(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 December 2012 (27.12.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/176004 A2

(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/GB2012/051483

(22) International Filing Date:
25 June 2012 (25.06.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1110712.5 23 June 2011 (23.06.2011) GB

(71) Applicant *(for all designated States except US)*: **UNIVERSITY OF ULSTER** [GB/GB]; Cromore Road, Coleraine, County Londonderry BT52 1SA (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TERNAN, Nigel George** [GB/GB]; 25 Primrose Park, Portrush, County Antrim BT56 8TD (GB). **MCMULLAN, Geoffrey** [GB/GB]; 7 Circular Road, Castlerock, County Londonderry BT51 4XA (GB). **GILL, Christopher Ian Richard** [GB/GB]; 41 Laurel Park, Coleraine, County Londonderry BT51 3AQ (GB). **JAIN, Shailesh** [IN/IN]; 603 Crystal Classic, Vile Parle (West), Mumbai 400056 (IN).

(74) Agent: **GARDNER, Rebecca**; Dehns, St Bride's House, 10 Salisbury Square, London, Greater London EC4Y 8JD (GB).

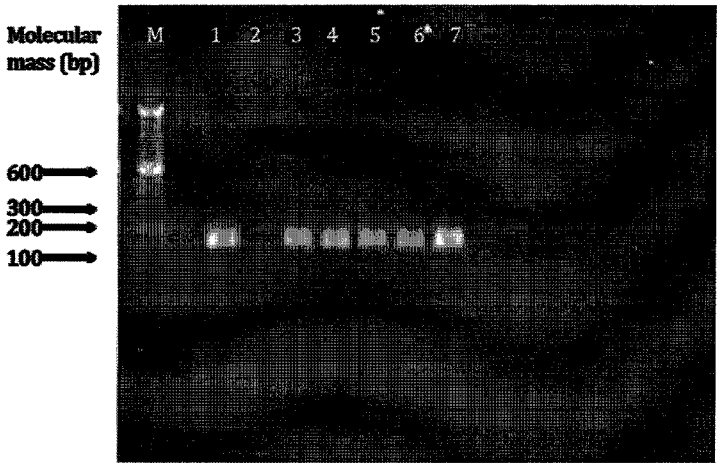
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

[Continued on next page]

(54) Title: DIAGNOSTIC METHODS FOR DETECTING CLOSTRIDIUM DIFFICILE

Figure 2C



(57) Abstract: The invention provides a method of detecting Clostridium difficile in a sample, comprising detecting the presence in said sample of one or more genes that have been identified as being specific to Clostridium difficile. Also provided is a method of diagnosing a Clostridium difficile infection in a subject, a method of determining the efficacy of a therapeutic regime being used to treat a Clostridium difficile infection and a method of testing for the presence of Clostridium difficile in a sample. Further provided are primer pairs and a kit suitable for use in such methods.

WO 2012/176004 A2

EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

Diagnostic methods for detecting *Clostridium difficile*

The present invention relates to methods of detecting *Clostridium difficile*, in particular in samples from a human or animal subject, such detection methods enable diagnosis of *Clostridium difficile* infections in said subject. The present methods rely on detection of certain genes which are specific for *Clostridium difficile*.

Clostridium difficile infection (CDI) has become a problematic nosocomial infection in hospitals and long term care facilities throughout the world. CDI is often associated with antibiotic treatment and causes diseases ranging from antibiotic associated diarrhoea to life threatening pseudomembraneous colitis. CDI is the leading cause of infectious diarrhoea among patients in hospitals worldwide.

CDI is a significant burden on the NHS and patients. It is estimated that the 1298 reported cases of CDI in Northern Ireland in 2008 will have cost the local economy a total of £39 million and resulted in the loss of 7139 bed days. In Northern Ireland, the yearly cost of CDI is the equivalent of 10.5% of the total drugs bill. The burden of CDI is not limited to the UK; CDI is also a significant burden on the Irish healthcare system and also on other healthcare providers worldwide. The ageing population, societal strategies to care for the elderly and healthcare management protocols have exacerbated the incidence of CDI. It is essential that the spread of this disease be contained, not least given the associated mortality rate of 6-15%.

Despite the fact that CDI is a problematic infection, there remain very few efficient and reliable methods available for the detection of *Clostridium difficile*. The most common methods currently used in hospitals for detecting *Clostridium difficile* are enzyme immunoassays which detect the presence of *Clostridium difficile* A and/or B toxins. Indeed, the current gold standard for *Clostridium difficile* testing is the cell culture cytotoxicity assay. However, this assay is not standardised and requires access to a continuous cell line and a certain level of technical expertise, in addition to taking up to 48h to yield a result. Consequently, many laboratories have switched to kit-based methods. However, these kits also rely on the detection of *Clostridium difficile* toxins.

Despite an abundance of *Clostridium difficile* detection kits on the market, a recent report by the NHS Centre for Evidence Based Purchasing states that of the nine kits tested "the poor PPVs of toxin detection kits, especially in the context of widespread testing raises doubts about their appropriateness when used as single tests for the laboratory detection of *C. difficile* toxins." (Wilcox and Eastwood, NHS Purchasing and Supplies Agency, Center for Evidence based Purchasing. *Clostridium difficile* toxin detection assays, CEP08054, 2009.). This affirms the sentiments expressed by Planche *et al.* (The Lancet: Infectious Disease (2008) 8:777-84) in which they conducted a meta analysis of the accuracy of available toxin detection kits and came to the conclusion that there was an unacceptably low predictive rate (<50% in some cases) when patient samples are presented with low toxin titre. In addition, certain strains of *Clostridium difficile* may be toxin A-/toxin B+; in this scenario, a detection method which relies on the detection of toxin A would give a false negative result. Also, the costs associated with toxin detection kits are high.

Some researchers have proposed methods for the detection of *Clostridium difficile* by testing for the presence of *Clostridium difficile* toxin genes (WO 2011/008942 and WO 2010/116290), rather than the toxins themselves.

Other methods of testing for *Clostridium difficile* include detection of glutamate dehydrogenase (GDH) by latex agglutination. However, this test is generally performed as an initial screening procedure and is followed by *Clostridium difficile* cell culture and a second step in which toxin detection is carried out. Such methods of detecting *Clostridium difficile* are time consuming, expensive, and prone to error. Furthermore, enzymes that are detected in some *Clostridium difficile* detection methods (e.g. GDH) are present in a variety of microorganisms and thus the specificity of such methods may not be absolute.

What is needed in the art is a cost-effective, toxin independent, high-sensitivity, high-specificity method of detecting a variety of *Clostridium difficile* strains, ribotypes and clinical isolates. Preferably such a method would be straight forward to perform and offer results in a short time-frame. Preferably the methods can be performed in a culture independent fashion.

The present inventors have identified certain *Clostridium difficile* specific genes (CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487,

CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961), the detection of each of which is indicative of the presence of *Clostridium difficile*. Surprisingly, the detection of each of these genes can reliably identify a large number of different strains, ribotypes and deposited isolates of *Clostridium difficile* and thus the methods of the present invention are particularly advantageous.

The present invention provides methods of detecting *Clostridium difficile*, or testing for the presence of *Clostridium difficile* in a sample, comprising detecting the presence in said sample of, or analysing said sample for the presence of, one more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes.

In one embodiment, the present invention provides a method of detecting *Clostridium difficile* in a sample, said method comprising detecting the presence in said sample of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample.

Viewed alternatively, the present invention provides a method of testing for the presence of *Clostridium difficile* in a sample, said method comprising analysing said sample for the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample.

All the methods of the invention described herein conveniently comprise contacting the sample with a detection moiety which can detect one of said genes. In certain embodiments, 2 or more moieties selective for 2 or more genes may be contacted with said sample or to a series of samples from the same source. The detection moieties will generally bind specifically to the gene or its product, for example based on nucleotide base-pair binding or antigen/antibody type interactions. Suitable detection moieties are discussed in more detail below. Thus methods may then involve a step of analysing the combination of sample plus

detection moiety in order to confirm the presence of detection moiety bound to said gene or gene product. The presence of such a bound conjugate may be confirmed *per se* or its presence derived, e.g. from the presence of the nucleic acid products of an amplification reaction enabled through binding of the detection moiety to the gene.

The complete genome (which includes the chromosome and the plasmid) sequence of *Clostridium difficile* strain 630, a virulent, and multidrug-resistant strain has been determined (Sebaihia *et al.*, Nature Genetics, 2006, volume 38, number 7, pages 779-786). The chromosome of *Clostridium difficile* strain 630 encodes 3,776 predicted protein sequences. The plasmid of *Clostridium difficile* strain 630 carries 11 predicted coding sequences. The sequence and annotation of the *Clostridium difficile* strain 630 chromosome and plasmid have been deposited in the EMBL database under accession numbers AM180355 and AM180356, respectively. In the above mentioned Sebaihia *et al.* publication, each coding sequence is assigned a name which begins "CD", for example CD0001. The same nomenclature is used in the present specification. Throughout this application, references to the genes "CD2961", "CD3617", "CD3618", "CD3635" or "CD3638" etc. include coding and non-coding nucleotide sequences of these genes, unless the context dictates otherwise. The coding nucleotide sequences of genes CD2961, CD3617, CD3618, CD3635 and CD3638 are set forth in this application as SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, respectively. Detection of one or more of SEQ ID NOs 1-5 or products thereof represents a preferred embodiment of the present invention.

As used herein, a "nucleic acid" is DNA or RNA, preferably DNA. As used herein, a "nucleotide" is a deoxyribonucleotide or a ribonucleotide, preferably a deoxyribonucleotide.

The nucleotide sequences of CD2961, CD3617, CD3618, CD3635 and CD3638 were determined in *Clostridium difficile* strain 630, but it will be understood in the art that modest sequence variation may occur between different strains and ribotypes of *Clostridium difficile*. The methods of the present invention are intended to detect one or more of these genes or gene products in all, or substantially all, strains, ribotypes and isolates of *Clostridium difficile*. The genes are defined with reference to strain 630 as discussed above and the equivalent gene sequences (homologous sequences) in other strains, ribotypes and isolates of *Clostridium*

difficile can be readily determined by the skilled man. Most preferably, the methods will positively identify 100% of *Clostridium difficile* strains, ribotypes and isolates, effective methods will positively identify at least 80%, preferably at least 90%, more preferably at least 95%, e.g. at least 98% of all available *Clostridium difficile* strains, ribotypes and isolates. Thus, nucleotide sequences that are homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 will preferably be detected by the methods of the present invention.

As referred to herein, "homologous" nucleotide sequences may have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

Sequence alignments and percent identity calculations may be determined using any method or tool known in the art including, but not limited to, the Megalign program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI), the Clustal V method of alignment (Higgins and Sharp (1989) CABIOS. 5:151-153) and the BLAST 2.0 suite of programs. Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information. The skilled man will be able to set the parameters of these tools to suit his desired purpose.

"Homologous" nucleotide sequences may be identified using oligonucleotide primer pairs directed to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5. Such oligonucleotide primer pairs may be capable of hybridising to, and, when combined with a nucleic acid amplification step, amplifying a portion of a nucleic acid that is homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5. The amplified portion of nucleic acid may then be sequenced and the sequence compared to an appropriate nucleic acid sequence database to identify nucleic acids homologous to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5. Methods of identifying genes using oligonucleotide primer pairs are well known in the art.

The nucleic acid of SEQ ID: NO: 1 may be detected using the primer pair as set forth in SEQ ID NO:11 and SEQ ID NO:12. Thus, sequences homologous to

SEQ ID NO: 1 may be identified using the primer pair as set forth in SEQ ID NO: 11 and SEQ ID NO:12.

The nucleic acid of SEQ ID: NO: 2 may be detected using the primer pair as set forth in SEQ ID NO:13 and SEQ ID NO:14. Thus, sequences homologous to SEQ ID NO: 2 may be identified using the primer pair as set forth in SEQ ID NO:13 and SEQ ID NO:14.

The nucleic acid of SEQ ID: NO: 3 may be detected using the primer pair as set forth in SEQ ID NO:15 and SEQ ID NO:16. Thus, sequences homologous to SEQ ID NO: 3 may be identified using the primer pair as set forth in SEQ ID NO:15 and SEQ ID NO:16.

The nucleic acid of SEQ ID: NO:4 may be detected using the primer pair as set forth in SEQ ID NO:17 and SEQ ID NO:18. Thus, sequences homologous to SEQ ID NO: 4 may be identified using the primer pair as set forth in SEQ ID NO:17 and SEQ ID NO:18.

The nucleic acid of SEQ ID: NO:5 may be detected using the primer pair as set forth in SEQ ID NO:19 and SEQ ID NO:20. Thus, sequences homologous to SEQ ID NO: 5 may be identified using the primer pair as set forth in SEQ ID NO: 19 and SEQ ID NO:20.

Thus methods of the invention which employ the above primers or sequences homologous thereto represent preferred embodiments.

It is well understood in the art that when detecting the presence of a gene in a sample, it is not necessary to detect the presence of the entire gene sequence; detecting the presence of a fragment of a gene may be indicative of the presence of the entire gene.

In a preferred method of the invention, the presence of one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 is detected.

As referred to herein "one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638" means one, two, three, four or five genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638. "One" gene means either CD2961, CD3617, CD3618, CD3635 or CD3638. "Two" genes may mean CD2961 and CD3617; CD2961 and CD3618; CD2961 and CD3635; CD2961 and CD3638; CD3617 and CD3618; CD3617 and CD3635; CD3617 and CD3638; CD3618 and CD3635; CD3618 and CD3638; or CD3635 and CD3638. "Three" genes may mean CD2961, CD3617

and CD3618; CD2961, CD3617 and CD3635; CD2961, CD3617 and CD3638; CD2961, CD3618 and CD3635; CD2961, CD3618 and CD3638; CD2961, CD3635 and CD3638; CD3617, CD3618 and CD3635; CD3617, CD3618 and CD3638; or CD3618, CD3635 and CD3638. "Four" genes may mean CD2961, CD3617, CD3618 and CD3635; CD2961, CD3618, CD3635 and CD3638; CD2961, CD3617, CD3635 and CD3638; CD2961, CD3617, CD3618, and CD3638; or CD3617, CD3618, CD3635 and CD3638. "Five" genes means CD2961, CD3617, CD3618, CD3635 and CD3638.

As referred to herein one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5 means one, two, three, four or five nucleotide sequences selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5. "One" nucleotide sequence means either SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 or SEQ ID NO:5. "Two" nucleotide sequences may mean SEQ ID NO:1 and SEQ ID NO:2; SEQ ID NO:1 and SEQ ID NO:3; SEQ ID NO:1 and SEQ ID NO:4; SEQ ID NO:1 and SEQ ID NO:5; SEQ ID NO:2 and SEQ ID NO:3; SEQ ID NO:2 and SEQ ID NO:4; SEQ ID NO:2 and SEQ ID NO:5; SEQ ID NO:3 and SEQ ID NO:4; SEQ ID NO:3 and SEQ ID NO:5; or SEQ ID NO:4 and SEQ ID NO:5. "Three" nucleotide sequences may mean SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:3; SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:4; SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:5; SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:4; SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5; SEQ ID NO:1, SEQ ID NO:4 and SEQ ID NO:5; SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4; SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:5; or SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5. "Four" nucleotide sequences may mean SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4; SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5; SEQ ID NO:1 , SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5; SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, and SEQ ID NO:5; or SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5. "Five" nucleotide sequences means SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

As referred to herein, a "product" of a gene includes mRNA molecules transcribed from the gene or polypeptides encoded by the gene. It will be appreciated that an mRNA molecule will comprise the same sequence as the DNA molecule from which it was transcribed, with the exception the mRNA molecule will

comprise uracil whereas the DNA molecule from which it was transcribed would instead comprise thymine at the corresponding positions.

In one embodiment, the gene product detected by the methods of the invention is an mRNA molecule. It is not necessary to detect the presence of the entire mRNA molecule (i.e. the entire mRNA nucleotide sequence); detecting the presence of a fragment of an mRNA molecule can be indicative of the presence of the entire mRNA molecule.

In another embodiment, the gene product detected by the methods of the invention is a polypeptide. A polypeptide of the sequence set forth in SEQ ID NO:6 is encoded by the nucleotide sequence of SEQ ID NO:1. A polypeptide having the sequence set forth in SEQ ID NO:7 is encoded by the nucleic acid sequence of SEQ ID NO:2. A polypeptide of the sequence set forth in SEQ ID NO:8 is encoded by the nucleotide sequence of SEQ ID NO:3. A polypeptide of the sequence set forth in SEQ ID NO:9 is encoded by the nucleotide sequence of SEQ ID NO:4. A polypeptide of the sequence set forth in SEQ ID NO:10 is encoded by the nucleotide sequence of SEQ ID NO:5. Thus, in a preferred embodiment, one or more of the polypeptides selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 and SEQ ID NO:10 are detected.

It will be appreciated that modest amino acid sequence variation may occur between different strains, ribotypes and isolates of *Clostridium difficile*. Thus, polypeptides homologous to SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 or SEQ ID NO:10 will preferably be detected in the methods of the present invention. Such homologous nucleotide sequences may have at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to the polypeptide sequences of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9 and SEQ ID NO:10.

It is not necessary to detect the presence of the entire polypeptide (i.e. the polypeptide's entire amino acid sequence); detecting the presence of a fragment of a polypeptide may be indicative of the presence of the entire polypeptide.

A number of different methods for detecting nucleic acids are known and described in the literature and any of these may be used according to the present invention. At its simplest, the nucleic acid may be detected by hybridisation to a probe (e.g. an oligonucleotide probe) and many such hybridisation protocols have been described (see e.g. Sambrook *et al.*, , Molecular cloning: A Laboratory Manual, 3rd Ed., 2001, Cold Spring Harbor Press, Cold Spring Harbor, NY).

Typically, the detection will involve a hybridisation step and/or an *in vitro* amplification step.

In one embodiment, the target nucleic acid in a sample may be detected by using an oligonucleotide with a label attached thereto, which can hybridise to the nucleic acid sequence of interest. Such a labelled oligonucleotide will allow detection by direct means or indirect means. In other words, such an oligonucleotide may be used simply as a conventional oligonucleotide probe. After contact of such a probe with the sample under conditions which allow hybridisation, and typically following a step (or steps) to remove unbound labelled oligonucleotide and/or non-specifically bound oligonucleotide, the signal from the label of the probe emanating from the sample may be detected. In preferred embodiments the label is selected such that it is detectable only when the probe is hybridised to its target.

