disclosed amplicons have also been designed to amplify in the same reaction and cycling conditions.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a polymerase chain reaction (PCR) based assay method for detection of diarrhoea causing pathogens, particularly ETEC and Campylobacter species. The present invention further provides materials such as primers, primer pairs and probes  
5 for use in the method of the invention. Particularly, the present invention provides a method for determining the presence of diarrhoea causing pathogens in a biological sample comprising the steps of: i) contacting the sample or nucleic acid isolated therefrom with primer pairs in a multiplex PCR assay comprising two or more separate PCR reactions, wherein the primers of said primer pairs amplify any of the amplicons as defined by SEQ  
10 ID NOS:55-72, preferably SEQ ID NOS:61-63 and 65-68, at least partly;

ii) performing a polymerase chain reaction with reaction mixes obtained from step i) so that the target sequences of diarrhoea causing pathogens are specifically amplified, if said sequences are present in the sample; and

iii) detecting the presence of amplified target sequences in the reaction mix, wherein the  
15 presence of any of the target sequences is indicative of the presence of diarrhoea causing pathogens in the sample.

Said biological sample can be a stool sample, a food sample, such as a meat sample, or any environmental sample. The sample may be enriched before step i).

Preferably, the primer pairs in step i) of the method are selected from the group consisting  
20 of primer pairs A) to R), more preferably G) to I) and K) to N), comprising or consisting of at least one of the following oligonucleotides:

- A) forward primer: 5'GCGTTCTTATGTAATGACTGCTGAAG-3' (SEQ ID NO:1),  
reverse primer: 5'-AGAAATTCTTCCTACACGAACAGAGTC-3' (SEQ ID NO:2);
- B) forward primer: 5'-TGCATCCAGAGCAGTTCTGC-3' (SEQ ID NO:3),  
25 reverse primer: 5'-CGGCGTCATCGTATACACAGG-3' (SEQ ID NO:4);
- C) forward primer: 5'-CCAGGCTTCGTCACAGTTGC-3' (SEQ ID NO:5),  
reverse primer: 5'-CAGTGA ACTACCGTCAAAGTTATTACC-3' (SEQ ID NO:6);

- D) forward primer: 5'-GCTCTTCGGCACAAGTAATATCAAC-3' (SEQ ID NO:7),  
reverse primer: 5'-TCTATTTTAAATTCCGTGAAGCAAACG-3' (SEQ ID NO:8);
- E) forward primer: 5'-TGGTCCATCAGGCATCAGAAGG-3' (SEQ ID NO:9),  
reverse primer: 5'-GGCAGTGCGGAGGTCATTTG-3' (SEQ ID NO:10);
- 5 F) forward primer: 5'-TGTCTTTATAGGACATCCCTGATACTTTC-3' (SEQ ID NO:11),  
reverse primer: 5'-TATCTACTCTTGATGCCAGAAACTAGC-3' (SEQ ID NO:12);
- G) forward primer: 5'-AAAATTGCAAATCCGTTTAACTAATC-3' (SEQ ID NO:13),  
reverse primer: 5'-GACTGACTAAAAGAGGGGAAAG-3' (SEQ ID NO:14);
- 10 H) forward primer: 5'-TCCTGAAAGCATGAATAGTAGC-3' (SEQ ID NO:15),  
reverse primer: 5'-TTATTAATAGCACCCGGTACAAG-3' (SEQ ID NO:16);
- I) forward primer: 5'-CCGGCAGAGGATGGTTACAG-3' (SEQ ID NO:17),  
reverse primer: 5'-TTGATTGATATCCCTGAGATATATTGTG-3' (SEQ ID NO:18);
- J) forward primer: 5'-GGAAGCAATACATATCTTAGAAATGAACTC-3' (SEQ ID NO:19),  
15 reverse primer: 5'-TCGGACAACCTGCAAGCATCTAC-3' (SEQ ID NO:20);
- K) forward primer: 5'-GAGTGAAAAAGATTTTGTTC AAGTTG-3' (SEQ ID NO:21),  
reverse primer: 5'-AAAAGTCGCTCAGGTTATGC-3' (SEQ ID NO:22);
- L) forward primer: 5'-AGTGCCTGAACCTCAATTTG-3' (SEQ ID NO:23),  
20 reverse primer: 5'-TCGATAGGATTTTCTTCAAATATTTAC-3' (SEQ ID NO:24);
- M) forward primer: 5'-GTTTGGTACAGTTTATGGCATTTCAC-3' (SEQ ID NO:25),  
reverse primer: 5'-CATGGCAATATCAACAATACTCATCTTAC-3' (SEQ ID NO:26);
- N) forward primer: 5'-CAGGAGCATGAGGTTACAGTATG-3' (SEQ ID NO:27),

reverse primer: 5'-TCTCTGGCCCCGCACAATG-3' (SEQ ID NO:28);

O) forward primer: 5'-GGGCTACAGAGATAGATATTACAGTAACTTAG-3' (SEQ ID NO:29),

reverse primer: 5'-CCACGGCTCTTCCCTCCAAG-3' (SEQ ID NO:30);

5 P) forward primer: 5'-TTCCGGTCGATCCTGCC-3' (SEQ ID NO:31),

reverse primer: 5'-GTTGTCCTGAGCCGTCC-3' (SEQ ID NO:32);

Q) forward primer: 5'-AGACGATCCAGTTTGTATTAG-3' (SEQ ID NO:33),

reverse primer: 5'GGCATCCTAACTCACTTAG-3' (SEQ ID NO:34); and

R) forward primer: 5'-TCTGGAAAACAATGTGTTC-3' (SEQ ID NO:35),

10 reverse primer: 5'-GGCATGTCGATTCTAATTC-3' (SEQ ID NO:36).

Preferred amplicons amplified in target organisms are listed in Table 6. However, a person skilled in the art knows that these amplicon sequences naturally vary in related strains.

This minor variation can be taken into account while designing primers suitable to amplify said amplicons in the method of the present invention. Preferably, at least 20, 25, 30 or 35  
15 nucleotides long sequence of each of the target amplicons selected from the group consisting of SEQ ID NOS:55-72, preferably SEQ ID NOS:61-63 and 65-68, are amplified in the method.

The method of the invention is characterized in that the presence of the amplified target sequence, i.e. the product, of each of primer pairs A) to R) in the PCR reaction in step iv)  
20 indicates the presence of diarrhoea causing pathogens in the sample in the following way:

- the product of primer pair A) or B) indicates the presence of EHEC;
- the product of primer pair C) indicates the presence of EHEC/EPEC;
- the product of primer pair D) indicates the presence of *Salmonella*;
- the product of primer pair E) or F) indicates the presence of *Shigella*/EIEC;
- 25 - the product of primer pair G), H), or I) indicates the presence of ETEC;

- the product of primer pair J) indicates the presence of EAEC;
- the product of primer pair K) indicates the presence of *Campylobacter jejuni*;
- the product of primer pair L) indicates the presence of *Campylobacter coli*;
- the product of primer pair M) indicates the presence of *Yersinia*  
5 *enterocolitica/pseudotuberculosis*;
- the product of primer pair N) indicates the presence of *Yersinia*  
*pseudotuberculosis/pestis*;
- the product of primer pair O) indicates the presence of *Vibrio cholerae*;
- the product of primer pair P) indicates the presence of *Giardia lamblia*;
- 10 - the product of primer pair Q) indicates the presence of *Entamoeba histolytica*; and
- the product of primer pair R) indicates the presence of *Cryptosporidium* sp.

Preferably, each primer of said primer pairs is less than 35, 40, 45, 50 or 55 nucleotides long, and more preferably, less than 50 nucleotides long. Each of the present primers can also be defined as consisting of at least 10 contiguous nucleotides present in one primer  
15 sequence selected from the group consisting of SEQ ID NOS:1-36.

One specific embodiment of the invention is to perform said method as a real-time polymerase chain reaction and in that case nucleic acid probes comprising or consisting of the following sequences are specifically used with each of primer pairs A) to R), preferably G) to I) and K) to N).

