toxins are available commercially. However, these tests are not perfect and can result in false positive tests (finding toxins when there is no *C. difficile*) and false negative tests (not finding toxins when *C. difficile* is present) can occur. For example, the possibility of a cross-reaction between *C. difficile* and *C. sordellii* is known. See, "Cloning and characterization of the cytotoxin L-encoding gene of *Clostridium sordellii*: homology with *Clostridium difficile* cytotoxin B", Green et. al., Gene, 1995, 161:57-61). Thus, there is still a significant clinical need to develop molecular testing for detection of *C. difficile* that is more sensitive than culture and less susceptible to false positive or false negative results.

SUMMARY ~~OF THE INVENTION~~

Embodiments of the present ~~invention~~ disclosure relate to methods for the rapid detection of the presence or absence of *C. difficile* in a biological or non-biological sample. The present disclosure ~~invention~~ includes methods of detection of *C. difficile* comprising performing at least one cycling step, which may include an amplifying step and a hybridizing step. Furthermore, the present disclosure ~~invention~~ relates to primers, probes, and kits that are designed for the detection of *C. difficile*. The gene targeted in the methods of the present disclosure ~~invention~~ for the detection of *C. difficile* is the *C. difficile* toxin B (*tcdB*) gene. For example, the *tcdB* gene encoding cytotoxin B was chosen because it was determined to be specific to toxigenic *C. difficile* and not present in other Clostridium species, with the exception of some enterotoxin-negative, cytotoxin-positive strains of *Clostridium sordellii* (Green, et. al., J. Med. Microbiol. 1996, 44:60-64). The heterogeneity of the *tcdB* gene among toxigenic *C. difficile* strains results in up to 31 toxinotypes thus far including Tox 0 which is the reference strain VPI 10463. The *tcdB* gene encodes a single-stranded protein having a C-terminal domain responsible for binding to the host cell membrane, a middle part involved in internalization, and the N-terminal catalytic (toxic) part (Rupnik, et al., Microbiology, 2005, 151(1):199-208).

~~In one aspect, t~~The present disclosure may ~~invention~~ provides a method for detecting *C. difficile* in a sample, the method comprising performing an amplifying step comprising contacting the sample with a set of *tcdB* primers to produce an amplification product if *C. difficile* is present in the sample; performing a hybridizing step comprising contacting the amplification product with one or more detectable *tcdB* probes; and detecting the presence or absence of the amplified product, wherein the presence of the amplified product is indicative of the presence of *C. difficile* in the sample and wherein the absence of the amplified product is indicative of the absence of *C. difficile* in the sample. In one embodiment, each primer of the set of *tcdB* primers comprises or consists of a sequence of nucleotides selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, and 6, or a complement thereof; and wherein the one or more detectable *tcdB* probes comprise or consists of a sequence of nucleotides selected from the group consisting SEQ ID NOs: 7, 8, and 9, or a complement thereof. Hence, in one aspect, there is provided a method of detecting *C. difficile* in a sample, the method comprising: performing an amplifying step comprising contacting the sample with a set of *tcdB* primers to produce an amplification product if a *tcdB* nucleic acid is present in the sample; performing a hybridizing step comprising contacting the amplification product with one or more detectable *tcdB* probes; and detecting the presence or absence of the amplified product, wherein the presence of the amplified product is indicative of the presence of *C. difficile* in the sample and wherein the absence of the amplified product is indicative of the absence of *C. difficile* in the sample; wherein each primer of the set of *tcdB* primers comprises a sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5 and 6, or a complement thereof; and wherein the one or more detectable *tcdB* probes comprise a sequence selected from the group consisting SEQ ID NOs: 7, 8, and 9, or a complement thereof. In some embodiments, a hybridizing step includes contacting the amplification product with a probe that is labeled with a donor fluorescent moiety and a corresponding acceptor fluorescent moiety. The method may further include detecting the presence or absence of fluorescence resonance energy transfer (FRET) between the donor fluorescent moiety and the acceptor fluorescent moiety of the probe. The presence or

absence of fluorescence is indicative of the presence or absence of *C. difficile* in the sample.

In one ~~aspect~~embodiment, amplification can employ a polymerase enzyme having 5' to 3' exonuclease activity. In certain ~~aspects~~embodiments, the first and second fluorescent moieties may be within no more than 5 nucleotides of each other along the length of the probe. In another ~~aspect~~embodiment, the *tcdB* probe includes a nucleic acid sequence that permits secondary structure formation. Such secondary structure formation generally results in spatial proximity between the first and second fluorescent moiety. In certain embodiments of this method, the second fluorescent moiety on the probe can be a quencher.

The present disclosure may also ~~In another aspect, the invention~~ provides for methods of detecting the presence or absence of *C. difficile* in a biological sample from an individual. Such methods generally include performing at least one cycling step, which includes an amplifying step and a dye-binding step. Typically, the amplifying step includes contacting the sample with a pair of *tcdB* primers to produce a *tcdB* amplification product if a *tcdB* nucleic acid molecule is present in the sample and the dye-binding step includes contacting the *tcdB* amplification product with a double-stranded DNA binding dye. Such methods also include detecting the presence or absence of binding of the double-stranded DNA binding dye into the amplification product, wherein the presence of binding is indicative of the presence of *C. difficile* in the sample, and wherein the absence of binding is indicative of the absence of *C. difficile* in the sample. A representative double-stranded DNA binding dye is ethidium bromide. In addition, such methods also can include determining the melting temperature between the *tcdB* amplification product and the double-stranded DNA binding dye, wherein the melting temperature confirms the presence or absence of the *C. difficile*.