In another embodiment, the target nucleic acid in a sample may be determined by using an oligonucleotide probe which is labelled only when hybridised to its target sequence, i.e. the probe may be selectively labelled. Conveniently, selective labelling may be achieved using labelled nucleotides, i.e. by incorporation into the oligonucleotide probe of a nucleotide carrying a label. In other words, selective labelling may occur by chain extension of the oligonucleotide probe using a polymerase enzyme which incorporates a labelled nucleotide, preferably a labelled dideoxynucleotide (e.g. ddATP, ddCTP, ddGTP, ddTTP, ddUTP). This approach to the detection of specific nucleotide sequences is sometimes referred to as primer extension analysis. Suitable primer extension analysis techniques are well known to the skilled man, e.g. those techniques disclosed in WO99/50448, the contents of which are incorporated herein by reference.

In a preferred embodiment of the present invention, the presence of genes, mRNA gene products, or fragments thereof, are detected by a primer-dependent nucleic acid amplification reaction. The amplification reaction is allowed to proceed for a duration (e.g. number of cycles) and under conditions that generate a sufficient amount of amplification product. Most conveniently the polymerase chain reaction (PCR) will be used, although the skilled man would be aware of other techniques. For instance LAR/LCR, SDA, Loop-mediated isothermal amplification and nucleic acid sequence based amplification (NASBA)/3SR (Self-Sustaining Sequence Replication) may be used. If an mRNA gene product is to be detected, it will first be converted into a cDNA molecule by reverse transcription using a reverse

transcriptase enzyme to generate a cDNA molecule. Upon completion of the reverse transcription reaction, the cDNA can be used as the template for the primer-dependent nucleic acid amplification reaction. A person skilled in the art will be well aware of how to generate cDNA molecules from mRNA molecules.

Many variations of PCR have been developed, for instance Real Time PCR (also known as quantitative PCR, qPCR), hot-start PCR, competitive PCR, and so on, and these may all be employed where appropriate to the needs of the skilled man.

In one basic embodiment using a PCR based amplification, the oligonucleotide primers of the invention are contacted with a reaction mixture containing the target sequence and free nucleotides in a suitable buffer. Thermal cycling of the resulting mixture in the presence of a DNA polymerase results in amplification of the sequence between the primers.

Optimal performance of the PCR process is influenced by choice of temperature, time at temperature, and length of time between temperatures for each step in the cycle. A typical cycling profile for PCR amplification is (a) 5 minutes of DNA melting (denaturation) at 95°C; (b) 30 seconds of DNA melting (denaturation) at 95°C; (c) 30 seconds of primer annealing at 50-65°C; (d) 30 seconds of primer extension at 68°C-72°C, preferably 72°C; and steps (b)-(d) are repeated as many times as necessary to obtain the desired level of amplification. A final primer extension step may also be performed. The final primer extension step may be performed at 68°C-72°C, preferably 72°C. In certain embodiments the annealing step is performed at 50-60°C, e.g. 50-58°C, 52-58°C, 54-58°C, 53-57°C, or 53-55°C. In other embodiments the annealing step is performed at about 55°C (e.g. 55°C±4°C, 55°C±3°C, 55°C±2°C 55°C±1°C or 55°C±0.5°C). The annealing step of other amplification reactions may also be performed at any of these temperatures.

The detection method of the present invention may be performed with any of the standard mastermixes and enzymes available.

Modifications of the basic PCR method such as qPCR (Real Time PCR) have been developed that can provide quantitative information on the template being amplified. Numerous approaches have been taken although the two most common techniques use double-stranded DNA binding fluorescent dyes or selective fluorescent reporter probes.

Double-stranded DNA binding fluorescent dyes, for instance SYBR Green, associate with the amplification product as it is produced and when associated the dye fluoresces. Accordingly, by measuring fluorescence after every PCR cycle, the relative amount of amplification product can be monitored in real time. Through the use of internal standards and controls, this information can be translated into quantitative data on the amount of template at the start of the reaction.

The fluorescent reporter probes used in qPCR are sequence specific oligonucleotides, typically RNA or DNA, that have a fluorescent reporter molecule at one end and a quencher molecule at the other (e.g. the reporter molecule is at the 5' end and a quencher molecule at the 3' end or vice versa). The probe is designed so that the reporter is quenched by the quencher. The probe is also designed to hybridise selectively to particular regions of complementary sequence which might be in the template. If these regions are between the annealed PCR primers the polymerase, if it has exonuclease activity, will degrade (depolymerise) the bound probe as it extends the nascent nucleic acid chain it is polymerising. This will relieve the quenching and fluorescence will rise. Accordingly, by measuring fluorescence after every PCR cycle, the relative amount of amplification product can be monitored in real time. Through the use of internal standard and controls, this information can be translated into quantitative data.

The amplification product may be detected, and amounts of amplification product can be determined by any convenient means. A vast number of techniques are routinely employed as standard laboratory techniques and the literature has descriptions of more specialised approaches. At its most simple the amplification product may be detected by visual inspection of the reaction mixture at the end of the reaction or at a desired time point. Typically the amplification product will be resolved with the aid of a label that may be preferentially bound to the amplification product. Typically a dye substance, e.g. a colorimetric, chromomeric fluorescent or luminescent dye (for instance ethidium bromide or SYBR green) is used. In other embodiments a labelled oligonucleotide probe that preferentially binds the amplification product is used.

The presence of gene CD2961 and of a nucleotide sequence of SEQ ID: NO: 1 may be detected using a primer-dependent nucleic acid amplification reaction with a forward primer comprising the sequence of SEQ ID NO: 11 and a reverse primer comprising the sequence of SEQ ID NO:12.

Thus, in a further aspect, the present invention provides a primer pair consisting of

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 11; and

(ii) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO 12 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 12.

The presence of gene CD3617 and of a nucleotide sequence of SEQ ID: NO: 2 may be detected using a primer-dependent nucleic acid amplification reaction with a forward primer comprising the sequence of SEQ ID NO:13 and a reverse primer comprising the sequence of SEQ ID NO:14.

Thus, in a further aspect, the present invention provides a primer pair consisting of

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 13; and

(ii) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO 14 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 14.

The presence of gene CD3618 and of a nucleic acid sequence of SEQ ID: NO: 3 may be detected using a primer-dependent nucleic acid amplification reaction with a forward primer comprising the sequence of SEQ ID NO: 15 and a reverse primer comprising the sequence of SEQ ID NO:16.

Thus, in a further aspect, the present invention provides a primer pair consisting of

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 15; and

(ii) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 16.

The presence of gene CD3635 and of a nucleic acid sequence of SEQ ID: NO: 4 may be detected using a primer-dependent nucleic acid amplification

reaction with a forward primer comprising the sequence of SEQ ID NO:17 and a reverse primer comprising the sequence of SEQ ID NO:18.

Thus, in a further aspect, the present invention provides a primer pair consisting of

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:17 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:17; and

(ii) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:18 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:18.

The presence of gene CD3638 and of a nucleic acid sequence of SEQ ID:NO:5 may be detected using a primer-dependent nucleic acid amplification reaction with a forward primer comprising the sequence of SEQ ID NO:19 and a reverse primer comprising the sequence of SEQ ID NO:20.

Thus, in a further aspect, the present invention provides a primer pair consisting of

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:19 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:19; and

(ii) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:20 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:20.

Throughout the text, references to SEQ ID NOs: 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 also include nucleotide sequences capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NOs: 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20, respectively.

The oligonucleotide primers of the invention may comprise up to 100 nucleotides, preferably up to 80, 60, 50, 40, 30 or 25 nucleotides. The oligonucleotide primers of the invention may comprise at least 18, preferably at least 19, 20, 21, 22, 23, 24 or at least 25 nucleotides, e.g. 20-40 nucleotides. The nucleotides of the oligonucleotide can be any type of nucleotide so long as hybridisation specificity or efficiency and amplification efficiency is not detrimentally effected. The oligonucleotide may therefore be a deoxyribonucleotide, a ribonucleotide, modifications thereof (e.g. PNA, morpholino-, LNA) and mixtures thereof. DNA oligonucleotides are preferred.

High stringency conditions for hybridisation are defined as 2x SSC/50% formamide at 50°C for binding conditions and 2 x SSC at 65°C for washing conditions (where SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2).

In preferred embodiments the nucleotide sequences that can hybridise to the nucleotide sequence complementary to SEQ ID NOs:11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 under high stringency conditions will hybridise to all, or substantially all, e.g. at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 contiguous nucleotides of the nucleotide sequence complementary to SEQ ID NOs:11, 12, 13, 14, 15, 16, 17, 18, 19 or 20, respectively.

In the methods of the present invention, polypeptide gene products, or fragments thereof may be detected by a suitable method known in the art. Suitable methods may include any antibody-mediated detection method. Suitable antibody-mediated detection methods include immunoblotting (e.g. western blotting), immunofluorescence assays, radioimmunoassays, or ELISAs.

Depending on the conditions employed, detection of a gene or product thereof may be a partially, semi-, or fully quantitative measurement, but can also be a qualitative (or relative) measure in which results from a sample which does not contain one or more of the genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635, or CD3638, or products thereof, are simply compared to results from the sample under investigation, with any differences between the two being noted without numerical values being affixed.

The methods of the invention are able to detect the presence of the genes CD2961, CD3617, CD3618, CD3635, and CD3638, or products thereof, in multiple clinically important *Clostridium difficile* strains and ribotypes. Preferred *Clostridium difficile* strains which can be detected include *Clostridium difficile* strain 630 (a *Clostridium difficile* strain of ribotype 12) and *Clostridium difficile* strain qcd32_g58 (a *Clostridium difficile* strain of ribotype 27). Preferred *Clostridium difficile* ribotypes which can be detected include 106, 078, 020, 001, 005, 026, 014 and 027. Other preferred *Clostridium difficile* ribotypes which can be detected include 078v, 015, 015-19, 023, 002, 053, 140.

The sample which is tested according to the methods of the invention is preferably a body fluid, swab or other cellular or non-cellular sample from a human. Such samples include, but are not limited to, bodily fluids which contain cellular materials and may or may not contain cells, e.g., blood, plasma, serum, urine, conjunctival secretions, seminal fluid, saliva, ocular lens fluid, lymphatic fluid,

amniotic fluid, faeces/stool and the like; endocervical, urethral, rectal, vaginal, vulva-vaginal, nasopharyngeal and pulmonary samples; and archival samples with known diagnosis. Test samples may also be sections of tissues such as frozen sections.

The sample may be any sample taken from the gastrointestinal GI tract. The GI tract, also referred to as the digestive tract or alimentary canal (and which terms may be used interchangeably with GI tract) is the continuous series of organs beginning at the mouth and ending at the anus. Specifically this sequence consists of the mouth, the pharynx, the oesophagus, the stomach, the duodenum, the small intestine, the large intestine and the anus. These organs can be subdivided into the upper GI tract, consisting of the mouth, pharynx, oesophagus, stomach, and duodenum, and the lower GI tract, consisting of the jejunum, the ileum (together the small intestine), the cecum, the colon, the rectum (together the large intestine) and the anus.

A GI tract sample of use in the invention may include, but is not limited to any fluid or solid taken from the lumen or surface of the GI tract or any sample of any of the tissues that form the organs of the GI tract. Thus the sample may be any luminal content of the GI tract (e.g. stomach contents, intestinal contents, mucus and faeces/stool, or combinations thereof) as well as samples obtained mechanically from the GI tract e.g. by swab, rinse, aspirate or scrape of a GI tract cavity or surface or by biopsy of a GI tract tissue/organ.

The sample can also be obtained from part of a GI tract tissue/organ which has been removed surgically. The sample may be a portion of the excised tissue/organ. In embodiments where the sample is a sample of a GI tract tissue/organ the sample may comprise a part of the mucosa, the submucosa, the muscularis externa, the adventitia and/or the serosa of the GI tract tissue/organ. Such tissue samples may be obtained by biopsy during an endoscopic procedure.

Samples may also be sections of tissues such as frozen sections.

Samples of use in the invention may also include environmental samples, preferably samples from a hospital or other clinical setting. Examples of such environmental samples include samples obtained from surfaces (e.g. floors), samples obtained from clothing, samples obtained from toilets, commodes, bedpans and the like, samples obtained from clinical devices (e.g. endoscopes), samples of the water supply, or air treatment apparatus of the hospital or other clinical setting, and samples obtained from the hands of healthcare workers.

The term "sample" also encompasses any material derived by processing a biological sample. Derived materials include, but are not limited to, cells (or their progeny) isolated from the sample (e.g. clinical isolates of *Clostridium difficile*), cell components, proteins/peptides and nucleic acid molecules (DNA or RNA) extracted from the sample. Processing of biological samples to obtain a test sample may involve one or more of: filtration, distillation, centrifugation, extraction, concentration, dilution, purification, inactivation of interfering components, addition of reagents, and the like.

The subject may be any human or non-human animal subject, but more particularly may be a vertebrate, e.g. an animal selected from mammals, birds, amphibians, fish and reptiles. The animal may be a livestock or a domestic animal or an animal of commercial value, including laboratory animals or an animal in a zoo or game park. Preferably the subject is a human. The subject may be of any age, e.g. an infant, a child, a juvenile, an adolescent or an adult.

As mentioned previously, the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635, and CD3638, or product thereof, is indicative of the presence of *Clostridium difficile* in a sample. Accordingly, the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635, and CD3638, or product thereof is indicative of the presence of *Clostridium difficile* and/or a *Clostridium difficile* infection in the subject from whom the sample was taken.

Thus, in a further aspect, the present invention provides a method of diagnosing a *Clostridium difficile* infection in a subject, said method comprising detecting the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes, in a sample that has been obtained from a subject. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample. As the sample has been obtained from said subject, the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes, in the sample is diagnostic of a *Clostridium difficile* infection in the subject from whom the sample has been obtained. All discussion of the various features of the methods of the invention and preferred embodiments apply *mutatis mutandis* to this aspect of the invention.

The methods of the present invention may be repeated over a period of time (e.g. one week or one month) on further samples that have been obtained from a subject undergoing treatment for a *Clostridium difficile* infection. Such repeated performance of the methods of the invention may yield information that is useful in determining the efficacy of the therapeutic regime being used to treat the *Clostridium difficile* infection. For example, failure to detect the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes, in a sample obtained from a subject being treated for a *Clostridium difficile* infection may indicate that the subject no longer has a *Clostridium difficile* infection. If quantitative methods are used, then a reduction in the amount of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes, in a sample obtained from a subject being treated for a *Clostridium difficile* infection may indicate that the therapeutic regime is being effective.

Thus, in another aspect, the present invention provides a method of determining the efficacy of a therapeutic regime being used to treat a *Clostridium difficile* infection, said method comprising:

(i) detecting the presence of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes, in a sample that has been obtained from a subject being treated for a *Clostridium difficile* infection; and

(ii) repeating step (i) on one or more further samples that have been obtained from the subject being treated for a *Clostridium difficile* infection.

Thus, for example, further samples will be obtained during the course of the treatment and/or after the treatment period has ended.

All discussion of the various features of the methods of the invention and preferred embodiments apply *mutatis mutandis* to this aspect of the invention.

In a further aspect the invention provides kits comprising one or more detection moieties for the detection of one or more genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638, or a product of said genes. Preferably the detection moiety is an oligonucleotide, which may be labelled or unlabelled and may form part of a primer pair of oligonucleotides designed for participation in an amplification reaction. Suitable moieties include,

but are not limited to antibodies directed against the polypeptide products of CD2961, CD3617, CD3618, CD3635 or CD3638, and the oligonucleotide primers described above. Preferably the kit comprises one or more of the primer pairs described above as detection moieties.

The kits of the invention are designed for use in the methods of the invention and may comprise further components. Each component may be provided in a separate compartment or vessel. Where convenient and practical, mixtures of components could be provided. The components may be provided in dry, e.g. crystallised, freeze dried or lyophilised, form or in solution, typically such liquid compositions will be aqueous and buffered with a standard buffer such as Tris, HEPES, etc.

The kit may also be provided with instructions for using the kit in the detection of *Clostridium difficile* (or for testing a sample for the presence of *Clostridium difficile*), or with directions for how such instructions may be obtained.

Further components might optionally be any or all of the means, e.g. buffers, enzymes etc. for performing an amplification and/or primer extension reaction with the oligonucleotides of the invention. For instance, the kits may optionally contain a PCR reaction buffer, nucleotide triphosphates (which may be labelled, e.g. labelled ddNTPs), further oligonucleotide primers, or DNA polymerases, preferably a thermostable polymerase such as Taq polymerase.

Further components might optionally be any or all of the means, e.g. buffers, enzymes etc. for performing a reverse transcription reaction. For instance a reverse transcriptase, RNA specific primers, an RT reaction buffer, and nucleotide triphosphates.

Further components might optionally be any or all of the means to take the sample. For instance such means might include dipsticks, biopsy apparatus, swabbing devices, pouches or vessels. Preferably these means will be provided in sterile form.

Further components might optionally be any or all of the means to purify or refine the sample. For instance means to isolate or concentrate cells in a sample, e.g. cell binding solid supports or filtration devices. In other embodiments the means to purify or refine the sample might be any or all of the means for extracting nucleic acid from a sample. For instance cell lysis reagents (e.g. chaotropic salts, alcohols, detergents, membrane altering compounds), nucleic acid binding solid supports or nucleic acid precipitating agents (e.g. salts, alcohols).

Further components might optionally be any or all of the means to detect amplified nucleic acid. For instance the labels described herein (e.g. double stranded DNA binding dyes, labelled oligonucleotide probes), apparatus to detect these labels, electrophoresis materials and apparatus, or chromatography materials and apparatus.