- 20 - the probe for primer pair A):

5'-TCCATGATARTCAGGCAGGACACTACTCAACCTTCC-3' (SEQ ID NO:37)

- the probe for primer pair B):

5'-TTGTCACTGTCACAGCAGAAGCCTTACGC-3' (SEQ ID NO:38)

- the probe for primer pair C):

- 25 5'-AGATTAACCTCTGCCGTTCCATAATGTTGTAACCA-3' (SEQ ID NO:39)

- the probe for primer pair D):

5'-CCAAACCTAAAACCAGTAAAGGCGAGCAGC-3' (SEQ ID NO:40)

- the probe for primer pair E):

5'-TCACTCCCGACACGCCATAGAAACGCATTT-3' (SEQ ID NO:41)

5 - the probe for primer pair F):

5'-ACAAACAGCAAAAGAGCATAGCATCCGAGAACT-3' (SEQ ID NO:42)

- the probe for primer pair G):

5'-CAAATATCCGTGAAACAACATGAC-3' (SEQ ID NO:43)

- the probe for primer pair H):

10 5'-AGGATTACAACAAATTCACAGCAGT-3' (SEQ ID NO:44)

- the probe for primer pair I):

5'-AGCAGGTTTCCCACCGGATCACCA-3' (SEQ ID NO:45)

- the probe for primer pair J):

5'-TCCGTATATTATCATCAGGGCATCCTTTAGGCGT-3' (SEQ ID NO:46)

15 - the probe for primer pair K):

5'-AAGACCCACAGTTTTACCAAGTTTT-3' (SEQ ID NO:47)

- the probe for primer pair L):

5'-AACTTGGCTTCTTATGTGCGT-3' (SEQ ID NO:48)

- the probe for primer pair M):

20 5'-CCTGGATAAGCGAGCGACGTATTCTCTATGC-3' (SEQ ID NO:49)

- the probe for primer pair N):

5'-AAACCAAAGCCGCCACACCACAG-3' (SEQ ID NO:50)

- the probe for primer pair O):

5'-AACCTGCCAATCCATAACCATCTGCTGCTG-3' (SEQ ID NO:51)

- the probe for primer pair P):

5'-ACGAAGCCATGCATGCCCGCT-3' (SEQ ID NO:52)

5 - the probe for primer pair Q):

5'-ACAAAATGGCCAATTCATTCAATGAA-3' (SEQ ID NO:53)

- the probe for primer pair R):

5'-CCTCCTAATCCAGAATGTCCTCCAG-3' (SEQ ID NO:54)

The melting temperature,  $T_m$ , of some of the probes (such as probes for primer pairs G),  
10 H), K) and L)) is preferably increased at least 5 degrees °C by addition of modified  
nucleotides. The amount of modified nucleotides in one probe is 1, 2, 3 or preferably 4.  
The underlined nucleotides in the above list are modified nucleotides each increasing the  
 $T_m$  of the probe. The modified nucleotide can be a LNA nucleotide (Exiqon A/S), minor  
groove binder (MGB™), SuperBase, or Peptide Nucleic Acid (PNA) or any other  
15 modification increasing the  $T_m$  of the probe.

Preferably, the above probes comprise the sequences as defined and are less than 40, 45, 50  
or 55 nucleotides long, and more preferably, less than 50 nucleotides long. Each of the  
present probes can also be defined as consisting of at least 10 contiguous nucleotides  
present in one probe sequence selected from the group consisting of SEQ ID NOS:37-54.

20 The method of the invention is based on multiplex PCR technique, wherein primer pairs  
are divided into separate PCR reactions. As a general guideline the multiplex assay should  
be designed so that the most frequently appearing pathogens (e.g. Antikainen et al, 2009)  
are in different multiplex reactions.

In one embodiment, the invention provides nucleotide primers comprising or consisting of  
25 any of the primer sequences from primer pairs G) to I) and K) to N) as defined above.

In another embodiment, the invention provides nucleotide primer pairs comprising or consisting of the sequences from any of primer pairs G) to I) and K) to N) as defined above.

In a further embodiment, the invention provides nucleotide probes comprising or  
5 consisting of any of the probe sequences as defined above.

The present invention is preferably directed to a method for determining the presence of diarrhoea causing pathogens in a sample, wherein the presence of at least pathogens ETEC, *Campylobacter jejuni*, and *Campylobacter coli* is checked in said sample. Further target pathogens may be *Yersinia enterocolitica/pseudotuberculosis*, and *Yersinia*  
10 *pseudotuberculosis/pestis*.

The present invention is further directed to the use of nucleotide primers, primer pairs or probes as defined above for determining the presence of diarrhoea causing pathogens in a sample.

The present invention also provides kits for the detection of the presence of diarrhoea  
15 causing pathogens in a sample. Such a kit comprises primer pairs selected from the group consisting of primer pairs A) to R), preferably G) to I) and K) to N), as defined above. The kit may further comprise a probe selected from the probes as defined above. The use of the primer pairs and probes are described above and in the Example below. Preferably, said kit comprises means for a real-time polymerase chain reaction, such as labelled probes,  
20 polymerase enzymes, buffers and nucleotides. Preferably, said kit is for the detection of the presence of at least pathogens ETEC, *Campylobacter jejuni*, and *Campylobacter coli* in a sample.

The publications and other materials used herein to illuminate the background of the invention, and in particular, to provide additional details with respect to its practice, are  
25 incorporated herein by reference. The present invention is further described in the following example, which is not intended to limit the scope of the invention.

## EXAMPLE

**Materials and methods**

*Patient samples.* Control stool samples were cultured at HUSLAB for *Salmonella*,  
*Yersinia*, *Shigella*, *Campylobacter* and EHEC with standard biochemical methods. A total  
5 of 146 travellers were recruited in Travel Clinic (Medicity, Helsinki, Finland) to participate  
in this study during six month period. The age ranged from 1 to 72 (mean 39.2 years); 84  
(57.5%) were females and 62 (42.5%) were males. The travel destinations were Europe in  
7.5%, Asia in 32.9%, Africa in 44.5%, Australia in 1.4% and America in 13.7% of cases.

Total nucleic acids were purified from the stool samples with NucliSENS kit using  
10 easyMAG platform as described in Antikainen et al., 2009. Briefly, stool swabs were  
suspended to 100 µl of Tris-EDTA buffer and purified by the general method of easyMAG  
platform and eluted to the volume of 25 µl. Eluate (0.5 µl) was used as a template in PCR.

Alternatively, the swaps can be suspended directly into lysis buffer. The samples are eluted  
to a volume of 100 ul and 2 ul of eluate is used as a template in PCR. This protocol is  
15 suitable for fully automated, integrated sample preparation and PCR plate setup steps.

*Identification of the isolates.* Faecal samples positive in PCR for *Salmonella*, *Shigella*,  
*Yersinia*, *Campylobacter* and EHEC were cultured and identified with normal diagnostics  
methods. Since for diarrhoeal *E. coli* strains no cultivation based routine method exists,  
positive samples were analysed by previously developed multiplex-PCR (Antikainen et al.,  
20 2009).

From those samples of which isolation of bacterial strains was unsuccessful, corresponding  
genes were separately amplified and sequenced in Sequence Core Facility in Haartman  
Institute (Helsinki, Finland) using primers listed in Table 1. Sequences were identified by  
Basic Local Alignment Search Tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

25

*Design of the Real-Time-PCR.* PCR was designed to identify specific virulence genes,  
species specific genes, or species specific regions within established universal genes (Table  
1). Real-Time PCR primers and probes were designed with Allele ID and Beacon Designer  
software (Palo Alto, CA) to recognize correct target genes and their global variants,  
30 including BLAST search and secondary structure prediction using NCBI data base.

RT-PCR was performed on Mx3005P detection system (Agilent Technologies, Garden Grove, CA) and thermocycling conditions were 95°C for 15 min, 40 cycles of 94°C for 1 min and 60°C for 1 min. Fluorescence was recorded at each annealing step. The 20- $\mu$ l reaction contained 1 x Qiagen Multitect NoROX master mix (Qiagen, Hilden, Germany), 1  
5  $\mu$ l of primer/probe mix (Tables 1 and 2) and 0.5  $\mu$ l of template DNA.

*Specificity of the PCR.* The analytical specificity of the PCR was analysed by using 249 bacterial strains as positive controls including *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* and *Vibrio* strains as well as diarrhoeal *E. coli* strains (Tables 1 and 2). The strains  
10 were originated from the Helsinki University Hospital Laboratory (HUSLAB), the National Institute of Health and Welfare (THL), and as a kind gift from M. Alexander Schmidt and Inga Benz (Westfälische Wilhelms-Universität, Münster, Germany), from Isabel Scaletsky (Universidade Federal de São Paulo, Brazil) as well as from Lin Thorstensen Brandal (The Norwegian Institute of Public Health, Norway). As negative  
15 controls, 243 bacterial strains from all major genera were used as described in Antikainen et al., 2009.

For PCR analysis, bacterial cells were collected to 100  $\mu$ l of water, boiled for 15 minutes, centrifuged one minute 13 000 rpm and the supernatant (0.5  $\mu$ l) was used in PCR reactions or bacterial DNA was purified with NucliSENS kit using easyMAG automatic nucleic acid  
20 purification platform as described by the manufacturer (bioMérieux, Marcy l'Etoile, France).

*Analytical sensitivity of the PCR.* To analyze sensitivity for clinical use, a mixture of DNAs containing all templates purified by easyMAG for each amplicon were diluted 10-  
25 fold and analyzed by PCR. In addition, the amplification of each reporter was separately analysed in 10-fold dilutions using boiled bacterial mass. Shortly, bacteria were grown on agar plates, collected to TE buffer and the viable count (colony forming unit (CFU)) was determined. Bacteria were diluted 10-folds and boiled for 15 minutes, centrifuged one minute 13 000 rpm and the supernatant (0.5  $\mu$ l) was used in PCR reactions.