~~In a further aspect, t~~The present ~~invention~~ disclosure may also provides a kit for detecting a nucleic acid of *C. difficile* (*tcdB* target). The kit can include a first oligonucleotide comprising or consisting of a sequence selected from the group consisting of SEQ ID NOs: 1, 2, and 3, or a complement thereof; a second oligonucleotide comprising or consisting of a sequence selected from the group consisting of SEQ ID NOs: 4, 5, and 6, or a complement thereof; and a third detectably labeled oligonucleotide comprising or consisting of a sequence selected from the group consisting of SEQ ID NOs: 7, 8, and 9, or a complement thereof. Hence, in another aspect, there is provided a kit for detecting a nucleic acid of *C. difficile* comprising: a first oligonucleotide comprising a sequence selected from the group consisting of SEQ ID NOs: 1, 2, and 3, or a complement thereof; a second oligonucleotide comprising a sequence selected from the group consisting of SEQ ID NOs: 4, 5, and 6, or a complement thereof; and a third detectably labeled oligonucleotide comprising a sequence selected from the group consisting of SEQ ID

NOs: 7, 8, and 9, or a complement thereof. In certain embodiments, the first and second oligonucleotides are primer oligonucleotides, while the third detectably labeled oligonucleotide is a probe oligonucleotide.

In one embodiment, the kit can include probes already labeled with donor and corresponding acceptor fluorescent moieties, or can include fluorophoric moieties for labeling the probes. In certain embodiments the acceptor fluorescent moiety is a quencher.

The kit can also include at least one of nucleoside triphosphates, nucleic acid polymerase, and buffers necessary for the function of the nucleic acid polymerase. The kit can also include a package insert and instructions for using the primers, probes, and fluorophoric moieties to detect the presence or absence of *C. difficile* in a sample.

~~In one aspect, t~~The present ~~invention~~ disclosure may also provides for an oligonucleotide comprising or consisting of a sequence of nucleotides selected from SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, and 9 or a complement thereof, which oligonucleotide has 100 or fewer nucleotides. In another aspect, there is provided an oligonucleotide comprising a nucleotide sequence selected from the group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, and 9 or a complement thereof. In certain embodiments, the oligonucleotide has 40 or fewer nucleotides. ~~In another aspect, t~~The present ~~invention~~ disclosure may also provides for an oligonucleotide that includes a nucleic acid having at least 70% sequence identity (e.g., at least 75%, 80%, 85%, 90% or 95%, etc.) to one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, and 9, or a complement thereof, which oligonucleotide has 100 or fewer, more specifically 40 or fewer nucleotides. In certain embodiments the oligonucleotides have 90% sequence identity to one of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, and 9, or a complement thereof. Generally, these oligonucleotides may be primer nucleic acids, probe nucleic acids, or the like in these embodiments. In certain of these embodiments, the oligonucleotides have 40 or fewer nucleotides (e.g., 35 or fewer nucleotides, 30 or fewer nucleotides, etc.). In some embodiments, the oligonucleotides comprise at least one modified nucleotide, e.g., to alter nucleic acid hybridization stability relative to unmodified nucleotides. Optionally, the oligonucleotides comprise at least one label and/or at least one quencher moiety. In some embodiments, the oligonucleotides include at least one conservatively modified variation.

The ~~In yet another aspect, the~~ present ~~invention~~ disclosure may provides for a pair of oligonucleotide primers, wherein a first oligonucleotide primer comprises or consists of a sequence selected from the group consisting of SEQ ID NOs: 1, 2, and 3, or a complement thereof and wherein a second oligonucleotide primer comprises or consists of a sequence selected from the group consisting of SEQ ID NOs: 4, 5, and 6, or a complement thereof. In certain of these

embodiments, at least one of the oligonucleotides has 40 or fewer nucleotides (e.g., 35 or fewer nucleotides, 30 or fewer nucleotides, etc.). In some embodiments, at least one of the oligonucleotides comprises at least one modified nucleotide, e.g., to alter nucleic acid hybridization stability relative to unmodified nucleotides. In some embodiments, at least one of the oligonucleotides includes at least one conservatively modified variation.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this ~~invention~~ disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure~~invention~~, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the disclosure ~~invention~~ are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the ~~invention~~ disclosure will be apparent from the drawings and detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

FIGURES 1A, 1B, and 1C show gel data of PCR products produced with various primer pairs for toxin B gene of *C. Difficile* and the level of cross reactivity with C. *sordellii*; CDB203BZ/CDB202BZ (Figure 1A), CDN211BZ/CDB214N (Figure 1B), and CDB205BZ/CDB204BZ (Figure 1C).

FIGURE 2A, 2B, and 2B show PCR growth curves of the CDB242HQ6 probe in amplification of genomic DNA of *C. difficile* Tox 0 and *C. sordellii* with various primer pairs: CDB203BZ/CDB202BZ (Figure 2A), CDN211BZ/CDB214N (Figure 2B), and CDB205BZ/CDB204BZ (Figure 2C).