In another aspect, as an alternative to the five target genes described in detail above, the methods described herein may be performed by analysing for, or detecting the presence of, one or more of the genes selected from the group consisting of, CD0588, CD0638, CD1234, CD1423, CD1424, CD1487, CD1543a, CD1728, CD1794, CD1897, CD1906, CD2046, CD2098, CD2216, CD2248, CD2264, CD2274, CD2300, CD2306, CD2309, CD2563, CD3188, CD3288, CD3321, CD3367, CD3369, CD3609 and CD3656, or a product of said genes. Of this further group of genes, one or more of the genes selected from the group consisting of CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD3609 are preferred. One or more of the genes selected from the group consisting of CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD3609, are especially preferred.

Preferred embodiments of the methods and kits described above apply, *mutatis mutandis*, to the detection of, or analysis for, one or more of these further groups of genes.

Thus, the invention provides a method of detecting *Clostridium difficile* in a sample, said method comprising detecting the presence in said sample of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample.

In a further aspect, the present invention also provides a method of diagnosing a *Clostridium difficile* infection in a subject, said method comprising detecting the presence of one or more genes selected from the group consisting of

CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes, in a sample that has been obtained from a subject. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample. As the sample has been obtained from said subject, the presence of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes, in the sample is diagnostic of a *Clostridium difficile* infection in the subject from whom the sample has been obtained.

In another aspect, the present invention provides a method of determining the efficacy of a therapeutic regime being used to treat a *Clostridium difficile* infection, said method comprising:

(i) detecting the presence of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes, in a sample that has been obtained from a subject being treated for a *Clostridium difficile* infection; and

(ii) repeating step (i) on one or more further samples that have been obtained from the subject being treated for a *Clostridium difficile* infection.

In a further aspect, the present invention provides a method of testing for the presence of *Clostridium difficile* in a sample, said method comprising analysing said sample for the presence of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes. The presence of said one or more genes or product thereof is indicative of the presence of *Clostridium difficile* in said sample.

In a further aspect the present invention provides a primer pair selected from the group consisting of

(a) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 67 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 67; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 68 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 68;

(b) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 13; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO 14 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 14;

(c) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 15; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 16;

(d) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:17 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:17; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:18 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:18;

(e) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:19 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:19; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:20 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:20;

(f) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:37 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 37; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:38 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:38 ;

(g) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 39 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 39; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 40 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 40;

(h) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 41 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 41; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 42 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 42;

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 43 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 43; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO; 44 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 44;

(j) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 45 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 45; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 46 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 46;

(k) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 47 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 47; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 48 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 48;

(l) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 49 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 49; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:50 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 50;

(m) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 51 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 51; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 52 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 52;

(n) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 53 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 53; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 54 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 54;

(o) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 55 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 55; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 56 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 56;

(p) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 57 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 57; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 58 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 58;

(q) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 59 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 59; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 60 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 60;

(r) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 61 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 61; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 62 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 62;

(s) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 63 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 63; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 64 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 64;

(t) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 65 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 65; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 66 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 66; and

(u) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 11; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 12 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 12.

In another aspect, the present invention provides a kit comprising one or more detection moieties for the detection of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638 CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes.

Preferred embodiments and other exemplification of the methods, kits and primers discussed above in relation to genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638 apply, *mutatis mutandis*, to the aspects of the invention relating to one or more of the genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961.

Likewise, all of the definitions and discussion above in relation to genes selected from the group consisting of CD2961, CD3617, CD3618, CD3635 and CD3638 apply, *mutatis mutandis*, to the aspects of the invention relating to one or more of the genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961.

LIST OF NUCLEOTIDE AND AMINO ACID SEQUENCES DISCLOSED HEREIN AND THEIR SEQUENCE IDENTIFIERS (SEQ ID NOs)

All nucleotide sequences are recited herein 5' to 3' in line with convention in this technical field.

SEQ ID NO:1 (coding nucleotide sequence of CD2961)

ATGGCTTTTGAAATAATAAAAAGCATTGTTGAGGCAGAGCAGACAGCAGACAGTATCAAAGTAAAAGC
TGTTACTGATGCAGAGTCTATCAGAGCTGATGCTGTAAACAAATGTGAAAGCATATTTGCTGATGTAA
AAAAACAAGCAAAGCTTATGGAAGAAACTCTTATTGAGAAGGCAGTCACCGACAGTAGAGCAGAGGTT
GATAAAATCTTAGCTAATGCTAAAAGTGAATGTCTGAAAATTGAAAAAACTGCTGAAGAAAGAAAAAG
TAAGGCTATTGAAGCGGTTATTGGAAAGGTAGTGAGATAA

SEQ ID NO:2 (coding nucleotide sequence of CD3617)

ATGGTAAATATGAATATTATAGAAATTCGCTCAGATAAAATATACAAGAAGATAATGGAT
GCACCAATAAACAAAAAAGAAGATATATACAGATATGAATTAATGAAGCCTTTTGAATTT
AAGTGGAAGTGTATGAATGTTCCAATAGTTGCTAGACAGAAAGGTGGATATGATGTAATT
ATAGCAAGTGAAATGTTAGGGGTTTTATCGCCTAAGGATATTGATGAAAAGCAAAAAAAG
AATATAAATGTGTTATCTGCTGATAAAATTTGGGCCACTTGTAAAGAAACCATAGAAAAC
TCTATAAATGCTTTTATAAAAGAAGGGTATGATTTAAACATTAAGGACTATAAATATTCA
ATATTATTGGCGAATCCAAATAGTCCTTATACAATATTAAGTGATGGATACTGGGGTGAT
GGTGGGATTCCTGGATATATATTTCTATCATTGGTTCCTAATGAATATACTATCAATAGA
TTACCAGTATTAATAGCACATGAATGTAATCACAATATTAGATTTCAGTTTATAGAGTGG
AATAATAATATAACATTAGAAGAAATGATGATAAATGAAGGTCTTGCAGAAAATTTTGCA
ACATGGATGTTTGGAGAGGAAATGTTAGGACCTTGGGTCAGTAGAACAGATATCGAAACA
TTAAATACTTATATAAAGCCAATAATAAAAAGTGCTTTAAAAGAAACTGGATTTCAAAAT
ATAACATCTTATCTTTATGGTGATGATATAGCTAAAATGCAAGGATATTTTCCAGTAGGG

TTGCCTTATTGTGCAGGATATGCTTGTGGATATTATATGATTAAGTATTATTTAGAAAAG
ACAAATAAATCAATAATCGAAGCGACTTTATTGCCTTATAGTGAGATAATCGAAGCAGTA
AAAGAGTTTTGGGAATAA

SEQ ID NO:3 (coding nucleotide sequence of CD3618)

TTGGTCATGCTAACTCCATATTTAATATTTAATGGTACTTGTGAAAAAGCATTTAATTTT
TATGCTGAGGCTTTCGGAGGAGGAAAAACTATATTTGCGCGATTAGACAGCAATCCAAAC
AATCCTGTTATGCACGCAAGTGTTACTTTCACAAAATACGAAGGTTGTATAATGGGTGCG
GATACAGACAAGCCTGTTGTAATTTCTGGCATGGCGATTTGTGTTGTTCTACCATCTCGA
GAAGCGATAGAAGAAATATCTGTAAAACTTGCCGAAGGTGGTACACTTGTACAAGAATTT
TTACCACACCCACCACCACATCAAAATGATGGCGCTGCTGAAGTACTTGATAGGTATGGG
TATACTTGGTATTTAAGTACATAG

SEQ ID NO:4 (coding nucleotide sequence of CD3635)

ATGGCTATGGGTTTTGAATTTAAAATAATGAGAAGTTTAATATATGTAGGACTTGCCAAG
GAAGAATATAGACCTAAGCTAATGGACTGGTTATATCGTCACCATATTCCAGATAGTATT
AGCACTTTTGGACCATATTGTACTAAATATGCCTTTTATCAAGCATATCCTACACCAAAT
GAAGGTGAGCGTTTTGGTGCACGTAAGATGCAACTAACAGAACATTATTGGCTTGTAGAT
GAACATATGCCTGAGATGGCAAATAGAATTATGACAGAATATATGCCTATGGATGTTCTA
CGTTGGCAAGGGTGTATACCAGATGTAGAAAATAAAAGGGTTCATGAAAATGCAGAAAGT
GGAGATGCAGGACGTGCAGTAGGTGGAGATAATGGATGTCCACCATTTATATTTGCCTTT
GTTCCAATAAACTGGGAAGAAGACTTTAGAGGAAAAGGACGTACTGTACAAGATGGACCA
AACTATCGTTGGCAATTTATGATTAAGTATCCAGATGGTATCTCTAAAGAAGAAGGAGAA
AAATGGTTCTATGATGAGGTAGTGCCATACTTTACAAACTGTTGCTATGTTAATCGTTTT
GTCAGTAGTAAAATAATGATTAATTATGGAGCAACTGCTTTTGACCGTGTATCAGAACTA
TGGTTTGAAGGGGAAGAAGAATGGTATAAAGCTGTGGTTGAAGAAACAAAGTCGTTTATT
AAAAAACCAGAATGGGCACAAGAAGAGGAGTTCCCATATTTAAAACCACAATTCAATATC
GCATCAGTATTCTTAGGTGATATAGCAACTATGGATGCATACTCACAGTATCGTGGATAT
ATACCAATGAGATAA

SEQ ID NO:5 (coding nucleotide sequence of CD3638)

ATGGAAGATAAATTTTATGCAAAAGGCAACGGAAATAACGGATATATTAAAAATCTTGAA
GTTTGTTCCTTTAATAACTTAGATGGAACTTGTGGAATGTTTCAAATGGCTCTGTACAAA
AGAGATGAAAAATACTATTTATATGGATGCTGTTTTGGAGGAAATAAAAAAAATGGAGTA
ATGATTAGCGATATTACAGACCCTTATAATCCACAATTTATAAAACATTTTCAAATGTTA
GACCCTAAAGAGTATCCTACAACAACAACTCCCAAAATTCAAATAGCAGATGATTTAATG
ATAGTAGCAATGAGTTGTGGAAGTGGACCAGGAGCACTTGTTGACCAAGCTAAATTAGCA
AATATTAAGTGTGAAGCAGGAATTAGAATATACAGTTTAAAAGAAGACCCTTTAAATCCT
AAGTTTTTAGGATATTGGGATTGTGGCTTAAAGCATGTAATGGGTGTTCATAGATTTATG
TACAATGGTGGAAGATATGTACATTTATCAAGTGATTGTGTTGGCTTTGAAGGTCTGATT
TATAGGGTCATAGATATAATAAATCCTACTAATCCAGTGGAAATAGGTAAATGGTGGAGA
CCAGACCAATATGCAGATGGATATCCAAATAGAACTTTTGATGCAGGAGCACCTCATTGC
CCAGAATTTATGGATAAAGGATGGCTTCATGGACCTCCATTTGTAAGAGACGGAAAAGCA
TATTGTGGTTATGGAGGAGCTGGTTTAGTTGTATTAGATGTTGAAGATTTAACAAGACCA
AGATGCTTAGGTGAATTGCCATTTACGCCTGCATTTTCTAGTAGACTTGCAGGTGCAAGA
ACTCATACAGCATTACCATTGCCAGGAAGAGATTTAGTCGTTGTTCAAAATGAGGGAGAA
AGATTCCAGTTCTTTAAACCAGATAACATTACAGATGTTCAAGCTATGAATAATATACAT
ATGGTTGATGTTAGTGACCCAACAAAACCAACATTAATTGCTCAATTTCCATATCCTGAA
GTTCCAAAAGATTTCCCTTATCCTAACTTTAATGTTGCGGGATTAGGAAAACCAGGGCCA
TTTGGCCCACATAATCTTCATGAACCAATGGATAATAAGCCATGGTTAGAGCAAAGAGGA
GATAGAGTATATTGCTGTTATTTCCATGCAGGGCTAAGGGTTTATGATGTATCAGACCCA
TATTATATCAAAGAGCTAGCATATTTTATACCACCAAATCCAAATAAAACACCAGAAGAA
TCTTATTTCCCAGGATTCCCAGGACCACGCTTGGCAGTAACAGAAGATCTTATCGTTGAT
GATAGAGGCTACATCATCATAGATGCTTTAGATGATGGATTCTATATATTAAAAATGAAA

GATGATTAA

SEQ ID NO:6 (amino acid sequence encoded by CD2961)

MAFEIIKSIVEAEQTADSIKVKAVTDAESIRADAVNKCESIFADVKKQAKLMEETLIEKAVTDSRAEV
DKILANAKSECLKIEKTAEERKSKAIEAVIGKVVR

SEQ ID NO:7 (amino acid sequence encoded by CD3617)

MVNMNIIEIRSDKIYKKIMDAPINKKEDIYRYELMKPFEFKWKCMNVPIVARQKGGYDVIIASEMLGV
LSPKDIDEKQKKNINVLSADKIWATCKETIENSINAFIKEGYDLNIKDYKYSILLANPNPYTILSDGY
WGDGGIPGYIFLSLVPNEYTINRLPVLIAHECNHNIRFQFIEWNNNITLEEMMINEGLAENFATWMFG
EEMLGPWVSRTDIETLNTYIKPIIKSALKETGFQNITSYLYGDDIAKMQGFPVGLPYCAGYACGYYMI
KYYLEKTNKSIIEATLLPYSEIIEAVKEFWE

SEQ ID NO:8 (amino acid sequence encoded by CD3618)

MVMLTPYLIFNGTCEKAFNFYAEAFGGGKTIFARLDSNPNNPVMHASVTFTKYEGCIMGADTDKPVVI
SGMAICVVLPSREAIEEISVKLAEGGTLVQEFLPHPPPHQNDGAAEVLDRYGYTWYLST

SEQ ID NO:9 (amino acid sequence encoded by CD3635)

MAMGFEFKIMRSLIYVGLAKEEYRPKLMDWLYRHHIPDSISTFGPYCTKYAFYQAYPTPNEGERFGAR
KMQLTEHYWLVDEHMPEMANRIMTEYMPMDVLRWQGCIPDVENKRVHENAESGDAGRAVGDNGCPPFI
FAFVPINWEEDFRGKGRTVQDGPNYRWQFMIKYPDGISKEEGEKWFYDEVVPYFTNCCYVNRFVSSKI
MINYGATAFDRVSELWFEGEEEWYKAVVEETKSFIKKPEWAQEEEFPYLKQFNIASVFLGDIATMDAY
SQYRGYIPMR

SEQ ID NO:10 (amino acid sequence encoded by CD3638)

MEDKFYAKGNGNNGYIKNLEVCSFNNLDGTCGMFQMALYKRDEKYYLYGCCFGGNKKNGVMISDITDP
YNPQFIKHFQMLDPKEYPTTTTPKIQIADDLMIVAMSCGSGPGALVDQAKLANIKCEAGRIYSLKEDP
LNPKFLGYWDCGLKHVMGVHRFMYNGGRYVHLSSDCVGFEGLIYRVIDIINPTNPVEIGKWWRPDQYA
DGYPNRTFDAGAPHCPEFMDKGWLHGPPFVRDGKAYCGYGGAGLVVLDVELTRPRCLGELPFTPAFSS
RLAGARTHTALPLPGRDLVVVQNEGERFQFFKPDNITDVQAMNNIHMVDVSDPTKPTLIAQFPYPEVP
KDFPYPNFNVAGLGKPGPFGPHNLHEPMDNKPWLEQRGDRVCCYFHAGLRVYDVSDPYYIKELAYFIP
PNPNKTPEESYFPGFPGPRLAVTEDLIVDDRGYIIIDALDDGFYILKMKDD

SEQ ID NO:11 (forward primer directed to SEQ ID NO: 1)

AGAAGGCAGTCACCGACAGT

SEQ ID NO:12 (reverse primer directed to SEQ ID NO: 1)

CCTTTCCAATAACCGCTTCA

SEQ ID NO:13 (forward primer directed to SEQ ID NO: 2)

GATGGATACTGGGGTGATGG

SEQ ID NO:14 (reverse primer directed to SEQ ID NO: 2)
AAGGCAATAAAGTCGCTTCG

SEQ ID NO:15 (forward primer directed to SEQ ID NO: 3)
TTTAATGGTACTTGTGAAAAAGCAT

SEQ ID NO:16 (reverse primer directed to SEQ ID NO: 3)
GCCATCATTTTGATGTGGTG

SEQ ID NO:17 (forward primer directed to SEQ ID NO: 4)
CATATGCCTGAGATGGCAAA

SEQ ID NO:18 (reverse primer directed to SEQ ID NO: 4)
CTTGTGCCCATTCTGGTTTT

SEQ ID NO:19 (forward primer directed to SEQ ID NO: 5)
GGATGCTGTTTTGGAGGAAA

SEQ ID NO:20 (reverse primer directed to SEQ ID NO: 5)
AAATTCTGGGCAATGAGGTG

SEQ ID NO: 21 (coding nucleotide sequence of CD0638)

TTGTTTATTTTGAATTTTGGAGGATTAATTATGGATTCAAATAATAATACTATAAAATCA
ACTGTTAAAAAGGGTATTTCTTTTGGTTCTTGTTTAGCAATGATTATTTCTTATACTGCA
TGGAAATCTATTCCATGGGCTATTTTTCATGGCTTAATGAGTTGGATATATGTACTTTAT
TATTGGGTTAAGTATGCATAG

SEQ ID NO: 22 (coding nucleotide sequence of CD1424)

ATGTTTAGAGATGAAATGGATAAATGTACACACATGTTAACTGCTTATATTAGTAGTTTA
TATGATTATTGTGATTTTATAGATACACAGCTAGATGATTTTATACTAGAGTACGGAGAA
AATGTAGTAGAATCTTGTTTACATCAAGTGATGGTATTGGTAAGTAAGTATAATTAA

SEQ ID NO: 23 (coding nucleotide sequence of CD1487)

ATGGAAAATGATACTATTAAGGCTGATGATATTCTCAATTATTGTCTATCAAACTTAGAT
GATGTTGTACTAATGGATAGTTGGGGGGAACGAGCAATTTACTACAATCCTAATGGTGTT