*Clinical sensitivity and specificity.* The clinical specificity and sensitivity was analysed with clinical samples (n=119) known to be positive for *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia* or EHEC by routine cultivation method in HUSLAB. In addition, 65 culture negative samples were analysed.

5

## Results

*Validation of the Real-Time-PCR method.* The Real-Time PCR assay was optimized and validated using the reference strains including 249 positive strains and 243 strains belonging to other major genera (Table 5). All positive control strains were correctly  
10 identified and no false positive amplification was obtained. Thus, the assay achieved 100% analytical specificity.

The clinical specificity and sensitivity was analyzed from faecal samples obtained from routine diagnostics. The routine samples positive in *Salmonella* (n=50), *Campylobacter* (n=50), *Yersinia* (n=4) and *Shigella* (n=6) as well as EHEC (n=9) were analysed in PCR  
15 and all but one gave correct amplification (Table 5). In addition, 64 culture negative samples were analysed and none were positive for *Salmonella*, *Campylobacter*, *Yersinia* or *Shigella*. Diarrhoeal *E. coli* strains could not be identified with this method since no cultivation method exists. Thus, the clinical sensitivity of the assay was 99.2% and clinical specificity was 100%.

20 Analytical sensitivity of PCR was defined by 10-fold dilutions of the template DNA mixture analyzed by PCR. The sensitivity with 40 amplification cycles was 0.1 ng/ml for EHEC and *Salmonella* and for others the sensitivity was 1 ng/ml. In addition, the sensitivity was measured from DNA obtained with boiling the bacterial strain. In that assay, the limit of detection was 5-50 CFU per reaction. These results represent the lowest  
25 concentration required for correct identification (>90% positive).

*Clinical validation of the PCR.* The real time PCR method was utilized in the analysis of the faecal samples of 146 travellers before and after the trip abroad. The data is presented in Tables 3 and 4. All samples were positive with the internal control; no PCR inhibition  
30 was detected. Of the pre-trip samples, only three (2.1%) were positive and those were

positive for EAEC. From these samples, the *E. coli* strain giving congruent PCR result was isolated. The most common findings were diarrhoeagenic *E. coli* strains (EPEC; 41.1%, EAEC; 38.4%, ETEC; 18.5%, EHEC; 7.5%), followed by *Campylobacter* (4.1%), *Salmonella* (2.1%) and *Shigella*/EIEC (1.4%).

- 5 All samples positive in *Campylobacter*, *Yersinia*, *Shigella*, *Salmonella* or EHEC were confirmed as positive either by cultivation or by sequencing of the PCR product

Two or more findings were found from 45 patients.

- 10 *Secretion of diarrhoeagenic E. coli species.* The kinetics of DEC was studied from 60 patients with no trip abroad in two months follow-up period. The same finding was found in seven out of 60 samples (four times EAEC, two times EPEC), different finding than previously was found from five patients, but all of these had travelled abroad during the follow-up period, whereas the other follow-up samples were negative. This kinetics is in line with other *Enterobacteriaceae* pathogens previously described.

15

A norovirus was detected in 5,5% patients suggesting that bacteria are predominant pathogens in traveller's diarrhoea.

## Discussion

- 20 This is the first systematic follow-up study analyzing all major pathogens associated with traveller's diarrhoea using the new molecular methods. The study design allowed the inventors to follow the consequences of travelling to the tropical countries case by case as a normal sample prior to the trip was available. The most important achievement of the study was that all the major pathogens within the patient group were able to be identified
- 25 using straight-forward modern methods, which eliminates inherent biases in comparison to results from different studies. As expected in high hygiene countries, such as Finland, there was a very low prevalence of diarrhoeal pathogens in the healthy individuals (2.1%). In a striking contrast, the inventors were able to identify a pathogen in 74% of symptomatic patients which is probably the best estimate of patients with traveller's diarrhoeal to date

which confirms that virtually all the pathogens are imported, and they do not belong to normal flora of Finnish patients. All the diarrhoeal pathogens were more frequent in the symptomatic patients than asymptomatic individuals, including all the diarrhoeagenic *E. coli* species suggesting that they all are relevant diarrhoeal pathogens, and not just  
5 reflecting a disturbance in normal flora. Of the samples from symptomatic patients, 26% were negative in all studied bacterial pathogens. This study is in line with other recent studies suggesting that diarrhoeagenic *E. coli* species are the most predominant bacterial pathogens in the patients with traveller's diarrhoea.

The study covers all the major bacterial pathogens, excluding *Aeromonas* sp, *Plesiomonas*,  
10 enterotoxigenic *Bacteroides fragilis*, *Arcobacter* and DAEC. Their relative proportion is low based on previous studies, and their pathogenic role and incidence is not fully understood yet (von Graevenitz, 2007). The Real-Time PCR method recognizes virulence genes or species specific genes of the pathogens. For example to identify the virulent EAEC, aggR gene was chosen since it is the best characterized gene contributing to  
15 aggregative pattern and diarrhoeal symptoms (Monteiro et al., 2009); (Huang et al., 2007; Mohamed et al., 2007). To cover all the possible clinically relevant target species, it was necessary to screen multiple different target genes and their conserved regions for optimal sensitivity and specificity of the assay. For example it was impossible to detect the pathogenic species among *Yersinia* and *Campylobacter* families using only one primer-  
20 probe set. The assay sensitivity and specificity were high, app. 100%, compared to independent reference methods suggesting that it could be possible to replace stool culture as primary screening method to traveller's diarrhoea. In any case, the high proportion of DEC in the patients with diarrhoea suggests that at least they should be analyzed by the method capable to identify DEC, such as PCR.

25 The assay design allows identification of 13 pathogens simultaneously using control samples in optimal conditions. The inventors were able to identify up to four different pathogens from one patient sample demonstrating that multiple pathogens can be identified in parallel. This is in line with the fact that there are often multiple pathogens causing the disease. Nevertheless, a typical PCR reaction inherently favours the most abundant target.  
30 A false negative result is most likely when there are one or two highly abundant pathogens among with one very low copy pathogen within the same multiplex reaction. This option must be controlled by other methods, and/or re-sampling and a warning reported when

applying this assay to any diagnostic purpose. To minimize the risk for false negative results, the multiplex composition was designed so that the most frequent pathogens were in the different multiplex reactions.

5 An internal positive control was tested with each sample to monitor presence of putative PCR inhibitors, but no inhibition was detected. This suggests that the semi-automated DNA extraction process is of sufficient quality, and it is suitable for stool pathogen analysis.

10 Taken together, this study is line with the other recent studies suggesting that diarrhoeagenic *E. coli* species are the predominant stool pathogens in traveller's diarrhoeae. Applying the new Real-Time PCR technology, they can be now successfully screened, among with the other stool pathogens, directly from stool samples.

Table 1. Primers. (SEQ ID NOS: 1-36 and 73-74)

Origin	Gene	Forward primer (5' ->3')	Reverse primer (5' ->3')
<b>Multiplex 1</b>			
EHEC	<i>stx1</i>	GCGTTCTTATGTAATGACTGCTGAAG	AGAAATTCCTTCCACACGAAACAGAGTC
EHEC	<i>stx2</i>	TGCATCCAGAGCAGTTCTG	CGCGTCAATCGTATACACAGG
EHEC / EPEC	<i>eae</i>	CCAGGCTTCGTCCACAGTTGC	CAGTGAACACTACCGTCAAAGTTATTACC
<i>Salmonella</i>	<i>invA</i>	GCTCTTCGGCACAAAGTAATATCAAC	TCTATTTTAAATTCCTCCGTGAAGCAAACG
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CTAATCCCAGCAACCCCAACC	CTAATCAATGTGAGACATATGATAGAAATC
<b>Multiplex 2</b>			
ETEC	<i>est</i>	AAAATTGCAAAAATCCGTTTAACTAATC	GACTGACTAAAAGAGGGGAAAG
ETEC	<i>est</i>	TCCTGAAAGCATGATAGTAGC	TTATTAATAGCACCCGGTACAAG
ETEC	<i>elt</i>	CCGGCAGAGGATGGTTACAG	TTGATTGATATTCCCTGAGATATATTGTG
<i>Yersinia</i> <i>enterocolitica</i> / <i>pseudotuberculosis</i>	<i>virF</i>	GTTTGGTACAGTTTATGGCATTTCAC	CATGGCAATATCAACAATACTCATCTTAC
<i>Yersinia</i> <i>pseudotuberculosis</i>	<i>rumB</i>	CAGGAGCATGAGGTTACAGTATG	TCTCTGGCCCCCGCACAAATG
<i>Campylobacter</i> <i>jejuni</i>	<i>rimM</i>	GAGTGAAAAGATTGTTCAAGTTG	AAAAGTCCCTCAGGTTATGC
<i>Campylobacter</i> <i>coli</i>	<i>gyrB</i>	AGTGCCCTGAACCTCAATTTG	TCGATAGGATTTTCTTCAAAAATATTTAC
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CTAATCCCAGCAACCCCAACC	CTAATCAATGTGAGACATATGATAGAAATC

