Comparative analysis of different methods to detect *Clostridium difficile* infection

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SUMMARY

The increased incidence and severity of *Clostridium difficile* infection, particularly in North America and Europe, have brought renewed focus on the most appropriate method to detect *C. difficile* and/or its toxins in stools. This prospective study evaluated the usefulness of the Illumigene™ *C. difficile* assay in diagnostic practice for the detection of toxigenic *C. difficile* DNA in clinical samples. A total of 88 out of 306 stool samples analysed were positive both by Illumigene and the combination of toxigenic *C. difficile* culture (TC) and immunochromatographic assay (IC) with a concordance of 100%. Of the 218 samples negative by the combination of TC and IC, 204 were negative also by Illumigene with a concordance of 93.57%. In our experience, compared to conventional assays Illumigene assay proved to be easy to perform, accurate and prompt giving results within 1 hour at a cost of 28 euro per sample.

KEY WORDS: Clostridium difficile, Diagnosis, LAMP, Toxins, Diarrhoea.

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INTRODUCTION

Clostridium difficile is the major causative agent of healthcare-associated diarrhoea and pseudomembranous colitis (Freeman *et al.,* 2010). Two large toxins, toxin A (TcdA, enterotoxin) and toxin B (TcdB, cytotoxin), are recognised as the main virulence factors of *C. difficile*. Both toxins are localised within a 19.6 kb region of the bacterial chromosome termed *locus* of pathogenicity (PaLoc) (Braun *et al.,* 1996).

For a long time, the distinction between toxigenic *C. difficile* strains, like those producing both toxins (A+ B+) and causing disease, and non toxigenic strains (A-B-), producing neither toxins nor causing disease (Rupnik, 2008), was commonly accepted. However, later studies showed that *C. difficile* is a very heterogeneous species and many types of variant isolates can be differentiated both among toxigenic and non toxigenic strains. The term "variant strain" describes any strain that differs from the reference *C. difficile* strain VPI 10463 (A+B+) either in the DNA sequence of the PaLoc and/or in the pattern of toxin production (Rupnik, 2008).

The incidence and the severity of *C. difficile* infections (CDI) have been increasing for the last decade, particularly in North America and European nations. The most virulent variant strains, such as 027/NAP1/BI strain and ribotype 078/toxinotype V strain, were isolated in these countries. The reported changes in CDI epidemiology have brought renewed focus on the most appropriate method to detect *C. difficile* and/or its toxins in faecal samples (Freeman *et al.,* 2010). The diagnosis of CDI is usually based on a clinical history of recent antimicrobial usage and diarrhoea in combination with the result of laboratory tests (Cohen *et al.,* 2010; Crobach *et al.,* 2009; Delmee *et al.,* 2005). Currently, the "gold standard" methods for the diagnosis of CDI are the stool cytotoxicity assay and the toxigenic

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C. difficile culture (www.cdc.gov/HAI/organisms/cdiff/Cdiff_clinicians.html, accessed on 11th June 2012). The stool cytotoxicity assay is not standardized, requires skill and facilities for cell culture, implies a long time to report and may be affected by the lability of toxin B (Cohen *et al.,* 2010; Crobach *et al.*, 2009; Delmee *et al.,* 2005; Doing *et al.,* 2011), so that its usefulness in the clinical setting is limited (www.cdc.gov/HAI/organisms/cdiff/Cdiff_clinicians.html, accessed on 11th June 2012). The toxigenic *C. difficile* culture is slow and laborious, often requires 72 to 96 h to complete, and therefore is unlikely to be adopted routinely in the current laboratory diagnosis of CDI (Cohen *et al.,* 2010; Crobach *et al.,* 2009; Delmee *et al.,* 2005). Nowadays, faster detection methods have been developed for the detection of *C. difficile* toxins and most laboratories have adopted enzyme immunoassays for toxins A and B as the routine method of testing. These techniques are easier to perform, faster than gold standard assays and do not require specific technical skills (Barbut *et al.,* 2009; Planche *et al.,* 2008; Wilkins *et al.,* 2003). However, they are not sensitive enough to be used as a stand-alone assay for CDI laboratory diagnosis (Lalande *et al.,* 2011).