TTAAAGCGAGGGGTATATGTTCTTACCATTAAGGAAAAAGACAGTAATAATGATAAAGGT
TCGTTAGTTAGTCGTCCAAATGTATACCGTGTGAATATAGGATTAAAAAAAGAAACTTTT
ATTGAAATGTTTGGATATATTCCAAAGCGTCCAGGTGTAGGTCAAATAGTTGATATGGAT
TTTGATTTTACAAAATTGGACACAATCATGCCACATCCTATCTACTCATGGATGGGATGG
ATATGCGTCCTAAGCCCTACTGAAAAGACATTTGAGAACTTTAAAACTTTAATAGGAGAA
TCTTACAATTTTGCAAAACAAAAATTTAAAAAGAGAAAAAACAAGTAA

SEQ ID NO: 24 (coding nucleotide sequence of CD1543a)

ATGCGAGAAGAAAAAAGTAATGAAAAGTATGATTGTTATTGGTGTAATCAAGAGAATAAT
TTCTGTGTAGAAATAAAAGATAATATAGTCATGATAGATGATGGCACTGGTACGTTAAAA
CAAGCCGTTTTCATAGGGTATAAACAAATCCAAATTAATTTAAACTGTTCACATTGTCAA
AACTTAAATAGAATAAAATTAAATTTGTAG

SEQ ID NO: 25 (coding nucleotide sequence of CD1794)

TTGTTTATAGATGAAGAATTAGAAGGTTATATATTAACATGTAAAATATCTGAAGACTTT
AAAAATATACCTGAATATAGTGATGAAGAGTTTTATGTTACAGTCTATAAAGATGAAAGT
TCTGACTCTGGGTACTATGCTTTATTAGAAAATAAAGAAGAAAGAGTTGTATGGGATGGA
GAAGTTGTTGCCAATAATATTTTTAATAACCTTTGGATTGTAGTAAATAAGGTTAAAACT
GGATAA

SEQ ID NO: 26 (coding nucleotide sequence of CD1906)

TTGCATATGGAAATCAATGTTATAGAAATTTTCCCTAAAGATAAAGCTAAACTTAATAAA
ATAGAAATGGATAAAGCTAGTTGGTTTGTAAATATAATAAGTAAAAAATATCCTAAAGAA
GCTTTAAATGAAGCATTTAGTACTTTAGAAAAAGAATTAAATATAAGTAAAGCTAATACA
TAA

SEQ ID NO: 27 (coding nucleotide sequence of CD2046)

GTGGATGAAATGCTTGTATATAATAAAAGTTTTTATCCTAATGACATATTTCCAAGATTA
GATTTTTCAAAAATAAAAAAACAGTTAAAATTGATAGATAATGACCTGTCAGATTTTGGA
AGCATATGTATAATAGAAAAAGAACATTATACGATAAGTGTAAACAGTATAGGTGAAATA
AATGTGTACTATGATTTAGAGTACGAAAATAAGGTGTATAGAATAGTTTATGAGATTGAA
AAGTTATTTAAATCTCAAGTTGGAAGGTTTAGCATATCTACATACAGAAATTGA

SEQ ID NO: 28 (coding nucleotide sequence of CD2098)

TTGGCTGGTAATCTAAATAATATGAGAGCAGTAAATAATTTTAGAGGAGATAAGAACATT
TTAGAATGTTTAGTCAGCTTTGAGGGTCGTTCAATAAGTCAGAGAAAAGTAAGGGTATTT
TTTAAAGAAAAACAAAATCAAATAGAAATTGATTTTGCAGAAGAGGAAATTTCTAAATTG
GTTGAAAATGTTGTTTTAAATACATCATATCAAGAAATGTTATATGATGAAATAGAGAAA
CAACTGGAAATTGATTGTATAGGTACTTGGATGATATTATCTAAATTAAAAGATGGTAGT
AGAGTTCACTGA

SEQ ID NO: 29 (coding nucleotide sequence of CD2216)

ATGATTGTGATTGAAGGTAGCGATAAATTTAAGATTGCAAAAGAATATATTGATGTAGAA
TATACTCTTTTTAGCAAAGTAACTTTTAGGTATGAAAAGTTGAAATTTAAAGATAATGCT
GAATTGGAAAAAATTAAGATGTTTAAATATAAAAATGGCTACATCCCTAATAAATTAAAC
CTTTCTTTTGGATATGGATTCTCTTCTTATAAAAAGCAAATAATTAGAGAAACTGTAGAT
ACTTTAAGATTGACAGAAATTTTTTCAAGCGAGAACATAGAAGATATTAAATTTATAAAA
GATGGTACAAAAAAATTAGAAATTAGCATAGAGAAAGTTGTGAAATTTAAACGTCGAAAA
AAAAGAAATTATGTTTGTTGCTATTGCCCTGATATGTATAGGGACATAAAACTCGACAAA
GAATCTATCAATAAGATATACAACAGAAAAATAAAAATAGAAAGAGAAGTTAATATTTTT
GAAGATGAGGATGTTATAATAAACAAAAAAGTATTGAAGTTTCCAAAGTCTTGGACAAAG
AATATGCAAAAATATTGGTTAAGTGAAAATAAGTATCCCATACATTCTACTGTAATTGAT
GATGATAGATATAAATGTTGTAATGTACAATATACAAAAAATAGAGTGATAATATTATAT
TACATATATAACCATTAA

SEQ ID NO: 30 (coding nucleotide sequence of CD2264)

GTGTTAAAAAAGTGGTTTGGTATTGTGAAAAAAAGACAAAAAAGTGAGTCAGTAAAAGAA
GAAGGTGAAGTAATATTAAAAAACGAAAAAATATTATCTGAAGAAAAGTTGATAGATGAA
GAAGGAGTTGTAGTTAATATTGATAATGAAATATTAACTAAAGTAGAAGTAGTAAATGAT
GACAATGAAATAGAAGAAAAAATAATAGAAGAAGATTGGTTAATTAGTGAAAATACCATA
AAATTAGATGATAAAGAAGCAATTATTAATGATAAAAACATAGAATTATGTAAAAAAGAA
GTTCAAGTTGAAGGTGAAAAAATAGATTTAAATAAGTTTGAAGGACTTGACCAAAATGAA
AATTATAATCTAGAAAAAAATGTTATTGAAGAAAAAGAAGTAAGTGAATGTTTGACAGAA
GAAGATTTAGAGTACATAAAAGAAATTAAAATAAAAAGAGGAAAAAGTATAAAAGCTATA
AACTTGTATACTAAAGAAGAGTGGGTTTTTGACACTCATATACAGTGTAGTAAAAAACTC
AAAGTTCCATTAGGGTACATAAGAGAAAATTTAAAATATGGATATATGGATTACTTTGGA
GATGCAATAAATTATTTAAGTGAAGTATTAAATATAGATGAATACTGCAAAAGTGAATGG
AGCTATCTCGATAATAGCAAATCTCCATCTGAGATATTTAATATTCTAAACAATAAAATA
TTTAGTATAAGGCTTTCTAATGAAAAAAGAAATGAAATTTTGACAAATGATAAAATTGAA
GCATTAAAAATGAATTATAGATTTGAATGTATTGATGAAGAATATGATGAATATTTTAAA
AAATATAAGTCTATAATCAAAAGAGGTGGAAAGAAAAAAGTTGAATTAGTAAATAAAAAA
GGTGACATTTTAGAAATATTTAAGTCTTTAGAAGAATGTGCCATTTATTTGCAAAAGGAA
AAGAATGAAGTTATACAAATGTTAAAATATGGAGATACAAAAGTAGGAAGAAACTTTATA
AGGTATAGTTTGAGAAGTATTTAA

SEQ ID NO: 31 (coding nucleotide sequence of CD2274)

ATGGATTTAAGTGGCATATTTAAATACTATTGTAAAGAGTGTGAAAATACATGGAATAAT
TCGAGTGTTGAATTATTTGAGAATATAGAAACGTATAGTAAAGATTCACAAAAAAAGAGG
GAAAAAGAATTAGATAAATTGCTAAATACAATATCAGTTCATTTAGAGAGGTATCCAAGT
GATGCTGTATTGAGAAAAATGTGGGTAAAAAAGGGCGAGGTTTTCTTACAAAAGACATTG
GAAAAAGAAAATATTTTTAAGTTAGAAAAAATGGATGTAGAGGATAGAAAAAAATTTTTA
GATATAACAAAACAGTTTATTAGAGATGCTAGAAAATTTGATGATGATTTACCTATAGGT
GATATTATGCAAGCTATGAGGAATGTATGGATTTCAAATGCATTACAATTATTATTTGGT
AAAGAAGTATATTATTCAAAAGCTAACTTTGCATATAGTATGTTATATCCATATACTGAT
AATTATTTAGACAATACAAATATAGATAAAAATGATAAGATTTTATTTAATAACTGGTTA
GAAAAAAGGCTCCTGGGAGAACACATTAAATCTAAGGATTATCATGAAAGTAAAGTATCT
CAAATGATAGATTATATTGAAAGTGTATACCCTAGAGAAAAGTTTACAGAAGTTTATGAA
TCGTTATTATTAATATTTAAAAGTCAAGTAAATAGTTTAAAACAACATGGTAAGGAAAAT
CATTTGTGTAAAGAAGATTTATTATCCATTTCTATTGAAAAAGGAGGTTCATCCGTTTTA
GTAGATGGATATTTAATAAGTGGATTGATGACAAAGGAAGAAATAGAGTTTTGTATAGGA
TATGGATTTTTATTACAAATATCTGATGATTTACAAGATATAAAAGAAGATTTAAAATAC
AACCATAAAACTATTATTACAGAGATGTCAAAAGAGGGTACTTTAGATAAAGTTGTAAAT
AAACTAATAAATTTTACTATTGAGTTAATAGATAGTTTTAAAATTAATAATAAAAATAAA

TCTGTAATAACTATGATAAAGAATGATTGCTTAATGTTAATTTTATTTTCTGTAGTTTAT
AATGCTGAATTTTTTTCTGTAGGATATATAAAAGAAGTAGAGAAATTTATTCCATATACA
ATAGATTATTCATTAGAGATTGAAGAAAAAATAAAAGAAAAATTTAAAAATATAGATGTT
TTAAATAATGAAAATGAATATAAAGAAATGATTGATATTATTTGTGCAGAGTAG

SEQ ID NO: 32 (coding nucleotide sequence of CD2309)

ATGTTTAGAGATAAAATGGATAAATGTACACATATGTTAACTGCTTATATTGGTAGTTCA
TATGATTATTGTGATTTTATAGATACACAGTTAGATGATTTTATATTAGAGTACGGAGAA
AAAGTTGTGGAATCTTGCTTGCATCAAGTGATGGTATTGGTAAGTAAGTATAATTAG

SEQ ID NO: 33 (coding nucleotide sequence of CD3188)

ATGAGCAAAAGTGAATTAACAGCAGAAACAACAGAAGAAATGTTAGAAGTACTAAGTGGT
AAAGATTATGATATTGCATGTCATTTACATGAACTTGGTAAATCATTAGATTGTAAAATT
GAACCAAAAACAGGTGCTCGTTCTTACAAAATAGTATATTCAACTAAGAAACCAAAACGT
AGCTTATTTACTATTGAATGTAATGAAAAAAAATGGAGAGTTAAAGCAAATCTTTTTCAT
CTAAATACATATAAAGATGCTGTGGAGGAATGCTCTAAAACTATTAAAGATAGTATAACT
AAAACTCGTACTTGTAAGAAATGTAATTCAAAGTGTATTGGAGGTTCTTCGTTTGAATTA
GATGGAAAGTCTTACCTGACTTGCATAGGAAGTGGTCATTATTTTGCAAATATGGAAGAA
ATGGATTGGAAAAACCTAGAAAAATTAATTACTAAAGAAAATAATATTATGCAGGAATCT
GTATAG

SEQ ID NO: 34 (coding nucleotide sequence of CD3288)

ATGGACTATATAGGAATAGAAAATATAACACCTTATGAAAATACATATGAATTTAGTGTA
TATGAATATGATGATGAAATCACCTTAGGTAGTGAAAAGTTATATGTATGTGAATTAAGG
GTTGTATTGATTAAAGTTAATTCTCTGTATGTTGAAAGATTGCATAAATCAGTTGAAGCA
ATGGTCTTAGTAAAAAATTTGAAAAAAGATTTAGATAAAACACTTGTTGTAAACAAAATA
AAGAATTTTGTGCTAGATGAGATTTGGGTAGAAAATCTAGTAAAAGAGAATATAGAAGTT
ATATTTGTAGAAAGCTAG

SEQ ID NO: 35 (coding nucleotide sequence of CD3367)

ATGAAAATATCTAGTCAATATAGAAGTCAATATTCATTTAGATATGAAAGTAATATAAAT
AATACAAGAATAAATGAAAGTATGGTTAAGAAAAATGAAACTGTAGGAAAAGACACTTAT
TTATCTAATATTATGAAACAAAAGCAAGAACTTAATGATAGAATTAGAGATTTAAAATAT
AGACAAGAGGTTTATACTAAAAAAATAAATGACGCAATTAAGAACTTATGTAAATCAGAA
ATAAGAGAAACAACTAATAATTTTTCTAATATAGAAATAGGTATTAAAAATAGCATTATA
GAAGAGAAAAATAAAAGTACAATGTTAGATGAAAATTCAACTTATCTAAATACAAATGAT
GAAAAAGAATCTTTAATCACTAAAGAGTCTAATGAAAAAATTGAAGAAGAAATATTAAAT
GATGAAAAATTAGAAGAGTTAGAACAAAAAAAGGATTATAAAGAGGATTCTAATAAAAAA
GAGAAAGTATCTGAGGACTTATCTTTAGTAGGTAAAACTCGTGAAGAGCTTGAAAATATG
CTTAAAAATTTTATAAATTTAACACAAGAAGAAATAATGAAACTTGAGTCGAGAATAGAA
AAGTTAGATAAAAATGCTGAAGAATACAAACAAAATTCAAAGACTAATATATTTGATAAA
ACAGATGAACAAAAAAAACATATAAATGTACTGATTTAA

SEQ ID NO: 36 (coding nucleotide sequence of CD3609)

ATGTTTAAGAAAATGGCAGTACTAAAAGATATAGCAACTAAAATAGGTCGTAAAAAAGCG
TATGAACTATTAGAAATGGTTGAAGGTAATGATGCCTTTGTAGCTGAGGTAAAGATAAAA

AAGAATGGAATAGAATCTAAAAAAGAAGAAATTATGTTAAAAGATAATCAAAAAATAATA
TTAGAGTATATAGAAGGTTAA

SEQ ID NO: 37 (forward primer directed to SEQ ID NO: 21)
TGTTTATTTTGAATTTTGGAGGATT

SEQ ID NO: 38 (reverse primer directed to SEQ ID NO: 21)
CATGAAAAATAGCCCATGGAA

SEQ ID NO: 39 (forward primer directed to SEQ ID NO: 22)
AGATGAAATGGATAAATGTACACACA

SEQ ID NO: 40 (reverse primer directed to SEQ ID NO: 22)
CCATCACTTGATGTAAACAAGATTC

SEQ ID NO: 41 (forward primer directed to SEQ ID NO: 23)
GGGGGAACGAGCAATTTACTA

SEQ ID NO: 42 (reverse primer directed to SEQ ID NO: 23)
ACCTACACCTGGACGCTTTG

SEQ ID NO: 43 (forward primer directed to SEQ ID NO: 24)
TGATTGTTATTGGTGTAATCAAGAGAA

SEQ ID NO: 44 (reverse primer directed to SEQ ID NO: 24)
ACCCTATGAAAACGGCTTGTT

SEQ ID NO: 45 (forward primer directed to SEQ ID NO: 25)
ATAAAGATGAAAGTTCTGACTCTGG

SEQ ID NO: 46 (reverse primer directed to SEQ ID NO: 25)
TTAACCTTATTTACTACAATCCAAAGG

SEQ ID NO: 47 (forward primer directed to SEQ ID NO: 26)
TGCATATGGAAATCAATGTTATAGAAA

SEQ ID NO: 48 (reverse primer directed to SEQ ID NO: 26)
TGCTTCATTTAAAGCTTCTTTAGGATA

SEQ ID NO: 49 (forward primer directed to SEQ ID NO: 27)
ACCTGTCAGATTTTGGAAGCA

SEQ ID NO: 50 (reverse primer directed to SEQ ID NO: 27)
TGCTAAACCTTCCAACTTGAGAT

SEQ ID NO: 51 (forward primer directed to SEQ ID NO: 28)
GTCAGCTTTGAGGGTCGTTC

SEQ ID NO: 52 (reverse primer directed to SEQ ID NO: 28)
ACAATCAATTTCCAGTTGTTTCTCT

SEQ ID NO: 53 (forward primer directed to SEQ ID NO: 29)
CCTTTCTTTTGGATATGGATTCTC

SEQ ID NO: 54 (reverse primer directed to SEQ ID NO: 29)
CAGGGCAATAGCAACAAACA

SEQ ID NO: 55 (forward primer directed to SEQ ID NO: 30)
AAGAGTGGGTTTTTGACACTCA

SEQ ID NO: 56 (reverse primer directed to SEQ ID NO: 30)
AGCTCCATTCACTTTTGCAGT

SEQ ID NO: 57 (forward primer directed to SEQ ID NO: 31)
AAAAAGGCTCCTGGGAGAAC

SEQ ID NO: 58 (reverse primer directed to SEQ ID NO: 31)
CGGATGAACCTCCTTTTTCA

SEQ ID NO: 59 (forward primer directed to SEQ ID NO: 32)

GGATAAATGTACACATATGTTAACTGC

SEQ ID NO: 60 (reverse primer directed to SEQ ID NO: 32)
GCAAGCAAGATTCCACAACT

SEQ ID NO: 61 (forward primer directed to SEQ ID NO: 33)
ACCAAAAACAGGTGCTCGTT

SEQ ID NO: 62 (reverse primer directed to SEQ ID NO: 33)
AGAGCATTCCTCCACAGCAT

SEQ ID NO: 63 (forward primer directed to SEQ ID NO: 34)
TGATGAAATCACCTTAGGTAGTGAA

SEQ ID NO: 64 (reverse primer directed to SEQ ID NO: 34)
CCCAAATCTCATCTAGCACAAA

SEQ ID NO: 65 (forward primer directed to SEQ ID NO: 35)
AAAACTCGTGAAGAGCTTGAAAA

SEQ ID NO: 66 (reverse primer directed to SEQ ID NO: 35)
TGAATTTTGTTTGTATTCTTCAGCA

SEQ ID NO: 67 (forward primer directed to SEQ ID NO: 36)
AAAATGGCAGTACTAAAAGATATAGCA

SEQ ID NO: 68 (reverse primer directed to SEQ ID NO: 36)
CCTCAGCTACAAAGGCATCA

The invention will now be further described in the following non-limiting Examples with reference to the following drawings in which:

Figure 1 shows the layout of *Clostridium difficile* genomic templates on an LC480 plate. 1-41 – clinical *C. difficile* isolates; 630 – *C. difficile* 630 (positive

control); E. coli – *Escherichia coli*; Staph – *Staphylococus epidermidis*; -ve – no template negative control.