<b>Multiplex 3</b>	
<i>Shigella</i> / EIEC	<i>ipaH</i>
<i>Shigella</i> / EIEC	<i>invE</i>
EAEC	<i>aggR</i>
<i>Vibrio cholerae</i>	<i>ctx</i>
<i>Oryza sativa</i> , terminal flower gene	
<p>TGGTCCATCAGGCATCAGAAAGG  TGTCTTTATAGGACATCCCTGATACTTTC  GGAAGCAATACATATCTTAGAAATGAACTC  GGGCTACAGAGATAGATATTACAGTAACTT  AG  CTAATCCCAGCAACCCAACC  CTAATCAATGTGAGACATATGATAGAAATC</p>	
<b>Multiplex 4</b>	
<i>Giardia</i> sp	<i>18S rRNA</i> <i>gene</i>
<i>Entamoeba</i> <i>histolytica</i>	<i>18S rRNA</i> <i>gene</i>
<i>Cryptosporidium</i> <i>sp.</i>	<i>cowp</i>
<i>Oryza sativa</i> , terminal flower gene	
<p>TTCCGGTCCGATCCTGCC  AGACGATCCAGTTTGTATTAG  TCTGGAAAACAATGTGTTC  CTAATCCCAGCAACCCAACC  GTTGTCCCTGAGCCGTCC  GGCATCCCTAACTCACTTAG  GGCATGTCGATTCTAATTC  CTAATCAATGTGAGACATATGATAGAAATC</p>	

Table 2. Probes for rtPCR.

Origin	Gene	Probe (5'→3') (SEQ ID NOS:37-54 and 75)	5' modification of the probe	3' modification of the probe
<b>Multiplex 1</b>				
EHEC	<i>stx1</i>	TCCATGATARTCAGGCAGGACACTACTCAACCTTCC	6-FAM	BHQ-1
EHEC	<i>stx2</i>	TTGTCACACTGTCACAGCAGAAAGCCTTACGC	6-FAM	BHQ-1
EHEC / EPEC	<i>eae</i>	AGATTAACCTCTGCCGTTCCATAATGTTGTAACCA	JOE	BHQ-1
<i>Salmonella</i>	<i>invA</i>	CCAAACCTAAAACCAGTAAAGCGGAGCAGC	TXR	BHQ-2
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CCTGCACTGGTAAGCTATG	CY5	BHQ-2
<b>Multiplex 2</b>				
ETEC	<i>est</i>	CAAATATCCCGTGAAACAACATGAC	6-FAM	BHQ-1
ETEC	<i>est</i>	AGGATTACAACACAATTCACAGCAGT	6-FAM	BHQ-1
ETEC	<i>elt</i>	AGCAGGTTTCCCACCGGATCACCA	6-FAM	BHQ-1
<i>Yersinia enterocolitica</i> / <i>pseudotuberculosis</i>	<i>virF</i>	CCTGGATAAGCGAGCGACGTATTCTCTATGC	JOE	BHQ-1
<i>Yersinia pseudotuberculosis</i> / <i>pestis</i>	<i>rumB</i>	AAACCAAAGCCGCCACACCACAG	JOE	BHQ-1
<i>Campylobacter jejuni</i>	<i>rimM</i>	AAGACCCACAGTTTACCAGT	TXR	BHQ-2
<i>Campylobacter coli</i>	<i>gyrB</i>	AACTTGGCTCTTCTTATGTGCGT	TXR	BHQ-2
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CCTGCACTGGTAAGCTATG	CY5	BHQ-2

<b>Multiplex 3</b>			
<i>Shigella</i> / EIEC	<i>ipaH</i>	TCACTCCCGACACGCCCATAGAAACGCATTT	BHQ-1
<i>Shigella</i> / EIEC	<i>invE</i>	ACAAACAGCAAAAGAGCATAGCATCCGAGAACT	BHQ-1
EAEC	<i>aggR</i>	TCCGTATATTATCATCAGGGCATCCTTTAGGCGT	BHQ-1
<i>Vibrio cholerae</i>	<i>ctx</i>	AACCTGCCAATCCATAACCAATCTGCTGCTG	BHQ-2
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CCTGCACTGGTAAGCTATG	BHQ-2
<b>Multiplex 4</b>			
<i>Giardia</i> sp	<i>18S rRNA</i> gene	ACGAAGCCCATGCATGCCCGCT	BHQ-1
<i>Entamoeba histolytica</i>	<i>18S rRNA</i> gene	ACAAAATGGCCAATTCAATCAATGAA	BHQ-1
<i>Cryptosporidium</i> sp.	<i>cowp</i>	CCTCCTAATCCAGAAATGTCCTCCAG	BHQ-2
<i>Oryza sativa</i> , terminal flower gene	<i>Ory</i> (a control)	CCTGCACTGGTAAGCTATG	BHQ-2

Table 3. Findings before and after the trip abroad.

	Before trip abroad	After trip abroad
	number (%)	number (%)
<i>Campylobacter</i>	0 (0)	6 (4.1)
<i>Salmonella</i>	0 (0)	3 (2.1)
<i>Shigella</i> / EIEC	0 (0)	2 (1.4)
<i>Yersinia</i>	0 (0)	0 (0)
EHEC	0 (0)	11 (7.5)
EAEC	3 (2.1)	56 (38.4)
EPEC	0 (0)	60 (41.1)
ETEC	0 (0)	27 (18.5)
<i>Vibrio</i>	0 (0)	0 (0)
Total	3 (2.1)	165 (113.0)

Table 4. Findings after trip abroad with or without symptoms.

	asymptomatic	symptomatic
	number (%)	number (%)
<i>Campylobacter</i>	0 (0)	6 (4.1)
<i>Salmonella</i>	1 (0.7)	2 (1.4)
<i>Shigella</i> / EIEC	0 (0)	2 (1.4)
<i>Yersinia</i>	0 (0)	0 (0)
<i>Vibrio</i>	0 (0)	0 (0)
EHEC	4 (2.7)	7 (4.8)
EAEC	15 (10.3)	40 (27.4)
EPEC	19 (13.0)	39 (26.7)
ETEC	5 (3.4)	22 (15.1)
Total	31 (60.8)	69 (74.2)

Table 5. A summary of known positive control strains and samples.

	PCR positive	Total
<b>Pure control strains</b>		
Positive control strains	246	246
Negative control strains	0	243
Total		489
<b>Feecal control samples</b>		
Positive		
<i>Campylobacter</i>	52	53
<i>Salmonella</i>	50	50
<i>Yersinia</i>	5	5
<i>Shigella</i>	6	6
EHEC	9	9
Negative	0	65

Table 6. Amplicons (5'-&gt;3') amplified in target organisms.

EHEC stx1

GCGTTCTTATGTAATGACTGCTGAAGATGTTGATCTTACATTGAACTGGGGAAGGTTGAGTAGTG  
 TCCTGCCTGATTATCATGGACAAGACTCTGTTTCGTGTAGGAAGAATTTCT  
 (SEQ ID NO:55)

EHEC stx2

TGCATCCAGAGCAGTTCTGCGTTTTGTCCTGTACAGCAGAAGCCTTACGCTTCAGGCAGATACA  
 GAGAGAATTTTCGTGAGGCACTGTCTGAACTGCTCCTGTGTATACGATGACGCCG (SEQ ID  
 NO:56)

EHEC/EPEC eae

CCAGGCTTCGTACAGTTGCAGGCCTGGTTACAACATTATGGAACGGCAGAGGTTAATCTGCAGA  
 GTGGTAATAACTTTGACGGTAGTTCCTG (SEQ ID NO:57)

Salmonella invA

GCTCTTCGGCACAAGTAATATCAACGGTACAGTCTCTGTAGAGACTTTATCGAGATCGCCAATCA  
GTCCTAACGACGACCCTTCTTTTTCTCAATACTGAGCGGCTGCTCGCCTTGCTGGTTTTAGGTTT  
GGCGGCGCTACGTTTTGCTTACGGAATTTAAAATAGA  
(SEQ ID NO:58)

Shigella / EIEC ipaH

TGGTCCATCAGGCATCAGAAGGCCTTTTCGATAATGATACCGGCGCTCTGCTCTCCCTGGGCAGG  
GAAATGTTCCGCCTCGAAATTCTGGAGGACATTGCCGGGATAAAAGTCAGAACTCTCCATTTTGT  
GGATGAGATAGAAGTCTACCTGGCCTTCCAGACCATGCTCGCAGAGAACTTCAGCTCTCCACTG  
CCGTGAAGGAAATGCGTTTCTATGGCGTGTGGGAGTGACAGCAAATGACCTCCGCACTGCC  
(SEQ ID NO:59)