Immunoenzymatic assays for the diagnosis of CDI other than those for toxins A and B have also been commercially available for more than 10 years, such as those for the detection of *C. difficile* glutamate dehydrogenase (GDH).

The commercial GDH tests offer a turnaround time of 15 to 45 min, and this is one of the reasons these tests are used in many laboratories. This antigen has proven to be a good screening marker for *C. difficile* because this essential enzyme is produced constitutively in large amounts by all *C. difficile* strains and can be readily detected in faecal specimens but it does not distinguish toxigenic from non toxigenic strains (Wilkins *et al.,* 2003).

The changes in CDI epidemiology have recently led to the development of commercial assays based on real-time PCR for the detection of toxins A and B. In December 2008, the Food and Drug Administration (FDA) approved the first commercial real-time PCR assay for the detection of *C. difficile* toxins (Kvach *et al.,* 2010). The majority of variant strains show mutations in *tcd*A and *tcd*B genes, encoding toxins A and B, respectively. The commercially available real-time PCR assays correctly detect the majority of variant strains of *C. difficile* on a genetic basis, and are designed to detect a conservative region of the toxin B gene (*tcd*B). However, it has been reported that in *C. difficile* variant strains *tcd*A is more conserved than *tcd*B (Rupnik, 2008). Recently, a new commercial assay based on loop-mediated isothermal amplification (LAMP) technology and targeting *tcd*A gene, the Illumigene™ *C. difficile* assay (Meridian Bioscience, Cincinnati, OH), was developed. The *tcd*A target region was selected as a conservative region in all known A+B+ and A-B+ toxinotypes of variant strains of *C. difficile*, which is intact in all toxigenic strains, including those with a large deletion in the *tcd*A gene (Lalande *et al.,* 2011).

The aim of this study was to assess the usefulness of the Illumigene™ *C. difficile* assay in diagnostic practice for the detection of toxigenic *C. difficile* DNA in clinical samples, compared to the combination of the toxigenic *C. difficile* culture and the detection of the toxin A/B and glutamate dehydrogenase (GDH) by an immunochromatographic assay. Its possible application as a primary test in the diagnosis of CDI was also evaluated after a subsequent 5-month period in the field.

MATERIALS AND METHODS

Samples and patients

Three-hundred and six faecal samples collected from 306 patients (291 Italians and 15 foreigners from developing countries) attending the University Hospital of Parma (Northern Italy) with a suspicion of CDI over a three-month period in the year 2011 were examined in a prospective study.

All faecal samples were subjected to an immunochromatographic assay for GDH and toxins A and B, toxigenic *C. difficile* culture and Illumigene™ *C. difficile* assay as described below.

Immunochromatographic assay (IC)

The simultaneous detection of *C. difficile* GDH and toxins A/B in faecal samples was performed by the immunochromatographic assay "C. DIFF QUIK CHEK COMPLETE" (TechLab, USA) according to the manufacturer's instructions (Planche *et al.,* 2009).

Toxigenic *C. difficile* culture (TC)
Specific medium plates, containing cycloserine-cefoxitin-fructose agar (CCFA) (Kima, Italy) and Schaedler agar (Kima, Italy), respectively, were streaked with stool samples and incubated anaerobically at 37°C for 48-72 h. To enhance the sensitivity of the TC, an aliquot from all the faecal samples tested was also inoculated in cooked-meat broth (CMB) (Kima, Italy) for 48 h in an anaerobic atmosphere and pre-treated by heat-shock at 100°C for 3 minutes before inoculation onto CCFA agar plates according to standard procedures (Jousimies-Somer *et al.*, 2002). The species identification of putative *C. difficile* colonies was based on the biochemical test Rapid ID 32A (bioMérieux, France) and/or performed by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) (Bruker Daltonics Microflex LT system, Becton Dickinson, Italia S.p.A.) according to the manufacturer's instructions. In order to investigate the *in vitro* production of toxins A and B, the identified *C. difficile* isolates were also cultured anaerobically at 37°C for 72 h in CMB, and 0.5 ml of the broth culture was tested by "C.DIFF QUIK CHEK COMPLETE", according to the manufacturer's instructions.