Figure 2A is an amplification graph of the real-time PCR reaction for the CD2961 gene.

Figure 2B is a melting curve of the real-time PCR reaction for the CD2961 gene.

Figure 2C is a gel photograph showing real-time PCR products from the CD2961 plate. Lane M: 100bp molecular mass ladder; Lane 1, positive control; Lane 2, blank, Lanes 3-7: clinical isolates.

Examples

Example 1

Introduction

Using an *in silico* comparative genomics approach the presence of proteins that are unique to *Clostridium difficile* have been identified. 53 genes annotated as encoding "hypothetical proteins" – i.e. those whose biological function has yet to be experimentally verified – in both *C. difficile* strain 630 and *C. difficile* strain qcd32_g58 (a hypervirulent strain which produces higher levels of toxins) are absent from all other *Clostridium* species and related organisms whose genome sequences are available at the Clostridb database (http://xbase.bham.ac.uk/clostridb/). Crucially, no significant matches to any other gene products were found when a BlastP search was made of the NCBI non redundant sequence database. This led the inventors to hypothesise that some of these 53 genes (and DNA molecules, RNA molecules or polypeptides derived therefrom) would be potential biomarkers unique to, and therefore likely to be extremely specific for, *C. difficile*.

In this investigation, PCR primer sets were designed against 52 potential biomarker genes. These 52 genes are CD0588, CD0589, CD0590, CD0638, CD1124, CD1234, CD1423, CD1424, CD1487, CD1543a, CD1581, CD1586, CD1597, CD1613, CD1728, CD1757, CD1794, CD1897, CD1906, CD2046, CD2098, CD2133, CD2216, CD2248, CD2264, CD2274, CD2300, CD2306, CD2309, CD2454, CD2547, CD2563, CD2815, CD2961, CD2972, CD3022,

CD3023, CD3024, CD3163, CD3188, CD3288, CD3321, CD3367, CD3369, CD3573, CD3609, CD3617, CD3618, CD3635, CD3638, CD3641, and CD3656.

The ability of these primer sets to direct primer directed amplification was assessed by carrying out PCR reactions on the genomic DNA of *C. difficile* strain 630. Some of these primer sets were then used to screen 41 clinical *C. difficile* isolates for the presence of the relevant biomarker (gene of interest).

Materials and Methods

Reagents:

All chemicals and reagents used were of Analar grade or better. Brain Heart Infusion (BHI) agar was purchased from Oxoid, UK. All reagents necessary for standard PCR master mix and 100 base pair ladder were purchased from Invitrogen, UK; Agarose QA was from Qbiogene. All reagents necessary for qPCR were purchased from Roche Diagnostics, UK. All apparatus was sterilised and cleaned with Virkon (Antec Intl Ltd., UK) and soaked in 2% Decon (Decon Labs Ltd., UK) overnight before use.

Growth of Bacterial Strains:

Clostridium difficile strain 630 was obtained from Dr Peter Mullany of the Eastman Dental Institute, London. A further 41 *Clostridium difficile* strains were obtained from Dr Derek Fairley of the Northern Ireland Ribotyping Network, based in the Bacteriology department of The Royal Victoria Hospital (RVH), Belfast. All strains were routinely grown on Brain Heart Infusion (BHI) agar plates within a Don Whitely MG500 anaerobic cabinet (Don Whitely Scientific Ltd, Yorkshire, UK), in addition to being kept frozen on cryobeads at -70°C. The anaerobic conditions within the cabinet were 80% N_2, 10% CO_2 and 10% H_2 at 37°C. The agar plates were incubated for 48 h to allow growth of the organisms. Resazurin (1 mg/L) was used as an anaerobic indicator and was added to the BHI agar prior to autoclaving. *Escherichia coli* and *Staphylococcus epidermidis* were used as negative controls in this study; they were grown on nutrient agar plates at 37°C under aerobic conditions.

DNA Extraction:

DNA was extracted using the Fast DNA Spin Kit for Soil (MP Biomedicals, USA) from a loop of bacterial biomass, according to the manufacturer's instructions. The extracted DNA was then quantified using a Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). For qPCR the extracted DNA was diluted to 1ng/µl and stored at -70°C until needed. Prior to use, genomic DNA was diluted four-fold to enable the addition of 4 µl template to each reaction well.

Primer Design and Validation:

The primers used in this study were designed using the OligoPerfect™ tool (Invitrogen, UK). The forward and reverse primers were designed against *C. difficile* gene sequences downloaded from NCBI. A total of 52 primer sets were ordered from Invitrogen, UK, i.e. a primer set was designed for each of the potential *Clostridium difficile* biomarker genes. The primers were then validated by standard endpoint PCR using *Clostridium difficile* 630 genomic DNA as template in a TGradient Thermocycler (Whatman Biometra, Goetigen, Germany). The PCR master mix (96.5 µl) was made up of PCR buffer, $MgCl_2$, dNTPs and millipore water as per the kit instructions; to this 2.5U Taq polymerase, 1µl forward and reverse primers and 1 µl genomic DNA template was added to make up a 100 µl PCR reaction. The PCR cycling conditions used were as follows: an initial denaturation stage for 5 minutes at 95°C followed by 30 cycles of 95°C for 30 s, an annealing stage at a range of temperatures between 54°C-58°C (depending on primers) for 30 s and extension at 72°C for 30 s; with a final extension at 72°C for 5 minutes. The thermocycler then held the temperature at 4°C. The PCR products were then mixed with 6x loading dye (Invitrogen) and visualised by gel electrophoresis/UV transillluminator on a 1% TBE agarose gel containing 4 µg ethidium bromide/100ml gel (Sigma-Aldrich, UK) to determine band size in comparison to 100bp markers (Invitrogen), and the image was recorded using Alpha-Imager 2200 software (Mason Technologies, Dublin, Ireland).

Quantitative PCR:

All quantitative PCR (qPCR) was carried out using the LightCycler480 (Roche Diagnostics, UK), following the manufacturer's instructions for cycling conditions and preparation of mastermix. A mix of Master SYBR Green 1 (Roche Diagnostics, UK) and the primers specific to that plate was prepared and 6µl of this was added to 90 of the 96 wells and to this 4µl of the appropriate DNA was added to the well. Figure 1 shows the order in which the genomic DNA templates were added to the LC480 plate.

The cycling conditions used for the qPCR were: an initial denaturation stage of 95°C for 5 minutes followed by 40 cycles of 95°C for 10 seconds, annealing at 55°C-60°C (depending on primers) for 10 seconds and extension at 72°C for between 10-20 seconds (dependent upon expected product size). Once the amplification programme had finished, a melting curve analysis was performed to determine if one or multiple products had been produced. Data was stored in the form of a printable pdf file generated by the LC480 instrument and contained all relevant details including crossing point in the amplification step and melting profile for the amplicons produced from each template. This allowed screening of a given gene against 41 *Clostridium difficile* genomes at once in a single lightcycler 480 run, thus aiding throughput.

Gel Electrophoresis of qPCR products:

As a confirmatory step, a number of samples from each plate, including the positive and negative controls were electrophoresed on a 1.5% TBE/agarose gel as described above.

Results

Primer design and validation.

The genome of *Clostridium difficile* 630 was downloaded from the NCBI website; from this, a total of 683 genes annotated as “conserved hypothetical protein” were identified. A BlastP search was then performed using the ClostriDB website to determine which of these 683 conserved hypothetical proteins were unique to *C. difficile*. A total of 53 of these “hypothetical” proteins were identified as being unique to *C. difficile*.

Primers were subsequently designed to amplify 100-500bp amplicons from 52 of these genes as per the Materials and Methods section of this example. Validation of the primers was by endpoint PCR using *C. difficile* 630 DNA. In the present study, PCR reactions were performed using 24 of the 52 primer sets and these 24 primer sets produced clear bands (amplicons) of the expected size on agarose gels following electrophoresis. These 24 genes and their associated primer sequences are identified in Table 1.

Clinical isolates.

42 clinical isolates of *C. difficile,* of varying ribotypes, including 106, 078, 020, 001, 005, 026 and 014, were obtained from The Royal Victoria Hospital (RVH), Belfast under the terms of a material transfer agreement between UUTech and the RVH. The strains were sub-cultured on BHI agar plates in an anaerobic cabinet at 37°C; one strain (clinical isolate) failed to grow, thus genomic DNA was extracted from 41 strains (clinical isolates). These 41 strains (clinical isolates) are listed in Table 2). The strains were also transferred onto cryobeads (TSC Ltd, UK) and archived at -70°C for future reference. The extracted DNA was diluted to 1ng/µl for real-time PCR use and stored in aliquots at -70°C.

Lightcycler 480 qPCR

Real-time (quantitative) PCR (qPCR) was performed using the Lightcycler480 instrument (Roche Diagnostics, UK). A 96 well plate was used to carry out the PCR with 90 of the wells containing master mix with the specific primers for the gene of interest; the template genomic DNA samples were then added to the appropriate well in a specific order (as per Figure 1), with each DNA sample on the plate being analysed in duplicate. Laboratory strains of *Escherichia coli* and *Staphylococcus epidermidis* were used as negative controls. Each plate was run on the Lightcyler480 with cycling conditions specific to the primers i.e. with an annealing temperature and an extension time specific to the primer set being used. Results were obtained in the form of amplification graphs and melting curves (see Figure 2A and 2B for representative examples for the gene CD2961). If a crossing point of greater than 40 was recorded in the analysis the result was left as a question mark (?) in the data Table (Table 3) instead of a definite negative result

as the late crossing point may have been due to other factors such as primer specificity, template variability, or not enough cycles. Agarose gel electrophoresis, to check for bands (amplicons) of expected size, was used as an extra confirmatory step to enhance the accuracy of the results obtained (see Figure 2C for a representative example for the gene CD2961).

From Table 3 it is evident that five of the genes of interest (CD2961, CD3617, CD3618, CD3635 and CD3638) yielded amplicons of the expected sizes from all the *C. difficile* genomic DNA templates tested.

Summary

In summary, 53 genes annotated as encoding "hypothetical proteins" that are unique to *Clostridium difficile* have been identified. It has been demonstrated CD2961, CD3617, CD3618, CD3635 and CD3638 are detectable in 41 clinical *C. difficile* isolates of varying ribotypes.

Table 1: 24 conserved hypothetical protein encoding genes and their associated primer sequences. Whether or not a primer set could direct amplification of the target gene was determined by endpoint PCR using *C. difficile* 630 genomic DNA template. The presence of a "Y" in the "Amplicon" column indicates that a clear band (amplicon) of the expected size was visualised on an agarose gel following electrophoresis of the PCR product.

Gene No.	CD No. (Gene)	CD630	CD-QCD-32g58	fwd primer	rev primer	Annealing temperature (°C)	Amplicon Size (bp)	Amplicon
2	**CD0589**	√	√ (97%)	TGGAAAGAGCGGAGAACTTG (SEQ ID NO: 69)	GATAGCCACCACTTCCTCCA (SEQ ID NO: 70)	57	470	Y
3	**CD0590**	√	√ (97%)	GGTGGAAATGGACAAGATGG (SEQ ID NO: 71)	TCTCCATCATCTGCTGCTTG (SEQ ID NO: 72)	57	476	Y
5	**CD1124**	√	√ (99%)	TCTGTGGCCAAAAGAAAACA (SEQ ID NO: 73)	CCACAATTAAATCAAAATGGTCT (SEQ ID NO: 74)	57	500	Y
11	**CD1581**	√	√ (95%)	TCAGAAGATTGGTATGAAAGAGGA (SEQ ID NO: 75)	TGGCATTTATGGCAACAATTA (SEQ ID NO: 76)	57	431	Y
12	**CD1586**	√	√ (98%)	TTTTGAGTTTTATTGCCCAAAT (SEQ ID NO: 77)	GGTAAACCAGCTGGAGCTTT (SEQ ID NO: 78)	57	531	Y
13	**CD1597**	√	√ (98%)	ATGGAGTGGCGAAACAAAAC (SEQ ID NO: 79)	GCATGTGCAGTTTCATGTAATTCT (SEQ ID NO: 80)	57	513	Y
14	**CD1613**	√	√ (100%)	GGTATAGATCTTTCAGCTCCTCCA (SEQ ID NO: 81)	CAACAGCAATCATCACAATCG (SEQ ID NO: 82)	57	452	Y
16	**CD1757**	√	√ (100%)	TGGCATAAGGATTTAATTGATGT (SEQ ID NO: 83)	AAACATGATATTTCCAGACCACAA (SEQ ID NO: 84)	57	450	Y
22	**CD2133**	√	√ (98%)	ACTATATGGAATTTGAAGATATTCCTG (SEQ ID NO: 85)	TTTGATTGTTCTCTTATTTCAACTGC (SEQ ID NO: 86)	54	397	V faint
30	**CD2454**	√	√ (98%)	TGGTTTTTGCATATACGAATGA (SEQ ID NO: 87)	CCTCCCTTCCATCTACAATCC (SEQ ID NO: 88)	58	453	Y
31	**CD2547**	√	√ (98%)	GGGCAGGGCAAAGTTGTTAT (SEQ ID NO: 89)	TTTTGGTCGTGAGTTGCTGA (SEQ ID NO: 90)	58	478	Y
33	**CD2815**	√	√ (98%)	GCATGCAAACATTTTGGTGA (SEQ ID NO: 91)	TTCAGATACCTTGTCATCATGGA (SEQ ID NO: 92)	58	438	Y
34	**CD2961**	√	√ (99%)	AGAAGGCAGTCACCGACAGT (SEQ ID NO: 11)	CCTTTCCAATAACCGCTTCA (SEQ ID NO: 12)	58	129	Y
35	**CD2972**	√	√ (99%)	CCTAGATGAAAGACCAATTTTAGATGA (SEQ ID NO: 93)	CAGAGTCACAATTTCCACAACAG (SEQ ID NO: 94)	58	413	Y
36	**CD3022**	√	√ (97%)	ATCTTGTGGGCTGGGTATTG (SEQ ID NO: 95)	CCTCCTCCATGTACCGATTT (SEQ ID NO: 96)	58	540	Y
37	**CD3023**	√	√ (96%)	CCTCCAACAGATGGAAAACC (SEQ ID NO: 97)	GTACTGCCCACACCTTGTGA (SEQ ID NO: 98)	58	456	Y
38	**CD3024**	√	√ (95%)	CAACCAATCATAGGAACAACCA (SEQ ID NO: 99)	TCCACAATATCCACATTGGTC (SEQ ID NO: 100)	58	480	Y
39	**CD3163**	√	√ (97%)	TGGGGATGATAGGATGTTATACTAAA (SEQ ID NO: 101)	TCCATCATCAGATGCTTCTTGTA (SEQ ID NO: 102)	58	464	Y
45	**CD3573**	√	√ (98%)	TGGATATAAGAGCGTTACCTATAAGA (SEQ ID NO: 103)	TCAACTCCACCTTTCCAAAAA (SEQ ID NO: 104)	57	474	Y
47	**CD3617**	√	√ (98%)	GATGGATACTGGGGTGATGG (SEQ ID NO: 13)	AAGGCAATAAAGTCGCTTCG (SEQ ID NO: 14)	57	475	Y
48	**CD3618**	√	√ (99%)	TTTAATGGTACTTGTGAAAAAGCAT (SEQ ID NO: 15)	GCCATCATTTTGATGTGGTG (SEQ ID NO: 16)	57	306	Y
49	**CD3635**	√	√ (95%)	CATATGCCTGAGATGGCAAA (SEQ ID NO: 17)	CTTGTGCCCATTCTGGTTTT (SEQ ID NO: 18)	57	499	Y
50	**CD3638**	√	√ (99%)	GGATGCTGTTTTGGAGGAAA (SEQ ID NO: 19)	AAATTCTGGGCAATGAGGTG (SEQ ID NO: 20)	57	525	Y
51	**CD3641**	√	√ (98%)	CTTGTGACGGGCATGTATTG (SEQ ID NO: 105)	TGTTTTAAGCCCTCCCATTG (SEQ ID NO: 106)	57	419	Y

Table 2 List of *Clostridium difficile* strains obtained from Royal Victoria Hospital

	RVH ref No	Ribotype
1.	100058-106	106
2.	100048-106	106
3.	090092-106	106
4.	100059-106	106
5.	100150-078	078
6.	090126-106	106
7.	090269-106	106
8.	100149-078	078
9.	090160-106	106
10.	100162-020	020
11.	090389-106	106
12.	090361-106	106
13.	090183-106	106
14.	090391-106	106
15.	090129-106	106
16.	090217-106	106
17.	090225-106	106
18.	090223-106	106
19.	090645-106	106
20.	090294-106	106
21.	090540-106	106
22.	100063-106	106
23.	100158-078	078
24.	100170-001	001
25.	100171-001	001
26.	100172-020	020
27.	100173-078v	078v
28.	100177-005	005
29.	100163-026	026
30.	100178-106	106
31.	100164-014	014
32.	100167-001	001
33.	100168-001	001
34.	100169-078	078
35.	100143-026	026
36.	100144-014	014
37.	100146-005	005
38.	100147-014	014
39.	100142-005	005
40.	100140ii-020	020
41.	100153-001	001

Table 3 (shown overleaf) – qPCR screening of CD2961, CD3617, CD3618, CD3635 and CD3638 across 41 strains of *C. difficile*, with *E. coli* and *Staphylococcus aureus* as negative controls and *C. difficile* strain 630 as a positive control. For all of the qPCR reactions for genes CD3617, CD3618, CD3635 and CD3638 and some of the PCR reactions for gene CD2961, the PCR product produced by the qPCR reaction was subjected to agarose gel electrophoresis to further assess whether an amplicon of the expected size was produced. In all cases in which the template DNA was *C. difficile* genome, bands of the expected size were observed (as shown by a "G+" in the "GEL" column). Note that no amplification from the *E.coli* or *Staphylococcus epidermidis* samples was observed.