Shigella / EIEC invE

TGTCTTTATAGGACATCCCTGATACTTTCAGAAAATTAAGACCAATACCAAGTTCTCGGATGCTAT  
GCTCTTTTGCTGTTTGTATATCGTTTGCTAGTTTTCTGGCATCAAGAGTAGATA (SEQ ID NO:60)

ETEC est

AAAATTGCAAATCCGTTTAACTAATCTCAAATATCCGTGAAACAACATGACGGGAGGTAACATG  
AAAAAGCTAATGTTGGCAATTTTTATTTCTGTATTATCTTCCCCTCTTTTAGTCAGTC (SEQ ID  
NO:61)

ETEC est

TCCTGAAAGCATGAATAGTAGCAATTACTGCTGTGAATTGTGTTGTAATCCTGCTTGTACCGGGTG  
CTATTAATAA (SEQ ID NO:62)

ETEC elt

CCGGCAGAGGATGGTTACAGATTAGCAGGTTTCCCACCGGATCACCAAGCTTGGAGAGAAGAAC  
CCTGGATTCATCATGCACCACAAGGTTGTGGAAATTCATCAAGAACAATTACAGGTGATACTTGT  
AATGAGGAGACCCAGAATCTGAGCACAATATATCTCAGGGAATATCAATCAA (SEQ ID NO:63)

EAEC aggR

GGAAGCAATACATATCTTAGAAATGAACTCATATTTCTTGAGAGAGGAATAAATATATCAGTAAG  
ATTGCAAAGAAGAAATCAACAGTAAATCCATTTATCGCAATCAGATTAAGCAGCGATACATTAA

GACGCCTAAAGGATGCCCTGATGATAATATACGGAATATCAAAAGTAGATGCTTGCAGTTGTCCG  
A (SEQ ID NO:64)

*Campylobacter jejuni* rimM

GAGTGAAAAAGATTTTGTTC AAGTTGCAAAACTTGGTAAA ACTGTGGGTCTTAAGGGTTATGTAA  
AATTGCATAACCTGAGCGACTTTT (SEQ ID NO:65)

*Campylobacter coli* gyrB

AGTGCCTGAACCTCAATTTGAAGGACAAACTAAAGGAAA ACTTGGCTCTTCTTATGTGCGTCCTAT  
AGTTTCAAAAGCAAGTTTTGAATATCTTAGTAAATATTTTGAAGAAAATCCTATCGA (SEQ ID  
NO:66)

*Yersinia enterocolitica / pseudotuberculosis* virF

GTTTGGTACAGTTTATGGCATTTCACCACGCGCCTGGATAAGCGAGCGACGTATTCTCTATGCTCA  
CCAATTACTTCTTAATTGTAAGATGAGTATTGTTGATATTGCCATG (SEQ ID NO:67)

*Yersinia pseudotuberculosis / pestis* rumB

CAGGAGCATGAGGTTACAGTATGTGGGATCTGTTCTGTGGTGTGGGCGGCTTTGGTTTACATTG  
TGCGGGGCCAGAGA (SEQ ID NO:68)

*Vibrio cholerae* ctx

GGGCTACAGAGATAGATATTACAGTAACTTAGATATTGCTCCAGCAGCAGATGGTTATGGATTGG  
CAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGG (SEQ ID NO:69)

*Giardia lamblia* 18S rRNA gene

TTCCGGTCGATCCTGCCGGAATCCGACGCTCTCCCAAGGACACAAGCCATGCATGCCCCGCGCAC  
CCGGGAGGCGGCGGACGGCTCAGGACAAC (SEQ ID NO:70)

*Entamoeba histolytica* 18S rRNA gene

AGACGATCCAGTTTGTATTAGTACAAAATGGCCAATTTATTTAAATGAATTGAGAAATGACATTCT  
AAGTGAGTTAGGATGCC (SEQ ID NO:71)

Cryptosporidium sp. cowp

TCTGGAAAACAATGTGTTCAATCAGACACAGCTCCTCCTAATCCAGAATGTCCTCCAGGCACTATA  
CTGGAGAATGGCACATGTAAATTAATTCAACAAATTGATACCGTTTGTCTTCTGGTTTTGTTGAA  
GAAGGAAATAGATGTGTTCAATATCTCCCTGCAAATAAAATCTGTCCTCCTGGATTCAATTTGTCA  
GGACAACAATGTATGGCACCAGAATCAGCTGAATTAGAATCGACATGCC (SEQ ID NO:72)

Table 7. Primers and a probe for *Oryza sativa*, terminal flower gene control

CTAATCCCAGCAACCCAACC (SEQ ID NO:73)

CTAATCAATGTGAGACATATGATAGAAATC (SEQ ID NO:74)

CCTGCACTGGTAAGCTATG (SEQ ID NO:75)

Table 8. Distribution of ETEC toxin variants in control strains and patient samples. The results show that all ETEC variants are detected by at least one of the present primer pairs.

ST= Heat Stable Toxin

LT= Heat Labile Toxin

Oligonucleotide pairs amplifying the target (strain/patient sample)  
ST variant 1                      ST variant 2                      Heat labile toxin (LT)

Strain name	Origin	est_005 (SEQ ID NOS:13 and 14)	estlab_004 (SEQ ID NOS:15 and 16)	elt_001 (SEQ ID NOS:17 and 18)
JA4	Reference strain THL	-	+	+
JA24	Reference strain THL	-	-	+
JA25	Reference strain THL	-	-	+
JA26	Reference strain THL	-	-	+
JA27	Reference strain THL	-	-	+
JA28	Reference strain THL	-	-	+
JA32	Reference strain THL	-	+	-
JA35	Control species, Germany	+	-	+
JA36	Control species, Germany	+	-	+
JA48	Patient sample	-	+	+
JA50	Patient sample	+	-	-
JA53	Patient sample	-	+	-
JA58	Patient sample	+	-	-
JA61	Patient sample	+	-	-
JA64	Patient sample	-	+	+
JA85	Patient sample	+	-	-
JA88	Patient sample	-	+	-
JA122	Patient sample	+	-	-
JA124	Patient sample	-	+	+
mixB	control DNA mixture	+	+	+

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## CLAIMS

1. Method for determining the presence of diarrhoea causing pathogens in a biological sample comprising the steps of:

i) contacting the sample or nucleic acid isolated therefrom with primer pairs in a multiplex PCR assay comprising two or more separate PCR reactions, wherein the primers of said primer pairs amplify each of the ETEC amplicons as defined by SEQ ID NOS:61-63 at least partly;

ii) performing a polymerase chain reaction with reaction mixes obtained from step i) so that the target sequences of diarrhoea causing pathogens are specifically amplified, if said sequences are present in the sample; and

iii) detecting the presence of amplified target sequences in the reaction mix, wherein the presence of any of the target sequences is indicative of the presence of diarrhoea causing pathogens in the sample.

2. The method according to claim 1, wherein in step i) the primers of said primer pairs further amplify each of the Campylobacter amplicons as defined by SEQ ID NOS:65-66 at least partly.

3. The method according to claim 1 or 2, wherein in step i) said primer pairs further amplify each of the Yersinia amplicons as defined by SEQ ID NOS: 67-68 at least partly.

4. The method according to any one of claims 1-3, wherein in step i) the sample or isolated nucleic acid therefrom is contacted with primer pairs comprising or consisting of at least one of the following sequences:

G) forward primer: 5'-AAAATTGCAAATCCGTTTAACTAATC-3' (SEQ ID NO:13),

reverse primer: 5'-GACTGACTAAAAGAGGGGAAAG-3' (SEQ ID NO:14);

H) forward primer: 5'-TCCTGAAAGCATGAATAGTAGC-3' (SEQ ID NO:15),

reverse primer: 5'-TTATTAATAGCACCCGGTACAAG-3' (SEQ ID NO:16);

I) forward primer: 5'-CCGGCAGAGGATGGTTACAG-3' (SEQ ID NO:17),

reverse primer: 5'-TTGATTGATATTCCTGAGATATATTGTG-3' (SEQ ID NO:18).

5. The method according to claim 4, wherein the sample or isolated nucleic acid therefrom is contacted with primers each consisting of at least 10 contiguous nucleotides present in nucleotide sequences as set forth in SEQ ID NOS: 13-18.

6. The method according to claim 4, wherein said primer pairs further comprise or consist of the following sequences

K) forward primer: 5'-GAGTGAAAAAGATTTTGTTC AAGTTG-3' (SEQ ID NO:21),

reverse primer: 5'-AAAAGTCGCTCAGGTTATGC-3' (SEQ ID NO:22); and

L) forward primer: 5'-AGTGCCTGAACCTCAATTTG-3' (SEQ ID NO:23),

reverse primer: 5'-TCGATAGGATTTTCTTCAA AATATTTAC-3' (SEQ ID NO:24).