Table 3

Strain/gene	CD2961		CD3617		CD3618		CD3635		CD3638	
	qPCR	GEL	qPCR	GEL	qPCR	GEL	qPCR	GEL	qPCR	GEL
1-106	YY		??	G+	Y?	G+	??	G+	??	G+
2-106	YY	G+	??	G+	?Y	G+	Y?	G+	??	G+
3-106	YY		??	G+	?Y	G+	Y?	G+	?N	G+
4-106	YY		YY	G+	YY	G+	??	G+	??	G+
5-078	YY		??	G+	YY	G+	??	G+	??	G+
6-106	YY		??	G+	YY	G+	??	G+	?N	G+
7-106	YY		Y?	G+	Y?	G+	YY	G+	??	G+
8-078	YY		YY	G+	YY	G+	??	G+	N?	G+
9-106	YY		??	G+	?Y	G+	??	G+	??	G+
10-020	YY		??	G+	YY	G+	Y?	G+	??	G+
11-106	YY		??	G+	YY	G+	YY	G+	??	G+
12-106	YY		?Y	G+	YY	G+	?Y	G+	?N	G+
13-106	YY		Y?	G+	YY	G+	YY	G+	??	G+
14-106	YY		YY	G+	YY	G+	Y?	G+	N?	G+
15-106	YY		??	G+	YY	G+	YY	G+	??	G+
16-106	YY		??	G+	YY	G+	YY	G+	??	G+
17-106	YY	G+	?Y	G+	YY	G+	YY	G+	??	G+
18-106	YY		??	G+	Y?	G+	??	G+	N?	G+
19-106	YY		?Y	G+	YY	G+	??	G+	??	G+
20-106	YY		??	G+	?Y	G+	??	G+	??	G+
21-106	YY		?Y	G+	YY	G+	??	G+	??	G+
22-106	YY		??	G+	YY	G+	YY	G+	??	G+

Table 3 (Continued)

Strain/gene	CD2961		CD3617		CD3618		CD3635		CD3638	
	qPCR	GEL	qPCR	GEL	qPCR	GEL	qPCR	GEL	qPCR	GEL
23-078	YY		YY	G+	YY	G+	??	G+	??	G+
24-001	YY		??	G+	YY	G+	Y?	G+	??	G+
25-001	YY		YY	G+	Y?	G+	YY	G+	??	G+
26-020	YY		?Y	G+	YY	G+	YN	G+	??	G+
27-078v	YY		YY	G+	YY	G+	??	G+	?Y	G+
28-005	YY		??	G+	YY	G+	YY	G+	??	G+
29-026	YY		?Y	G+	YY	G+	YY	G+	??	G+
30-106	YY		??	G+	YY	G+	YY	G+	??	G+
31-014	YY	G+	YY	G+	YY	G+	YY	G+	??	G+
32-001	YY		YY	G+	YY	G+	Y?	G+	??	G+
33-001	YY		YY	G+	YY	G+	YY	G+	?Y	G+
34-078	YY		YY	G+	YY	G+	??	G+	??	G+
35-026	YY		YY	G+	YY	G+	YY	G+	??	G+
36-014	YY		YY	G+	YY	G+	YY	G+	??	G+
37-005	YY		Y?	G+	YY	G+	YY	G+	??	G+
38-014	YY		?Y	G+	YY	G+	YY	G+	??	G+
39-005	YY		YY	G+	YY	G+	YY	G+	??	G+
40-020	YY		YY	G+	YY	G+	YY	G+	??	G+
41-001	YY		YY	G+	YY	G+	YY	G+	??	G+
630	YY	G+	YY	G+	YY	G+	YY	G+	YY	G+
E. coli	NN		NN	G-	NN	G-	NN	G-	NN	G-
Staph	NN		NN	G-	NN	G-	NN	G-	NN	G-

Example 2 (Prophetic Example)

The methods of Example 1 are performed with primer pairs designed to target each of genes CD0588, CD0638, CD1234, CD1423, CD1424, CD1487, CD1543a, CD1728, CD1794, CD1897, CD1906, CD2046, CD2098, CD2216, CD2248, CD2264, CD2274, CD2300, CD2306, CD2309, CD2563, CD3188, CD3288, CD3321, CD3367, CD3369, CD3609 and CD3656. Primers for each gene are described in Table 4.

Table 4 - Nucleotide sequences of primers directed to the genes listed in Example 2

CD No.	fwd primer	rev primer
CD0588	AGGTTGAAAATAGTAGAAAAGAAGATG (SEQ ID NO: 107)	TGGCTTAAACATTATACTACCATGA (SEQ ID NO: 108)
CD0638	TGTTTATTTTGAATTTTGGAGGA (SEQ ID NO: 109)	CATATATCCAACTCATTAAGCCATGA (SEQ ID NO: 110)
CD1234	TTCATATTTTATAACAAGGGGTGATG (SEQ ID NO: 111)	TTCTACTGTCTCAACTTTCTTCATAGC (SEQ ID NO: 112)
CD1423	TGAAATGTTCTAATTGTGGAAGTGT (SEQ ID NO: 113)	TTCTTATCTTTACACCAATTCCTATCA (SEQ ID NO: 114)
CD1424	GATGAAATGGATAAATGTACACACA (SEQ ID NO: 115)	CCAATACCATCACTTGATGTAAAC (SEQ ID NO: 116)
CD1487	TGGAAAATGATACTATTAAGGCTGA (SEQ ID NO: 117)	TTGCAAAATTGTAAGATTCTCCT (SEQ ID NO: 118)
CD1543a	AATGAAAAGTATGATTGTTATTGGTG (SEQ ID NO: 119)	TTAATTTGGATTTGTTTATACCCTATG (SEQ ID NO: 120)
CD1728	AAAATTACACCCTTAGAGGCACA (SEQ ID NO: 121)	TTACTCTTTTAAGTAAATTTCCACCTG (SEQ ID NO: 122)
CD1794	AAGATGAAAGTTCTGACTCTGGGTA (SEQ ID NO: 123)	ACCTTATTTACTACAATCCAAAGGTT (SEQ ID NO: 124)
CD1897	TCAGGTTGTGGATTATTTTGGA (SEQ ID NO: 125)	TGGTAATATTCCTCTTTATCATTTGAA (SEQ ID NO: 126)
CD1906	TGCATATGGAAATCAATGTTATAGAAA (SEQ ID NO: 127)	TTTACAAACCAACTAGCTTTATCCA (SEQ ID NO: 128)
CD2046	TTTTTATCCTAATGACATATTTCCAA (SEQ ID NO: 129)	AGATATGCTAAACCTTCCAACTTGA (SEQ ID NO: 130)
CD2098	GGCTGGTAATCTAAATAATATGAGAGC (SEQ ID NO: 131)	CCAAGTACCTATACAATCAATTTCCA (SEQ ID NO: 132)
CD2216	TTGTGATTGAAGGTAGCGATAAA (SEQ ID NO: 133)	TCCAAGACTTTGGAAACTTCA (SEQ ID NO: 134)
CD2248	GCATTGGATAAAGGACTGTGC (SEQ ID NO: 135)	CAAGCTCTGTCTTTGGAGCA (SEQ ID NO: 136)
CD2264	TGAAGGACTTGACCAAAATGAA (SEQ ID NO: 137)	TTTTTCTTTCCACCTCTTTTGA (SEQ ID NO: 138)
CD2274	CATGGAATAATTCGAGTGTTGAA (SEQ ID NO: 139)	GTTCTCCCAGGAGCCTTTTT (SEQ ID NO: 140)
CD2300	TGAATGATATGGCAAGAGATGT (SEQ ID NO: 141)	CCTGTTCCCCAATCAATCTG (SEQ ID NO: 142)
CD2306	TGCACCATAATTGTTAGAGCAAA (SEQ ID NO: 143)	TTTTTATTTTTAGTGCACACTCTCC (SEQ ID NO: 144)
CD2309	GGATAAATGTACACATATGTTAACTGC (SEQ ID NO: 145)	CTTGATGCAAGCAAGATTCC (SEQ ID NO: 146)
CD2563	AAAGAAGCAATGAAAAACGAGAA (SEQ ID NO: 147)	TTTTCTACTTAACCTTTCAGGTCCA (SEQ ID NO: 148)
CD3188	TGAGCAAAAGTGAATTAACAGCA (SEQ ID NO: 149)	TTTTCCAATCCATTTCTTCCA (SEQ ID NO: 150)
CD3288	GATGATGAAATCACCTTAGGTAGTGA (SEQ ID NO: 151)	ACCCAAATCTCATCTAGCACAAA (SEQ ID NO: 152)
CD3321	AATGTTCCATTTGACTATGTTCG (SEQ ID NO: 153)	GGAGGAAATTCATCATCTCCA (SEQ ID NO: 154)
CD3367	TGGTTAAGAAAAATGAAACTGTAGGA (SEQ ID NO: 155)	GCATATTTTCAAGCTCTTCACG (SEQ ID NO: 156)
CD3369	TTCAAGAAAGCATTCCTATCACA (SEQ ID NO: 157)	TCTTTGCTTACAACTATACCACCTTTT (SEQ ID NO: 158)
CD3609	TGTTTAAGAAAATGGCAGTACTAAAAG (SEQ ID NO: 159)	TTTTTATCTTTACCTCAGCTACAAAGG (SEQ ID NO: 160)
CD3656	TTGTTGTTTATGCTAATAATGTGGA (SEQ ID NO: 161)	TTCTATTTTTGAAAACTCTTCTTTCTC (SEQ ID NO: 162)

Example 3

Introduction

Using microarray analysis, expression of CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD3609 was assessed in *Clostridium difficile* strain 630.

Materials and Methods

Bacterial Cell Culture.

Clostridium difficile strain 630 was routinely maintained on BHI agar or grown in BHI broth (Oxoid) at 37°C in a MACS MG500 Anaerobic workstation fitted with an airlock (Don Whitley Scientific, UK). Heat stress was induced in broth cultures in the early exponential phase of growth using a water bath set at 41°C and cells were harvested in biological triplicates at late log phase (D_{650} = 1.1) of anaerobic growth as described by Jain et al (Jain S, Graham C, Graham RLJ, McMullan G, Ternan, NG (2011) A quantitative proteomic analysis of the heat stress response in *Clostridium difficile* strain 630. J Proteome Res 10(9): 3880-3890).

Total RNA Isolation.

RNA was extracted from aliquots of 4 × 10^8 cells from both control and heat-stressed triplicate cultures of *C. difficile* strain 630 using a Qiagen RNEasy mini kit. The Qiagen protocol was modified to include a mechanical lysis step – cells in TE buffer with proteinase K and lysozyme were added to a Lysing Matrix A tube (MP Biomedicals) and treated in a Fastprep FP120 machine (MP Biomedical) at speed 5.5 for 30s to break open the cells. Following both on-column and in-solution DNAse digestions, and a final on column cleanup, RNA samples were confirmed free of contaminating genomic DNA by performing PCR with *tpi* primers (Lemeé L, Dhalluin A, Pestel-Caron M, Lemeland JF, Pons JL (2004) Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. J Clin Microbiol 42: 2609-2617). RNA Samples were stored at -70°C until required for microarray experiments or for qRT-PCR.

Template Labelling and Microarray Hybridisations.

Microarray experimentation was out-sourced to Oxford Gene Technology (OGT; Begbroke Science Park, Oxford, UK). RNA samples were sent on dry ice to OGT where the quality and integrity of the 16S and 23S ribosomal RNA (rRNA) subunits was verified by using the 2100 Bioanalyzer system (Agilent Technologies). For all six RNA samples, an RNA integrity number of >9.6 was obtained, with A260/280 values of > 2.0, and 23S:16S rRNA ratios of ≥ 1.4. Using Ambion's MessageAmp™ II-Bacteria RNA Amplification Kit, the template mRNA samples were: (a) polyadenylated; (b) the mRNA samples with a stable poly(A) tail were reverse-transcribed into first strand cDNA using an oligo(dT)-primer bearing a T7 promoter; (c) the cDNA samples were then converted into double-stranded DNA (dsDNA); (d) dsDNA was then used as a template for *in vitro* transcription with T7 RNA polymerase to generate antisense RNA (aRNA); (e) aRNA was then finally labelled with fluorescent dyes (Cy3 and Cy5) to create labelled probes for hybridisation. In this investigation, a dye-swap (i.e. control samples labelled with Cy3 and heat-stress samples labelled with Cy5 and vice versa) was performed in order to generate technical replicates and to compensate for any potential bias introduced as a result of inherent discrepancies in Cy dye incorporation (Do JH, Choi DK (2007) cDNA Labeling Strategies for Microarrays Using Fluorescent Dyes. Eng Life Sci 7(1): 26-34). Prior to hybridisation, labelled aRNA was purified using Qiagen's RNeasy® MinElute Cleanup Kit as per the manufacturer's instructions. The labelled probes were then hybridised to a *C. difficile* strain 630 array (BUGS CD630 gene expression array plus Plasmid pCD630, 8 x 15k array, v2.01) comprising 3,776 genes using the Gene Expression Hybridisation Kit (Agilent Technologies) as described in the manufacturer's protocol.

Microarray Data Analysis.

The hybridised arrays were subsequently scanned at 532 nm and 635 nm, corresponding to Cy3 and Cy5 excitation maxima, using an Agilent C Microarray Scanner equipped with the extended dynamic range (XDR) software for improved resolution. The data was then extracted from raw microarray image files and the probe signals were subsequently quantified using Agilent's Feature Extraction Software version 10.5.1.1. Upon normalisation by the locally weighted scatterplot smoothing (LOWESS) algorithm, the data was imported to GeneSpring GX version 11.0 (Agilent Technologies) where the minimum fluorescence intensity was set to 1.

The mean normalised log_2 fluorescence ratios and standard errors of mean were then calculated across all probes for an individual gene and concatenated to gene level. The microarray data has been deposited in NCBI's Gene Expression Omnibus (Edgar R, Domrachev M, Lash AE (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucl Acid Res 30(1): 207-210) and is accessible through GEO Series accession number GSE37442 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37442).

Results and Conclusion

The data in Table 5 below shows the raw average fluorescence (raw avg fluor) and the expression relative to triosephosphate isomerase (tpi) expression (relative exp) for each of the genes CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD3609. These data show that each of these genes is expressed in *Clostridium difficile* strain 630.

Table 5

Gene	Name	raw avg fluor	relative exp
tpi	triosephosphate isomerase (reference gene)	1946	1.00
CD3609	hypothetical protein	40428	20.77
CD2309	hypothetical protein	28239	14.51
CD1543A	hypothetical protein	4363	2.24
CD2046	hypothetical protein	2593	1.33
CD1424	hypothetical protein	1028	0.53
CD2098	hypothetical protein	567	0.29
CD3288	hypothetical protein	522	0.27
CD0638	hypothetical protein	278	0.14
CD2274	hypothetical protein	231	0.12
CD2216	hypothetical protein	71	0.04
CD3188	hypothetical protein	39	0.02
CD1487	hypothetical protein	33	0.02
CD3635	hypothetical protein	30	0.02
CD2264	hypothetical protein	26	0.01
CD3367	hypothetical protein	22	0.01
CD1794	hypothetical protein	19	0.01
CD3638	hypothetical protein	18	0.01
CD1906	hypothetical protein	17	0.01

Example 4

Introduction

The study described in Example 1 was expanded. In this expanded study, the presence of each of CD0588, CD0638, CD1234, CD1423, CD1424, CD1487, CD1543a, CD1728, CD1794, CD1897, CD1906, CD2046, CD2098, CD2216, CD2248, CD2264, CD2274, CD2300, CD2306, CD2309, CD2563, CD3188, CD3288, CD3321, CD3367, CD3369, CD3609, CD3656, CD3617, CD3618, CD3635 and CD3638 was screened for in the 41 *Clostridium difficile* clinical isolates of Example 1 and, where a gene was detected as being present in those 41 clinical isolates, the presence of that gene was screened for in 41 further *Clostridium difficile* clinical isolates.

Methodology

In this expanded study, the materials and methods used were the same as the materials and methods described in Example 1.

For the genes detected in all of the clinical isolates tested, the nucleotide sequences of the primers used, the coding nucleotide sequences of the genes, the amplicon sizes and the melting temperatures of the primers are set forth in Table 6.

A list of the 82 *Clostridium difficile* clinical isolates obtained from the Royal Victoria Hospital (RVH) which were used in this study and the ribotypes thereof are set forth in Table 7. Note that the clinical isolates numbered 1-41 are the clinical isolates (strains) used in Example 1 (as listed in Table 2).

A list of the negative control strains used are set forth in Table 8.

In a first screen, each of the *Clostridium difficile* clinical isolates numbered 1-41 in Table 7 was screened for the presence of each of the genes CD0588, CD0638, CD1234, CD1423, CD1424, CD1487, CD1543a, CD1728, CD1794, CD1897, CD1906, CD2046, CD2098, CD2216, CD2248, CD2264, CD2274, CD2300, CD2306, CD2309, CD2563, CD3188, CD3288, CD3321, CD3367, CD3369,

CD3609, CD3656, CD3617, CD3618, CD3635 and CD3638. If a given gene was detected as present in all of the *Clostridium difficile* clinical isolates numbered 1-41, then, in a second screen, each of the *Clostridium difficile* clinical isolates numbered 42-82 in Table 7 was screened for the presence of that gene. If a given gene was detected in all 82 *Clostridium difficile* clinical isolates, negative control strains as set forth in Table 8 were screened (in a third screen) for the presence of that gene.

Results and Discussion

The results of this study are presented in Table 9. In Table 9, a "+" sign indicates that a gene was detected as present in all of the clinical isolates in the relevant screen, and a "-" sign indicates that a gene was not detected in all of the clinical isolates in the relevant screen. "Low sensitivity levels" means that the sensitivity of the PCR was such that a conclusion could not be drawn as to whether a gene was present or not; in these cases optimization of PCR cycling conditions will be required in order to determine whether these genes are present in the tested *Clostridium difficile* clinical isolates.

In this study it has been demonstrated that CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367, CD3609, CD3635 and CD3638 are detectable in 82 *Clostridium difficile* clinical isolates of varying ribotypes.

Table 6 - Nucleotide sequences of primers, coding nucleotide sequences of genes, amplicon sizes and melting temperatures (Tm) of primers

Gene	Sequence	F primer	R primer	Amp size	Tm
CD0638	TTGTTTATTTTGAATTTTGGAGGATTAATTATGGATTCAAATAATAATACTATAAAATCA ACTGTTAAAAAGGGTATTTCTTTTGGTTCTTGTTTAGCAATGATTATTTCTTATACTGCA TGGAAATCTATTCCATGGGCTATTTTTCATGGCTTAATGAGTTGGATATATGTACTTTAT TATTGGGTTAAGTATGCATAG	TGTTTATT TTGAATTT TGGAGGA TT	CATGAAA AATAGCCC ATGGAA	150	59
CD1424	ATGTTTAGAGATGAAATGGATAAATGTACACACATGTTAACTGCTTATATTAGTAGTTTA TATGATTATTGTGATTTTATAGATACACAGCTAGATGATTTTATACTAGAGTACGGAGAA AATGTAGTAGAATCTTGTTTACATCAAGTGATGGTATTGGTAAGTAAGTATAATTAA	AGATGAA ATGGATA AATGTAC ACACA	CCATCACT TGATGTAA ACAAGATT C	146	59
CD1487	ATGGAAAATGATACTATTAAGGCTGATGATATTCTCAATTATTGTCTATCAAACTTAGAT GATGTTGTACTAATGGATAGTTGGGGGGAACGAGCAATTTACTACAATCCTAATGGTGTT TTAAAGCGAGGGGTATATGTTCTTACCATTAAGGAAAAAGACAGTAATAATGATAAAGGT TCGTTAGTTAGTCGTCCAAATGTATACCGTGTGAATATAGGATTAAAAAAAGAAACTTTT ATTGAAATGTTTGGATATATTCCAAAGCGTCCAGGTGTAGGTCAAATAGTTGATATGGAT TTTGATTTTACAAAATTGGACACAATCATGCCACATCCTATCTACTCATGGATGGGATGG ATATGCGTCCTAAGCCCTACTGAAAAGACATTTGAGAACTTTAAAACTTTAATAGGAGAA TCTTACAATTTTGCAAAACAAAAATTTAAAAAGAGAAAAAACAAGTAA	GGGGGAA CGAGCAA TTTACTA	ACCTACAC CTGGACGC TTTG	199	60
CD1543 a	ATGCGAGAAGAAAAAAGTAATGAAAAGTATGATTGTTATTGGTGTAATCAAGAGAATAAT TTCTGTGTAGAAATAAAAGATAATATAGTCATGATAGATGATGGCACTGGTACGTTAAAA CAAGCCGTTTTCATAGGGTATAAACAAATCCAAATTAATTTAAACTGTTCACATTGTCAA AACTTAAATAGAATAAAATTAAATTTGTAG	TGATTGTT ATTGGTGT AATCAAG AGAA	ACCCTATG AAAACGG CTTGTT	110	60
CD1794	TTGTTTATAGATGAAGAATTAGAAGGTTATATATTAACATGTAAAATATCTGAAGACTTT AAAAATATACCTGAATATAGTGATGAAGAGTTTTATGTTACAGTCTATAAAGATGAAAGT TCTGACTCTGGGTACTATGCTTTATTAGAAAATAAAGAAGAAAGAGTTGTATGGGATGGA	ATAAAGA TGAAAGT	TTAACCTT ATTTACTA	130	57

	GAAGTTGTTGCCAATAATATTTTTAATAACCTTTGGATTGTAGTAAATAAGGTTAAAACT GGATAA	TCTGACTC TGG	CAATCCAA AGG		
CD1906	TTGCATATGGAAATCAATGTTATAGAAATTTTCCCTAAAGATAAAGCTAAACTTAATAAA ATAGAAATGGATAAAGCTAGTTGGTTTGTAAATATAATAAGTAAAAAATATCCTAAAGAA GCTTTAAATGAAGCATTTAGTACTTTAGAAAAAGAATTAAATATAAGTAAAGCTAATACA TAA	TGCATAT GGAAATC AATGTTAT AGAAA	TGCTTCAT TTAAAGCT TCTTTAGG ATA	134	60
CD2046	GTGGATGAAATGCTTGTATATAATAAAAGTTTTTATCCTAATGACATATTTCCAAGATTA GATTTTTCAAAAATAAAAAAACAGTTAAAATTGATAGATAATGACCTGTCAGATTTTGGA AGCATATGTATAATAGAAAAAGAACATTATACGATAAGTGTAAACAGTATAGGTGAAATA AATGTGTACTATGATTTAGAGTACGAAAATAAGGTGTATAGAATAGTTTATGAGATTGAA AAGTTATTTAAATCTCAAGTTGGAAGGTTTAGCATATCTACATACAGAAATTGA	ACCTGTC AGATTTTG GAAGCA	TGCTAAAC CTTCCAAC TTGAGAT	171	59
CD2098	TTGGCTGGTAATCTAAATAATATGAGAGCAGTAAATAATTTTAGAGGAGATAAGAACATT TTAGAATGTTTAGTCAGCTTTGAGGGTCGTTCAATAAGTCAGAGAAAAGTAAGGGTATTT TTTAAAGAAAAACAAAATCAAATAGAAATTGATTTTGCAGAAGAGGAAATTTCTAAATTG GTTGAAAATGTTGTTTTAAATACATCATATCAAGAAATGTTATATGATGAAATAGAGAAA CAACTGGAAATTGATTGTATAGGTACTTGGATGATATTATCTAAATTAAAAGATGGTAGT AGAGTTCACTGA	GTCAGCTT TGAGGGT CGTTC	ACAATCA ATTTCCAG TTGTTTCT CT	186	59
CD2216	ATGATTGTGATTGAAGGTAGCGATAAATTTAAGATTGCAAAAGAATATATTGATGTAGAA TATACTCTTTTTAGCAAAGTAACTTTTAGGTATGAAAAGTTGAAATTTAAAGATAATGCT GAATTGGAAAAAATTAAGATGTTTAAATATAAAAATGGCTACATCCCTAATAAATTAAAC CTTTCTTTTGGATATGGATTCTCTTCTTATAAAAAGCAAATAATTAGAGAAACTGTAGAT	CCTTTCTT TTGGATAT GGATTCTC	CAGGGCA ATAGCAA CAAACA	212	59

	ACTTTAAGATTGACAGAAATTTTTTCAAGCGAGAACATAGAAGATATTAAATTTATAAAA GATGGTACAAAAAAATTAGAAATTAGCATAGAGAAAGTTGTGAAATTTAAACGTCGAAAA AAAAGAAATTATGTTTGTTGCTATTGCCCTGATATGTATAGGGACATAAAACTCGACAAA GAATCTATCAATAAGATATACAACAGAAAAATAAAAATAGAAAGAGAAGTTAATATTTTT GAAGATGAGGATGTTATAATAAACAAAAAAGTATTGAAGTTTCCAAAGTCTTGGACAAAG AATATGCAAAAATATTGGTTAAGTGAAAATAAGTATCCCATACATTCTACTGTAATTGAT GATGATAGATATAAATGTTGTAATGTACAATATACAAAAAATAGAGTGATAATATTATAT TACATATATAACCATTAA				
CD2264	GTGTTAAAAAAGTGGTTTGGTATTGTGAAAAAAAGACAAAAAAGTGAGTCAGTAAAAGAA GAAGGTGAAGTAATATTAAAAAACGAAAAAATATTATCTGAAGAAAAGTTGATAGATGAA GAAGGAGTTGTAGTTAATATTGATAATGAAATATTAACTAAAGTAGAAGTAGTAAATGAT GACAATGAAATAGAAGAAAAAATAATAGAAGAAGATTGGTTAATTAGTGAAAATACCATA AAATTAGATGATAAAGAAGCAATTATTAATGATAAAAACATAGAATTATGTAAAAAAGAA GTTCAAGTTGAAGGTGAAAAAATAGATTTAAATAAGTTTGAAGGACTTGACCAAAATGAA AATTATAATCTAGAAAAAAATGTTATTGAAGAAAAAGAAGTAAGTGAATGTTTGACAGAA GAAGATTTAGAGTACATAAAAGAAATTAAAATAAAAAGAGGAAAAAGTATAAAAGCTATA AACTTGTATACTAAAGAAGAGTGGGTTTTTGACACTCATATACAGTGTAGTAAAAAACTC AAAGTTCCATTAGGGTACATAAGAGAAAATTTAAAATATGGATATATGGATTACTTTGGA GATGCAATAAATTATTTAAGTGAAGTATTAAATATAGATGAATACTGCAAAAGTGAATGG AGCTATCTCGATAATAGCAAATCTCCATCTGAGATATTTAATATTCTAAACAATAAAATA TTTAGTATAAGGCTTTCTAATGAAAAAAGAAATGAAATTTTGACAAATGATAAAATTGAA GCATTAAAAATGAATTATAGATTTGAATGTATTGATGAAGAATATGATGAATATTTTAAA AAATATAAGTCTATAATCAAAAGAGGTGGAAAGAAAAAAGTTGAATTAGTAAATAAAAAA GGTGACATTTTAGAAATATTTAAGTCTTTAGAAGAATGTGCCATTTATTTGCAAAAGGAA AAGAATGAAGTTATACAAATGTTAAAATATGGAGATACAAAAGTAGGAAGAAACTTTATA AGGTATAGTTTGAGAAGTATTTAA	AAGAGTG GGTTTTTG ACACTCA	AGCTCCAT TCACTTTT GCAGT	168	59
CD2274	ATGGATTTAAGTGGCATATTTAAATACTATTGTAAAGAGTGTGAAAATACATGGAATAAT TCGAGTGTTGAATTATTTGAGAATATAGAAACGTATAGTAAAGATTCACAAAAAAAGAGG GAAAAAGAATTAGATAAATTGCTAAATACAATATCAGTTCATTTAGAGAGGTATCCAAGT GATGCTGTATTGAGAAAAATGTGGGTAAAAAAGGGCGAGGTTTTCTTACAAAAGACATTG GAAAAAGAAAATATTTTTAAGTTAGAAAAAATGGATGTAGAGGATAGAAAAAAATTTTTA GATATAACAAAACAGTTTATTAGAGATGCTAGAAAATTTGATGATGATTTACCTATAGGT GATATTATGCAAGCTATGAGGAATGTATGGATTTCAAATGCATTACAATTATTATTTGGT	AAAAAGG CTCCTGG GAGAAC	CGGATGA ACCTCCTT TTTCA	228	60

	AAAGAAGTATATTATTCAAAAGCTAACTTTGCATATAGTATGTTATATCCATATACTGAT AATTATTTAGACAATACAAATATAGATAAAAATGATAAGATTTTATTTAATAACTGGTTA GAAAAAAGGCTCCTGGGAGAACACATTAAATCTAAGGATTATCATGAAAGTAAAGTATCT CAAATGATAGATTATATTGAAAGTGTATACCCTAGAGAAAAGTTTACAGAAGTTTATGAA TCGTTATTATTAATATTTAAAAGTCAAGTAAATAGTTTAAAACAACATGGTAAGGAAAAT CATTTGTGTAAAGAAGATTTATTATCCATTTCTATTGAAAAAGGAGGTTCATCCGTTTTA GTAGATGGATATTTAATAAGTGGATTGATGACAAAGGAAGAAATAGAGTTTTGTATAGGA TATGGATTTTTATTACAAATATCTGATGATTTACAAGATATAAAAGAAGATTTAAAATAC AACCATAAAACTATTATTACAGAGATGTCAAAAGAGGGTACTTTAGATAAAGTTGTAAAT AAACTAATAAATTTTACTATTGAGTTAATAGATAGTTTTAAAATTAATAATAAAAATAAA TCTGTAATAACTATGATAAAGAATGATTGCTTAATGTTAATTTTATTTTCTGTAGTTTAT AATGCTGAATTTTTTTCTGTAGGATATATAAAAGAAGTAGAGAAATTTATTCCATATACA ATAGATTATTCATTAGAGATTGAAGAAAAAATAAAAGAAAAATTTAAAAATATAGATGTT TTAAATAATGAAAATGAATATAAAGAAATGATTGATATTATTTGTGCAGAGTAG				
CD2309	ATGTTTAGAGATAAAATGGATAAATGTACACATATGTTAACTGCTTATATTGGTAGTTCA TATGATTATTGTGATTTTATAGATACACAGTTAGATGATTTTATATTAGAGTACGGAGAA AAAGTTGTGGAATCTTGCTTGCATCAAGTGATGGTATTGGTAAGTAAGTATAATTAG	GGATAAA TGTACAC ATATGTTA ACTGC	GCAAGCA AGATTCCA CAACT	125	57
CD3188	ATGAGCAAAAGTGAATTAACAGCAGAAACAACAGAAGAAATGTTAGAAGTACTAAGTGGT AAAGATTATGATATTGCATGTCATTTACATGAACTTGGTAAATCATTAGATTGTAAAATT GAACCAAAAACAGGTGCTCGTTCTTACAAAATAGTATATTCAACTAAGAAACCAAAACGT AGCTTATTTACTATTGAATGTAATGAAAAAAAATGGAGAGTTAAAGCAAATCTTTTTCAT CTAAATACATATAAAGATGCTGTGGAGGAATGCTCTAAAACTATTAAAGATAGTATAACT AAAACTCGTACTTGTAAGAAATGTAATTCAAAGTGTATTGGAGGTTCTTCGTTTGAATTA GATGGAAAGTCTTACCTGACTTGCATAGGAAGTGGTCATTATTTTGCAAATATGGAAGAA ATGGATTGGAAAAACCTAGAAAAATTAATTACTAAAGAAAATAATATTATGCAGGAATCT	ACCAAAA ACAGGTG CTCGTT	AGAGCATT CCTCCACA GCAT	154	60

	GTATAG				
CD3288	ATGGACTATATAGGAATAGAAAATATAACACCTTATGAAAATACATATGAATTTAGTGTA TATGAATATGATGATGAAATCACCTTAGGTAGTGAAAAGTTATATGTATGTGAATTAAGG GTTGTATTGATTAAAGTTAATTCTCTGTATGTTGAAAGATTGCATAAATCAGTTGAAGCA ATGGTCTTAGTAAAAAATTTGAAAAAAGATTTAGATAAAACACTTGTTGTAAACAAAATA AAGAATTTTGTGCTAGATGAGATTTGGGTAGAAAATCTAGTAAAAGAGAATATAGAAGTT ATATTTGTAGAAAGCTAG	TGATGAA ATCACCTT AGGTAGT GAA	CCCAAATC TCATCTAG CACAAA	197	59
CD3367	ATGAAAATATCTAGTCAATATAGAAGTCAATATTCATTTAGATATGAAAGTAATATAAAT AATACAAGAATAAATGAAAGTATGGTTAAGAAAAATGAAACTGTAGGAAAAGACACTTAT TTATCTAATATTATGAAACAAAAGCAAGAACTTAATGATAGAATTAGAGATTTAAAATAT AGACAAGAGGTTTATACTAAAAAAATAAATGACGCAATTAAGAACTTATGTAAATCAGAA ATAAGAGAAACAACTAATAATTTTTCTAATATAGAAATAGGTATTAAAAATAGCATTATA GAAGAGAAAAATAAAAGTACAATGTTAGATGAAAATTCAACTTATCTAAATACAAATGAT GAAAAAGAATCTTTAATCACTAAAGAGTCTAATGAAAAAATTGAAGAAGAAATATTAAAT GATGAAAAATTAGAAGAGTTAGAACAAAAAAAGGATTATAAAGAGGATTCTAATAAAAAA GAGAAAGTATCTGAGGACTTATCTTTAGTAGGTAAAACTCGTGAAGAGCTTGAAAATATG CTTAAAAATTTTATAAATTTAACACAAGAAGAAATAATGAAACTTGAGTCGAGAATAGAA AAGTTAGATAAAAATGCTGAAGAATACAAACAAAATTCAAAGACTAATATATTTGATAAA ACAGATGAACAAAAAAAACATATAAATGTACTGATTTAA	AAAACTC GTGAAGA GCTTGAA AA	TGAATTTT GTTTGTAT TCTTCAGC A	126	60
CD3609	ATGTTTAAGAAAATGGCAGTACTAAAAGATATAGCAACTAAAATAGGTCGTAAAAAAGCG TATGAACTATTAGAAATGGTTGAAGGTAATGATGCCTTTGTAGCTGAGGTAAAGATAAAA AAGAATGGAATAGAATCTAAAAAAGAAGAAATTATGTTAAAAGATAATCAAAAAATAATA TTAGAGTATATAGAAGGTTAA	AAAATGG CAGTACT AAAAGAT ATAGCA	CCTCAGCT ACAAAGG CATCA	100	59
CD3635	ATGGCTATGGGTTTTGAATTTAAAATAATGAGAAGTTTAATATATGTAGGACTTGCCAAG GAAGAATATAGACCTAAGCTAATGGACTGGTTATATCGTCACCATATTCCAGATAGTATT AGCACTTTTGGACCATATTGTACTAAATATGCCTTTTATCAAGCATATCCTACACCAAAT GAAGGTGAGCGTTTTGGTGCACGTAAGATGCAACTAACAGAACATTATTGGCTTGTAGAT GAACATATGCCTGAGATGGCAAATAGAATTATGACAGAATATATGCCTATGGATGTTCTA CGTTGGCAAGGGTGTATACCAGATGTAGAAAATAAAAGGGTTCATGAAAATGCAGAAAGT GGAGATGCAGGACGTGCAGTAGGTGGAGATAATGGATGTCCACCATTTATATTTGCCTTT	CATATGC CTGAGAT GGCAAA	CTTGTGCC CATTCTGG TTTT	499	