7. The method according to any one of claims 4-6, wherein in step i) the sample or isolated nucleic acid therefrom is contacted with further primer pairs comprising or consisting of at least one of the following sequences:

A) forward primer: 5'-GCGTTCTTATGTAATGACTGCTGAAG-3' (SEQ ID NO:1),

reverse primer: 5'-AGAAATTCTTCCTACACGAACAGAGTC-3' (SEQ ID NO:2);

B) forward primer: 5'-TGCATCCAGAGCAGTTCTGC-3' (SEQ ID NO:3),

reverse primer: 5'-CGGCGTCATCGTATACACAGG-3' (SEQ ID NO:4);

C) forward primer: 5'-CCAGGCTTCGTCACAGTTGC-3' (SEQ ID NO:5),

reverse primer: 5'-CAGTGA ACTACCGTCAAAGTTATTACC-3' (SEQ ID NO:6);

D) forward primer: 5'-GCTCTTCGGCACAAGTAATATCAAC-3' (SEQ ID NO:7),  
reverse primer: 5'-TCTATTTTAAATTCCGTGAAGCAAACG-3' (SEQ ID NO:8);

E) forward primer: 5'-TGGTCCATCAGGCATCAGAAGG-3' (SEQ ID NO:9),  
reverse primer: 5'-GGCAGTGCGGAGGTCATTTG-3' (SEQ ID NO:10);

F) forward primer: 5'-TGTCTTTATAGGACATCCCTGATACTTTC-3' (SEQ ID  
NO:11),

reverse primer: 5'-TATCTACTCTTGATGCCAGAAACTAGC-3' (SEQ ID NO:12);

J) forward primer: 5'-GGAAGCAATACATATCTTAGAAATGAACTC-3' (SEQ ID  
NO:19),

reverse primer: 5'-TCGGACAACGCAAGCATCTAC-3' (SEQ ID NO:20);

M) forward primer: 5'-GTTTGGTACAGTTTATGGCATTTCAC-3' (SEQ ID NO:25),

reverse primer: 5'-CATGGCAATATCAACAATACTCATCTTAC-3' (SEQ ID NO:26);

N) forward primer: 5'-CAGGAGCATGAGGTTACAGTATG-3' (SEQ ID NO:27),

reverse primer: 5'-TCTCTGGCCCCGCACAATG-3' (SEQ ID NO:28);

O) forward primer: 5'-GGGCTACAGAGATAGATATTACAGTAACTTAG-3' (SEQ ID  
NO:29),

reverse primer: 5'-CCACGGCTCTTCCCTCCAAG-3' (SEQ ID NO:30);

P) forward primer: 5'-TTCCGGTCGATCCTGCC-3' (SEQ ID NO:31),

reverse primer: 5'-GTTGTCCTGAGCCGTCC-3' (SEQ ID NO:32);

Q) forward primer: 5'-AGACGATCCAGTTTGTATTAG-3' (SEQ ID NO:33),

reverse primer: 5'-GGCATCCTAACTCACTTAG-3' (SEQ ID NO:34); and

R) forward primer: 5'-TCTGGAAAACAATGTGTTC-3' (SEQ ID NO:35),

reverse primer: 5'-GGCATGTCGATTCTAATTC-3' (SEQ ID NO:36).

8. The method according to claim 7, wherein each of said nucleic acid primers consists of at least 10 contiguous nucleotides present in nucleotide sequences as set forth in SEQ ID NOS: 1-36.

9. The method according to claim 7 or 8, wherein the presence of the amplified target sequence, i.e. the product, of each of primer pairs in the PCR reaction indicates the presence of diarrhoea causing pathogens in the sample in the following way:

- the product of primer pair A) or B) indicates the presence of EHEC;
- the product of primer pair C) indicates the presence of EHEC/EPEC;
- the product of primer pair D) indicates the presence of *Salmonella*;
- the product of primer pair E) or F) indicates the presence of *Shigella*/EIEC;
- the product of primer pair G), H), or I) indicates the presence of ETEC;
- the product of primer pair J) indicates the presence of EAEC;
- the product of primer pair K) indicates the presence of *Campylobacter jejuni*;
- the product of primer pair L) indicates the presence of *Campylobacter coli*;
- the product of primer pair M) indicates the presence of *Yersinia enterocolitica/pseudotuberculosis*;
- the product of primer pair N) indicates the presence of *Yersinia pseudotuberculosis/pestis*;
- the product of primer pair O) indicates the presence of *Vibrio cholerae*;
- the product of primer pair P) indicates the presence of *Giardia lamblia*;
- the product of primer pair Q) indicates the presence of *Entamoeba histolytica*; and

- the product of primer pair R) indicates the presence of *Cryptosporidium* sp.

10. The method according to claim 1, wherein said biological sample is a stool sample or a food sample.

11. The method according to claim 1, wherein the primers comprise the sequences of primer pairs and each primer of said primer pairs is less than 50 nucleotides long.

12. The method according to any one of claims 1-8, wherein said multiplex PCR assay is performed as a real-time polymerase chain reaction and nucleic acid probes comprising or consisting of the following sequences are specifically used with each of primer pairs:

- the probe for primer pair A):

5'-TCCATGATARTCAGGCAGGACACTACTCAACCTTCC-3' (SEQ ID NO:37)

- the probe for primer pair B):

5'-TTGTCACTGTCCACAGCAGAAGCCTTACGC-3' (SEQ ID NO:38)

- the probe for primer pair C):

5'-AGATTAACCTCTGCCGTTCCATAATGTTGTAACCA-3' (SEQ ID NO:39)

- the probe for primer pair D):

5'-CCAAACCTAAAACCAGTAAAGGCGAGCAGC-3' (SEQ ID NO:40)

- the probe for primer pair E):

5'-TCACTCCCGACACGCCATAGAAACGCATTT-3' (SEQ ID NO:41)

- the probe for primer pair F):

5'-ACAAACAGCAAAGAGCATAGCATCCGAGAACT-3' (SEQ ID NO:42)

- the probe for primer pair G):

5'-CAAATATCCGTGAAACAACATGAC-3' (SEQ ID NO:43)

- the probe for primer pair H):

5'-AGGATTACAACACAAATTCACAGCAGT-3' (SEQ ID NO:44)

- the probe for primer pair I):

5'-AGCAGGTTTCCCACCGGATCACCA-3' (SEQ ID NO:45)

- the probe for primer pair J):

5'-TCCGTATATTATCATCAGGGCATCCTTTAGGCGT-3' (SEQ ID NO:46)

- the probe for primer pair K):

5'-AAGACCCACAGTTTTACCAAGTTTT-3' (SEQ ID NO:47)

- the probe for primer pair L):

5'-AACTTGGCTTCTTATGTGCGT-3' (SEQ ID NO:48)

- the probe for primer pair M):

5'-CCTGGATAAGCGAGCGACGTATTCTCTATGC-3' (SEQ ID NO:49)

- the probe for primer pair N):

5'-AAACCAAAGCCGCCACACCACAG-3' (SEQ ID NO:50)

- the probe for primer pair O):

5'-AACCTGCCAATCCATAACCATCTGCTGCTG-3' (SEQ ID NO:51)

- the probe for primer pair P):

5'-ACGAAGCCATGCATGCCCGCT-3' (SEQ ID NO:52)

- the probe for primer pair Q):

5'-ACAAAATGGCCAATTCATTCAATGAA-3' (SEQ ID NO:53)

- the probe for primer pair R):

5'-CCTCCTAATCCAGAATGTCCTCCAG-3' (SEQ ID NO:54)

wherein the underlined nucleotides are preferably modified nucleotides increasing melting temperature,  $T_m$ , of the probes.

13. The method according to claim 12, wherein each of said nucleic acid probes consists of at least 10 contiguous nucleotides present in nucleotide sequences as set forth in SEQ ID NOS: 37-54.

14. The method according to claim 9, wherein primer pairs A) to F) and G) to N) are in separate PCR reactions.

15. The method according to claim 9, wherein the method comprises the following PCR reactions: the first reaction with primer pairs A) to D), the second reaction with primer pairs K) to L), the third reaction with primer pairs E), F), M), N) and O) and the fourth reaction with primer pairs P) to R).

16. The method according to claim 9, wherein the method comprises the following PCR reactions: the first reaction with primer pairs A) to D), the second reaction with primer pairs G) to I and K) to N), the third reaction with primer pairs E), F), J) and O) and the fourth reaction with primer pairs P) to R) .