	GTTCCAATAAACTGGGAAGAAGACTTTAGAGGAAAAGGACGTACTGTACAAGATGGACCA AACTATCGTTGGCAATTTATGATTAAGTATCCAGATGGTATCTCTAAAGAAGAAGGAGAA AAATGGTTCTATGATGAGGTAGTGCCATACTTTACAAACTGTTGCTATGTTAATCGTTTT GTCAGTAGTAAAATAATGATTAATTATGGAGCAACTGCTTTTGACCGTGTATCAGAACTA TGGTTTGAAGGGGAAGAAGAATGGTATAAAGCTGTGGTTGAAGAAACAAAGTCGTTTATT AAAAAACCAGAATGGGCACAAGAAGAGGAGTTCCCATATTTAAAACCACAATTCAATATC GCATCAGTATTCTTAGGTGATATAGCAACTATGGATGCATACTCACAGTATCGTGGATAT ATACCAATGAGATAA				
CD3638	ATGGAAGATAAATTTTATGCAAAAGGCAACGGAAATAACGGATATATTAAAAATCTTGAA GTTTGTTCCTTTAATAACTTAGATGGAACTTGTGGAATGTTTCAAATGGCTCTGTACAAA AGAGATGAAAAATACTATTTATATGGATGCTGTTTTGGAGGAAATAAAAAAAATGGAGTA ATGATTAGCGATATTACAGACCCTTATAATCCACAATTTATAAAACATTTTCAAATGTTA GACCCTAAAGAGTATCCTACAACAACAACTCCCAAAATTCAAATAGCAGATGATTTAATG ATAGTAGCAATGAGTTGTGGAAGTGGACCAGGAGCACTTGTTGACCAAGCTAAATTAGCA AATATTAAGTGTGAAGCAGGAATTAGAATATACAGTTTAAAAGAAGACCCTTTAAATCCT AAGTTTTTAGGATATTGGGATTGTGGCTTAAAGCATGTAATGGGTGTTCATAGATTTATG TACAATGGTGGAAGATATGTACATTTATCAAGTGATTGTGTTGGCTTTGAAGGTCTGATT TATAGGGTCATAGATATAATAAATCCTACTAATCCAGTGGAAATAGGTAAATGGTGGAGA CCAGACCAATATGCAGATGGATATCCAAATAGAACTTTTGATGCAGGAGCACCTCATTGC CCAGAATTTATGGATAAAGGATGGCTTCATGGACCTCCATTTGTAAGAGACGGAAAAGCA TATTGTGGTTATGGAGGAGCTGGTTTAGTTGTATTAGATGTTGAAGATTTAACAAGACCA AGATGCTTAGGTGAATTGCCATTTACGCCTGCATTTTCTAGTAGACTTGCAGGTGCAAGA ACTCATACAGCATTACCATTGCCAGGAAGAGATTTAGTCGTTGTTCAAAATGAGGGAGAA AGATTCCAGTTCTTTAAACCAGATAACATTACAGATGTTCAAGCTATGAATAATATACAT ATGGTTGATGTTAGTGACCCAACAAAACCAACATTAATTGCTCAATTTCCATATCCTGAA GTTCCAAAAGATTTCCCTTATCCTAACTTTAATGTTGCGGGATTAGGAAAACCAGGGCCA TTTGGCCCACATAATCTTCATGAACCAATGGATAATAAGCCATGGTTAGAGCAAAGAGGA GATAGAGTATATTGCTGTTATTTCCATGCAGGGCTAAGGGTTTATGATGTATCAGACCCA TATTATATCAAAGAGCTAGCATATTTTATACCACCAAATCCAAATAAAACACCAGAAGAA TCTTATTTCCCAGGATTCCCAGGACCACGCTTGGCAGTAACAGAAGATCTTATCGTTGAT GATAGAGGCTACATCATCATAGATGCTTTAGATGATGGATTCTATATATTAAAAATGAAA GATGATTAA	GGATGCT GTTTTGGA GGAAA	AAATTCTG GGCAATG AGGTG	525	

Table 7 - List of 82 *Clostridium difficile* clinical isolates

	RVH ref No.	Ribotype
1	100058-106	106
2	100048-106	106
3	090092-106	106
4	100059-106	106
5	100150-078	078
6	090126-106	106
7	090269-106	106
8	100149-078	078
9	090160-106	106
10	100162-020	020
11	090389-106	106
12	090361-106	106
13	090183-106	106
14	090391-106	106
15	090129-106	106
16	090217-106	106
17	090225-106	106
18	090223-106	106
19	090645-106	106
20	090294-106	106
21	090540-106	106
22	100063-106	106
23	100158-078	078
24	100170-001	001
25	100171-001	001
26	100172-020	020
27	100173-078v	078v
28	100177-005	005
29	100163-026	026
30	100178-106	106
31	100164-014	014
32	100167-001	001
33	100168-001	001
34	100169-078	078
35	100143-026	026
36	100144-014	014
37	100146-005	005
38	100147-014	014
39	100142-005	005
40	100140ii-020	020
41	100153-001	001
42	CD110020 015	015

43	CD110040 015	015
44	CD110050 015	015
45	CD110055 015	015
46	CD110060 027	027
47	CD110072 026	026
48	CD110107 015-19	015-19
49	CD110119 026	026
50	CD110147 026	026
51	CD110166 015	015
52	CD110172 023	023
53	CD110183 023	023
54	CD110185 023	023
55	CD110235 027	027
56	CD110243 026	026
57	CD110244 027	027
58	CD110251 015-19	015-19
59	CD110272 023	023
60	CD110373 027	027
61	CD110379 015	015
62	CD110425 027	027
63	CD110441 015	015
64	CD110446 023	023
65	CD110460 015	015
66	CD110465 023	023
67	CD110729 002	002
68	CD110732 002	002
69	CD110779 002	002
70	CD110798 002	002
71	CD110800 023	023
72	CD110811 002	002
73	CD110830 053	053
74	CD110831 053	053
75	CD110835 053	053
76	CD110837 002	002
77	CD110840 015-19	015-19
78	CD110849 015-19	015-19
79	CD110851 002	002
80	CD110856 002	002
81	CD110862 ?tox-	140
82	CD110863 ?tox-	140

Table 8 - List of negative control strains

Name	Number
Staphylococcus epidermidis	DSM 20044
Staphylococcus aureus	ATCC 12600
Escherichia coli	ATCC 11775
Salmonella typhimurium	ATCC 14028
Bacillus subtilis	ATCC 6051
Clostridium acetobutylicum	DSM 792
Roseburia inulinivorans	DSM 16841
Eubacterium rectale	DSM 17629
Clostridium novyi	DSM 14992
Clostridium sporogenes	DSM 795
Faecalibacterium prausnitzii	DSM 17677
Streptococcus thermophilus	NCIMB 702681
Bifidobacterium adolescentis	DSMZ 20083
Bifidobacterium longum subsp. *longum*	DSMZ 20219
Ruminococcus gauvreauii	DSMZ 19829
Ruminococcus luti (Blautia luti)	DSMZ 14534
Lactobacillus casei	DSMZ 20011
Lactobacillus reuteri	DSMZ 20016
Bacteroides vulgatus	DSMZ 1447
Bacteroides intestinalis	DSMZ 17393

Table 9 - Results of the Study described in Example 4

Gene	First Screen (Clinical isolates 1-41)	Second Screen (Clinical isolates 42-82)	Third Screen (negative control)
CD0588	Low Sensitivity Level		
CD0638	+	+	-
CD1234	Low Sensitivity Level		
CD1423	-		
CD1424	+	+	-
CD1487	+	+	-
CD1543a	+	+	-
CD1728	Low Sensitivity Level		
CD1794	+	+	-
CD1897	+	-	
CD1906	+	+	-
CD2046	+	+	-
CD2098	+	+	-
CD2216	+	+	-
CD2248	Low Sensitivity Level		
CD2264	+	+	-
CD2274	+	+	-
CD2300	Low Sensitivity Level		
CD2306	Low Sensitivity Level		

CD2309	+	+	-
CD2563	Low Sensitivity Level		
CD3188	+	+	-
CD3288	+	+	-
CD3321	Low Sensitivity Level		
CD3367	+	+	-
CD3369	Low Sensitivity Level		
CD3609	+	+	-
CD3656	Low Sensitivity Level		
CD3617	+	-	
CD3618	+	-	
CD3635	+	+	-
CD3638	+	+	-

CLAIMS

1. A method of testing for the presence of *Clostridium difficile* in a sample, said method comprising analysing said sample for the presence of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes.

2. A method of detecting *Clostridium difficile* in a sample, said method comprising detecting the presence in said sample of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes.

3. A method of diagnosing a *Clostridium difficile* infection in a subject, said method comprising performing the method of claim 1 or claim 2, wherein the sample has been obtained from said subject and the presence of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes, in the sample is diagnostic of a *Clostridium difficile* infection in said subject.

4. A method of determining the efficacy of a therapeutic regime being used to treat a *Clostridium difficile* infection, said method comprising

(i) performing the method of claim 1 or claim 2 on a sample that has been obtained from a subject being treated for a *Clostridium difficile* infection; and

(ii) repeating step (i) on one or more further samples that have been obtained from the subject being treated for a *Clostridium difficile* infection.

5. A primer pair selected from the group consisting of

(a) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 67 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 67; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 68 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 68;

(b) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 13 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 13; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO 14 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 14;

(c) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 15 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 15; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 16 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 16;

(d) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:17 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:17; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:18 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:18;

(e) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:19 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:19; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:20 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:20;

(f) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:37 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 37; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:38 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO:38 ;

(g) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 39 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 39; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 40 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 40;

(h) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 41 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 41; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 42 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 42;

(i) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 43 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 43; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO; 44 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 44;

(j) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 45 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 45; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 46 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 46;

(k) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 47 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 47; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 48 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 48;

(l) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 49 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 49; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO:50 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 50;

(m) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 51 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 51; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 52 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 52;

(n) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 53 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 53; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 54 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 54;

(o) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 55 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 55; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 56 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 56;

(p) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 57 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 57; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 58 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 58;

(q) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 59 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 59; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 60 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 60;

(r) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 61 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 61; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 62 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 62;

(s) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 63 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 63; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 64 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 64;

(t) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 65 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 65; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 66 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 66; and

(u) an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO: 11 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO: 11; and

an isolated oligonucleotide comprising the nucleotide sequence of SEQ ID NO 12 or a nucleotide sequence capable of hybridising under high stringency conditions to the sequence complementary to SEQ ID NO 12.

6. A kit comprising one or more detection moieties for the detection of one or more genes selected from the group consisting of CD3609, CD3617, CD3618, CD3635, CD3638, CD0638, CD1424, CD1487, CD1543a, CD1794, CD1906, CD2046, CD2098, CD2216, CD2264, CD2274, CD2309, CD3188, CD3288, CD3367 and CD2961, or a product of said genes.

7. The method of any one of claims 1 to 4 or the kit of claim 6, wherein the coding nucleotide sequence of CD3609 is that of SEQ ID NO:36 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3617 is that of SEQ ID NO:2 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3618 is that of SEQ ID NO:3 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3635 is that of SEQ ID NO:4 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3638 is that of SEQ ID NO:5 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD0638 is that of SEQ ID NO:21 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD1424 is that of SEQ ID NO:22 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD1487 is that of SEQ ID NO:23 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD1543a is that of SEQ ID NO:24 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD1794 is that of SEQ ID NO:25 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD1906 is that of SEQ ID NO:26 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2046 is that of SEQ ID NO:27 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2098 is that of SEQ ID NO:28 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2216 is that of SEQ ID NO:29 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2264 is that of SEQ ID NO:30 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2274 is that of SEQ ID NO:31 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD2309 is that of SEQ ID NO:32 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3188 is that of SEQ ID NO:33 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3288 is that of SEQ ID NO:34 or a sequence at least 80% identical thereto, the coding nucleotide sequence of CD3367 is that of SEQ ID NO:35 or a sequence at least 80% identical thereto, or the coding nucleotide sequence of CD2961 is that of SEQ ID NO:1 or a sequence at least 80% identical thereto.

8. The method of any one of claims 1 to 4, wherein the presence of one or more of said genes, or products of one or more of said genes, is detected by a primer-directed amplification reaction.

9. The method of claim 8, wherein said primer-directed amplification reaction is a polymerase chain reaction.

10. The method of any one of claims 1 to 4 or 7 to 9, wherein the *Clostridium difficile* is of a ribotype selected from the group consisting of 106, 078, 020, 001, 005, 026, 014, 027, 078v, 015, 015-19, 023, 002, 053 and 140.

11. The method of any one of claims 1-4, 7 or 10, wherein the presence of said product of said genes is determined by an antibody-mediated detection method.

12. The method of any one of claims 1 to 4 or 7 to 11 which comprises contacting said sample with a detection moiety which can detect one of said genes or gene products.

Figure 1

1	1	2	2	3	3	4	4	5	5	6	6
7	7	8	8	9	9	10	10	11	11	12	12
13	13	14	14	15	15	16	16	17	17	18	18
19	19	20	20	21	21	22	22	23	23	24	24
25	25	26	26	27	27	28	28	29	29	30	30
31	31	32	32	33	33	34	34	35	35	36	36
37	37	38	38	39	39	40	40	41	41	630	630
E. coli	E. coli	Staph	Staph	-ve	-ve						

Figure 2A

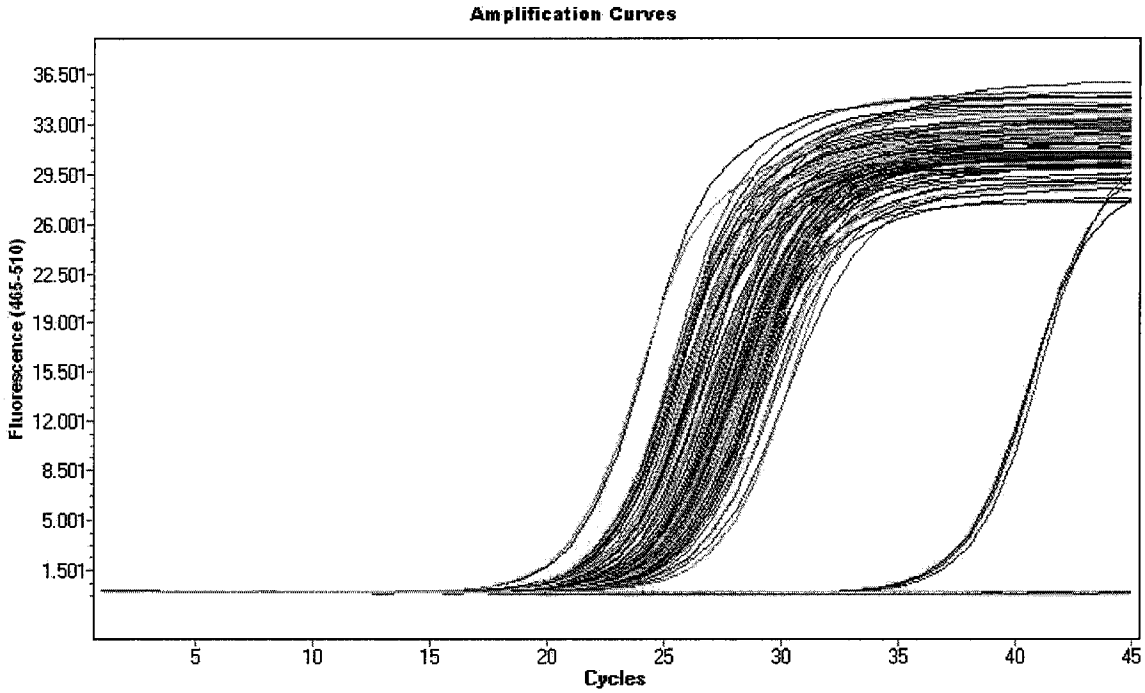


Figure 2B

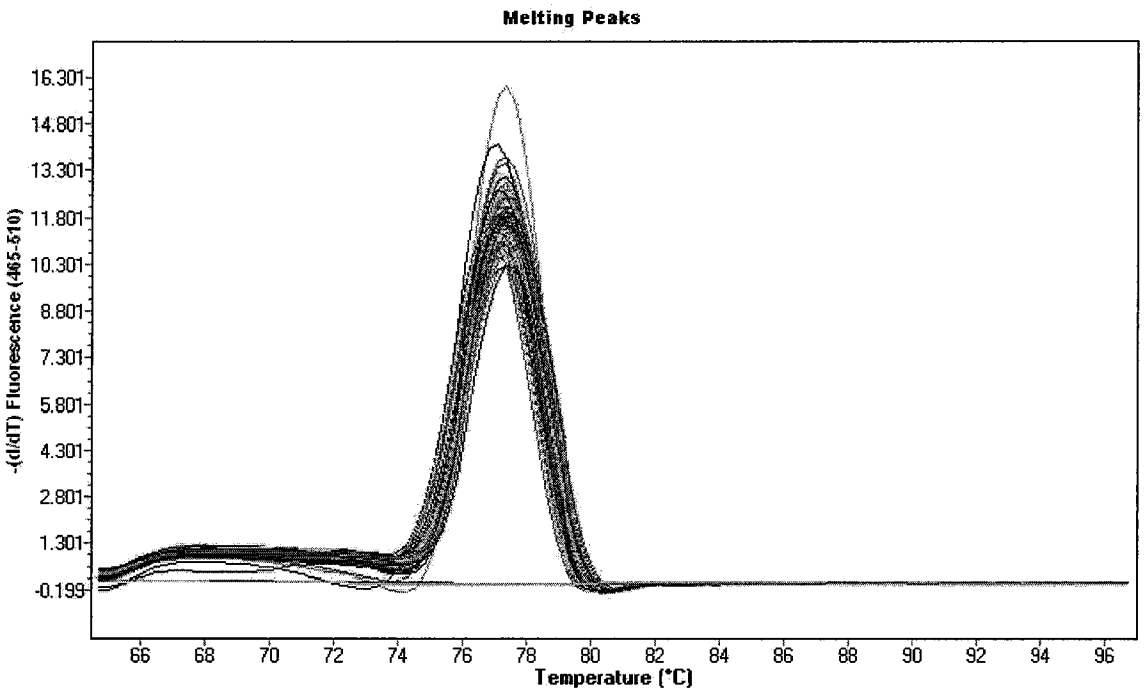


Figure 2C