17. Use of primer pairs for the detection of diarrhoea causing pathogens in a sample, wherein the primers of said primer pairs comprise or consist of any one of the following sequences:

A) forward primer: 5'-GCGTTCTTATGTAATGACTGCTGAAG-3' (SEQ ID NO:1),

reverse primer: 5'-AGAAATTCTTCCTACACGAACAGAGTC-3' (SEQ ID NO:2);

- B) forward primer: 5'-TGCATCCAGAGCAGTTCTGC-3' (SEQ ID NO:3),  
reverse primer: 5'-CGGCGTCATCGTATACACAGG-3' (SEQ ID NO:4);
- C) forward primer: 5'-CCAGGCTTCGTCACAGTTGC-3' (SEQ ID NO:5),  
reverse primer: 5'-CAGTGA ACTACCGTCAAAGTTATTACC-3' (SEQ ID NO:6);
- D) forward primer: 5'-GCTCTTCGGCACAAGTAATATCAAC-3' (SEQ ID NO:7),  
reverse primer: 5'-TCTATTTTAAATTCCGTGAAGCAAACG-3' (SEQ ID NO:8);
- E) forward primer: 5'-TGGTCCATCAGGCATCAGAAGG-3' (SEQ ID NO:9),  
reverse primer: 5'-GGCAGTGCGGAGGTCATTTG-3' (SEQ ID NO:10);
- F) forward primer: 5'-TGTCTTTATAGGACATCCCTGATACTTTC-3' (SEQ ID NO:11),  
reverse primer: 5'-TATCTACTCTTGATGCCAGAAAAGTAGC-3' (SEQ ID NO:12);
- G) forward primer: 5'-AAAATTGCAAATCCGTTTAACTAATC-3' (SEQ ID NO:13),  
reverse primer: 5'-GACTGACTAAAAGAGGGGAAAG-3' (SEQ ID NO:14);
- H) forward primer: 5'-TCCTGAAAGCATGAATAGTAGC-3' (SEQ ID NO:15),  
reverse primer: 5'-TTATTAATAGCACCCGGTACAAG-3' (SEQ ID NO:16);
- I) forward primer: 5'-CCGGCAGAGGATGGTTACAG-3' (SEQ ID NO:17),  
reverse primer: 5'-TTGATTGATATCCCTGAGATATATTGTG-3' (SEQ ID NO:18);
- J) forward primer: 5'-GGAAGCAATACATATCTTAGAAATGAACTC-3' (SEQ ID NO:19),  
reverse primer: 5'-TCGGACA ACTGCAAGCATCTAC-3' (SEQ ID NO:20);
- K) forward primer: 5'-GAGTGAAAAAGATTTTGTTC AAGTTG-3' (SEQ ID NO:21),  
reverse primer: 5'-AAAAGTCGCTCAGGTTATGC-3' (SEQ ID NO:22);
- L) forward primer: 5'-AGTGCCTGAACCTCAATTTG-3' (SEQ ID NO:23),

reverse primer: 5'-TCGATAGGATTTTCTTCAAATATTTAC-3' (SEQ ID NO:24);

M) forward primer: 5'-GTTTGGTACAGTTTATGGCATTTCAC-3' (SEQ ID NO:25),

reverse primer: 5'-CATGGCAATATCAACAATACTCATCTTAC-3' (SEQ ID NO:26);

N) forward primer: 5'-CAGGAGCATGAGGTTTACAGTATG-3' (SEQ ID NO:27),

reverse primer: 5'-TCTCTGGCCCCGCACAATG-3' (SEQ ID NO:28);

O) forward primer: 5'-GGGCTACAGAGATAGATATTACAGTAACTTAG-3' (SEQ ID NO:29),

reverse primer: 5'-CCACGGCTCTTCCCTCCAAG-3' (SEQ ID NO:30);

P) forward primer: 5'-TTCCGGTCGATCCTGCC-3' (SEQ ID NO:31),

reverse primer: 5'-GTTGTCCTGAGCCGTCC-3' (SEQ ID NO:32);

Q) forward primer: 5'-AGACGATCCAGTTTGTATTAG-3' (SEQ ID NO:33),

reverse primer: 5'-GGCATCCTAACTCACTTAG-3' (SEQ ID NO:34); and

R) forward primer: 5'-TCTGGAAAACAATGTGTTC-3' (SEQ ID NO:35),

reverse primer: 5'-GGCATGTCGATTCTAATTC-3' (SEQ ID NO:36);

wherein said detection is a multiplex PCR assay comprising two or more separate PCR reactions.

18. The use of claim 17, wherein said sample is a stool sample or a food sample.

19. The use of claim 17 or 18, wherein at least the primer pairs detecting the presence of ETEC, *Campylobacter jejuni*, and *Campylobacter coli* are used for the detection.

20. A nucleotide primer consisting of any of the primer sequences as set forth in SEQ ID NOS:1-36.

21. A nucleotide primer pair consisting of the sequences from any of primer pairs as defined in claim 17 and SEQ ID NOS:1-36.

22. A nucleotide probe consisting of any of the probe sequences as set forth in SEQ ID NOS:37-54.

23. Use of a nucleotide primer, primer pair or probe as defined in any of claims 17-19 for determining the presence of diarrhoea causing pathogens in a sample.

24. A kit for determining the presence of diarrhoea causing pathogens in a sample, wherein said kit comprises a nucleotide primer according to claim 20, a nucleotide primer pair according to claim 21 or a nucleotide probe according to claim 22.

25. The kit according to claim 24, comprising other PCR reagent components selected from the group consisting of: a polymerase, nucleotides, buffer, salts, detergents and/or other additives.

26. The kit according to claim 24 or 25, further comprising one or more control primers, probes or nucleotide sequences.

27. The kit according to any one of claims 24-26 for the detection of the presence of at least pathogens ETEC, *Campylobacter jejuni*, and *Campylobacter coli*, the kit comprising or consisting of any of the primer sequences from primer pairs

G) forward primer: 5'-AAAATTGCAAAATCCGTTTAACTAATC-3' (SEQ ID NO:13),  
reverse primer: 5'-GACTGACTAAAAGAGGGGAAAG-3' (SEQ ID NO:14);

H) forward primer: 5'-TCCTGAAAGCATGAATAGTAGC-3' (SEQ ID NO:15),  
reverse primer: 5'-TTATTAATAGCACCCGGTACAAG-3' (SEQ ID NO:16);

I) forward primer: 5'-CCGGCAGAGGATGGTTACAG-3' (SEQ ID NO:17),  
reverse primer: 5'-TTGATTGATATTCCTGAGATATATTGTG-3' (SEQ ID NO:18);

K) forward primer: 5'-GAGTGAAAAAGATTTTGTTC AAGTTG-3' (SEQ ID NO:21),  
reverse primer: 5'-AAAAGTCGCTCAGGTTATGC-3' (SEQ ID NO:22);

L) forward primer: 5'-AGTGCCTGAACCTCAATTTG-3' (SEQ ID NO:23),  
reverse primer: 5'-TCGATAGGATTTTCTTCAAATATTTAC-3' (SEQ ID NO:24);

M) forward primer: 5'-GTTTGGTACAGTTTATGGCATTTCAC-3' (SEQ ID NO:25),  
reverse primer: 5'-CATGGCAATATCAACAATACTCATCTTAC-3' (SEQ ID NO:26);  
and

N) forward primer: 5'-CAGGAGCATGAGGTTACAGTATG-3' (SEQ ID NO:27),  
reverse primer: 5'-TCTCTGGCCCCGCACAATG-3' (SEQ ID NO:28).

28. Method for determining the presence of diarrhoea causing pathogens in a biological sample comprising the steps of:

i) contacting the sample or nucleic acid isolated therefrom with primer pairs in a multiplex PCR assay comprising two or more separate PCR reactions, wherein the primers of said primer pairs comprise or consist of any one of the following sequences:

A) forward primer: 5'-GCGTTCTTATGTAATGACTGCTGAAG-3' (SEQ ID NO:1),  
reverse primer: 5'-AGAAATTCTTCCTACACGAACAGAGTC-3' (SEQ ID NO:2);

B) forward primer: 5'-TGCATCCAGAGCAGTTCTGC-3' (SEQ ID NO:3),

reverse primer: 5'-CGGCGTCATCGTATACACAGG-3' (SEQ ID NO:4);

C) forward primer: 5'-CCAGGCTTCGTCACAGTTGC-3' (SEQ ID NO:5),

reverse primer: 5'-CAGTGA ACTACCGTCAAAGTTATTACC-3' (SEQ ID NO:6);

D) forward primer: 5'-GCTCTTCGGCACAAGTAATATCAAC-3' (SEQ ID NO:7),

reverse primer: 5'-TCTATTTTAAATTCCGTGAAGCAAACG-3' (SEQ ID NO:8);

E) forward primer: 5'-TGGTCCATCAGGCATCAGAAGG-3' (SEQ ID NO:9),

reverse primer: 5'-GGCAGTGCGGAGGTCATTTG-3' (SEQ ID NO:10);

F) forward primer: 5'-TGTCTTTATAGGACATCCCTGATACTTTC-3' (SEQ ID NO:11),

reverse primer: 5'-TATCTACTCTTGATGCCAGAAACTAGC-3' (SEQ ID NO:12);

G) forward primer: 5'-AAAATTGCAAATCCGTTTAACTAATC-3' (SEQ ID NO:13),

reverse primer: 5'-GACTGACTAAAAGAGGGGAAAG-3' (SEQ ID NO:14);

H) forward primer: 5'-TCCTGAAAGCATGAATAGTAGC-3' (SEQ ID NO:15),

reverse primer: 5'-TTATTAATAGCACCCGGTACAAG-3' (SEQ ID NO:16);

I) forward primer: 5'-CCGGCAGAGGATGGTTACAG-3' (SEQ ID NO:17),

reverse primer: 5'-TTGATTGATATCCCTGAGATATATTGTG-3' (SEQ ID NO:18);

J) forward primer: 5'-GGAAGCAATACATATCTTAGAAATGAACTC-3' (SEQ ID NO:19),

reverse primer: 5'-TCGGACA ACTGCAAGCATCTAC-3' (SEQ ID NO:20);

K) forward primer: 5'-GAGTGAAAAAGATTTTGTTC AAGTTG-3' (SEQ ID NO:21),

reverse primer: 5'-AAAAGTCGCTCAGGTTATGC-3' (SEQ ID NO:22);

L) forward primer: 5'-AGTGCCTGAACCTCAATTTG-3' (SEQ ID NO:23),

reverse primer: 5'-TCGATAGGATTTTCTTCAAATATTTAC-3' (SEQ ID NO:24);

M) forward primer: 5'-GTTTGGTACAGTTTATGGCATTTCAC-3' (SEQ ID NO:25),  
reverse primer: 5'-CATGGCAATATCAACAATACTCATCTTAC-3' (SEQ ID NO:26);

N) forward primer: 5'-CAGGAGCATGAGGTTACAGTATG-3' (SEQ ID NO:27),  
reverse primer: 5'-TCTCTGGCCCCGCACAATG-3' (SEQ ID NO:28);

O) forward primer: 5'-GGGCTACAGAGATAGATATTACAGTAACTTAG-3' (SEQ ID NO:29),

reverse primer: 5'-CCACGGCTCTTCCCTCCAAG-3' (SEQ ID NO:30);

P) forward primer: 5'-TTCCGGTCGATCCTGCC-3' (SEQ ID NO:31),

reverse primer: 5'-GTTGTCCTGAGCCGTCC-3' (SEQ ID NO:32);

Q) forward primer: 5'-AGACGATCCAGTTTGTATTAG-3' (SEQ ID NO:33),

reverse primer: 5'-GGCATCCTAACTCACTTAG-3' (SEQ ID NO:34); and

R) forward primer: 5'-TCTGGAAAACAATGTGTTC-3' (SEQ ID NO:35),

reverse primer: 5'-GGCATGTCTGATTCTAATTC-3' (SEQ ID NO:36);

ii) performing a polymerase chain reaction with reaction mixes obtained from step i) so that the target sequences of diarrhoea causing pathogens are specifically amplified, if said sequences are present in the sample; and

iii) detecting the presence of amplified target sequences in the reaction mix, wherein the presence of any of the target sequences is indicative of the presence of diarrhoea causing pathogens in the sample.

29. The method according to claim 28, wherein each of said nucleic acid probes consists of at least 10 contiguous nucleotides present in nucleotide sequences as set forth in SEQ ID NOS: 1-36.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI2013/050716

A. CLASSIFICATION OF SUBJECT MATTER		
IPC: see extra sheet		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC: C07H, C12N, C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE, DK, FI, NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EPO-Internal, PAJ, WPI data, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, INSPEC, MEDLINE, Registry, DGENE		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	West D.M et al., "Rapid detection of Escherichia coli virulence factor genes using multiplex real-time TaqMan PCR assays", Veterinary Microbiology, 2007, 122, pages 323-331; whole document; especially the abstract; table 2, the primers used for amplifying LT	20, 23-27
A	--	1-19, 21-22, 28-29
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
22-11-2013		25-11-2013
Name and mailing address of the ISA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86		Authorized officer Terese Sandström Telephone No. + 46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI2013/050716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Zhang H. et al., "Taqman triplex real-time PCR assay for rapid detection of diarrheagenic Escherichia coli in raw milk", Journal of Northeast Agricultural University, 2010, Vol. 41, pages 108-115; whole document; especially the abstract; table 1, primer ItF	20, 23-27
A	--	1-19, 21-22, 28-29
X	Fujioka M. et al., "Direct detection of diarrheagenic Escherichia coli in patient stool specimens by developed Multiplex PCRs. For the establishment of surveillance system of diarrheagenic Escherichia coli", Igaku Kensa, 2008, Vol. 57, pages 1041-1046; whole document; especially the abstract and primer LT-1 at page 1042	22-26
A	--	1-21, 27-28
X	Fukushima H. et al., "Duplex real-time SYBR green PCR assays for detection of 17 species of food- or waterborne pathogens in stools", Journal of Clinical Microbiology, 2003, Vol. 41, pages 5134-5146; whole document; especially the abstract; table 2, primer LT-1	22-26
A	--	1-21, 27-28
A	Lothigius Å. et al., "Enterotoxigenic Escherichia coli is detectable in water samples from an endemic area by real-time PCR, Journal of Applied Microbiology, 2008, Vol. 104, pages 1128-1136; whole document; especially table 2	1-29
A	--	
A	Kodama H. et al., "Study on real-time PCR assays for simultaneous detection of food-borne pathogens", Hokuriku Koshu Eisei Gakkaishi, 2008, Vol. 34, pages 65-72; whole document; especially table 4	1-29
A	--	
A	Hidaka A. et al., "Multiplex real-time PCR for exhaustive detection of diarrhoeagenic Escherichia coli", Journal of Applied Microbiology, 2009, Vol.106, pages 410-420; whole document; especially table 4	1-29
	--	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI2013/050716

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Reischl U. et al., "Real-Time Fluorescence PCR Assays for Detection and Characterization of Heat-Labile I and Heat-Stable I Enterotoxin Genes from Enterotoxigenic Escherichia coli", Journal of Clinical Microbiolog, 2004, Vol. 42, pages 4092-4100; whole document; especially table 2 --	1-29
A	CN 101831491 A (BEIJING TAG ARRAY MOLECULAR TEST CO LTD), 15 September 2010 (2010-09-15); (abstract) Retrieved from: WPI database; Original document: page 24 --	1-29
P, X	Antikainen J. et al., "A quantitative polymerase chain reaction assay for rapid detection of 9 pathogens directly from stools of travelers with diarrhea", Clinical Gastroenterology and Hepatology, published online in May 2013, Vol. 11, pages 1300-1307; whole document	1-7, 9-12, 14, 17-28
P, A	-- -----	8, 13, 15-16, 29

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/FI2013/050716**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3.  Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

The following separate inventions were identified:

1: Claims 1-16, 17-18 (partly), 19, 20-28 (partly) and 29 directed to a method for determining the presence of diarrhoea causing pathogens in a biological sample comprising a multiplex PCR assay comprising two or more separate PCR reactions, involving primer pairs amplifying each of the enterotoxigenic E. coli (ETEC) amplicons as defined by SEQ ID NOS: 61-63 at least partly.

.../...

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
  
1-16, 17-18 (partly), 19, 20-28 (partly) and 29

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

**Continuation of:** Box No. III

The invention is further directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 13-18 and 43-45.

2: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 1-4 and 37-38, which are useful in the detection of EHEC amplicons defined by SEQ ID NOS: 55 and 56.

3: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 5-6 and 39, which are useful in the detection of EHEC/EPEC amplicon defined by SEQ ID NO: 57.

4: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 7-8 and 40, which are useful in the detection of Salmonella amplicon defined by SEQ ID NO: 58.

5: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 9-12 and 41-42, which are useful in the detection of Shigella/EIEC amplicons defined by SEQ ID NOS: 59 and 60.

6: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 19-20 and 46, which are useful in the detection of EAEC amplicon defined by SEQ ID NO: 64.

7: Claims 17-18 (partly) and 20-28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 21-24 and 47-48, which are useful in the detection of Campylobacter amplicons defined by SEQ ID NOS: 65 and 66.

8: Claims 17-18 (partly) and 20-28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 25-28 and 49-50, which are useful in the detection of Yersinia amplicons defined by SEQ ID NOS: 67 and 68.

9: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 29-30 and 51, which are useful in the detection of Vibrio cholerae amplicon defined by SEQ ID NOS: 69.

10: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 31-32 and 52, which are useful in the detection of Giardia lamblia amplicon defined by SEQ ID NO: 70.

.../...

**Continuation of:** Box No. III

11: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 33-34 and 53, which are useful in the detection of *Entamoeba histolytica* amplicon defined by SEQ ID NO: 71.

12: Claims 17-18 (partly), 20-26 (partly) and 28 (partly) directed to use/nucleotide primer/nucleotide primer pair/nucleotide probe/kit/method related to SEQ ID NOS: 35-36 and 54, which are useful in the detection of *Cryptosporidium* sp. amplicon defined by SEQ ID NO: 72.

**Continuation of:** second sheet

**International Patent Classification (IPC)**

**C12Q 1/68** (2006.01)

**C12N 15/11** (2006.01)

**C12Q 1/04** (2006.01)

**C12Q 1/10** (2006.01)

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/FI2013/050716

CN 101831491 A 15/09/2010 NONE

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