Illumigene™ *C. difficile* (Illumigene) assay
The Illumigene (Meridian Bioscience, Cincinnati, OH) assay uses LAMP technology to amplify and detect a conserved 204-bp sequence in the 5′ portion of the *tcd*A gene (Lalande *et al.*, 2011). Genomic DNA was extracted from faecal samples by heat at 95°C for 10 minutes. The assay was run according to the manufacturer's instructions. In each vial provided, the Illumigene assay contains an internal control of extraction and inhibition of amplification constituted by formalin-inactivated *Staphylococcus aureus* and its specific primers set. External quality controls supplied by the manufacturer, including a positive control (a solution containing plasmidic DNA with *S. aureus* and *C. difficile* DNA insert) and a negative control (a solution containing plasmidic DNA with *S. aureus* DNA insert), were run for each new reagent batch. The Illumigene assay reports a positive, negative, or invalid result. In case of an invalid result (that may be caused by inhibition of amplification, incorrect sample preparation, or failure in internal control preparation) the Illumigene assay was repeated according to the manufacturer's instructions.

Analytic sensitivity and specificity of the Illumigene assay
The analytic sensitivity of the Illumigene assay was tested using faecal samples experimentally seeded with a toxigenic *C. difficile* reference strain (ATCC VPI10463/ToxA+ Tox B+/wild type/toxinotype 0) cultured in CCFA medium. Serial dilutions of a known concentration of *C. difficile* were mixed with an equal quantity of human faeces obtaining experimentally seeded samples from 1.5×10^7 c.f.u./g (colony forming units per gram) of faeces to 1 c.f.u./g of faeces. The same samples were also used for a comparative evaluation of the detection limit of the IC, both for GDH and toxins A/B.
The analytic specificity of the Illumigene assay was tested using faecal samples containing enteropathogenic bacteria other than *C. difficile* such as *Salmonella* spp., *Helicobacter pylori* and *Staphylococcus aureus*, or intestinal protozoa such as *Giardia intestinalis*, *Entamoeba coli*, *Entamoeba dispar*, and *Blastocystis hominis*. Moreover, experimentally seeded samples containing bacteria/parasites together with *C. difficile* VPI10463 were tested by the Illumigene assay to verify the absence of their influence on the detection of *C. difficile* DNA. Analytic specificity was also determined by testing a non toxigenic *C. difficile* strain (ATCC 70057/Tox A- Tox B-). Moreover, the toxigenic *C. difficile* strain ribotype 078/toxinotype V was tested by the Illumigene assay.

Comparative evaluation of the results of Illumigene assay, TC, and IC
The results of the Illumigene assay were compared with those obtained by the combination of TC and the detection of toxins A/B by IC. A sample was defined as positive by the combination of IC and TC when TC was positive and/or when IC assay for toxins A/B was positive.
Discordant results between the Illumigene assay and the combination of IC and TC were investigated by an additional test, a PCR assay for the detection of *tcd*A/*tcd*B genes (Spigaglia *et al.*, 2002), as described below.
On the basis of the results of the comparative evaluation, a primary test for the detection of *C. difficile* was selected among those evaluated and a new algorithm was developed for the diagnosis

of CDI. This algorithm was applied to a total of 718 samples belonging to 718 patients (681 Italians and 37 foreigners from developing countries) over a five-month period.

Duplex PCR for the detection of *tcdA*/*tcdB* genes

A *tcdA* fragment of 624 bp and a *tcdB* fragment of 412 bp were amplified using the primers set described by Spigaglia and Mastrantonio (Spigaglia *et al.,* 2002). The PCR reaction mixture in a volume of 50 µl contained: 10x PCR reaction buffer, 1.5 mM $MgCl_2$, each deoxynucleotide (Società Italiana Chimici, Italy) at a concentration of 200 µM, 125 nM of primers TA1 and TA2 and 62.5 nM of primers TB1 and TB2 (Primm, Italy), 1.5 units of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). PCR conditions were: 2 min at 95°C, followed by 30 cycles consisting of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C and a final step at 72°C for 5 min. Duplex PCR products were analysed by gel electrophoresis.
The analytic sensitivity of the *tcd*A/*tcd*B PCR assay was evaluated using the same experimentally seeded faecal samples used to assess the detection limit of the Illumigene assay.

RESULTS

Analytic sensitivity and specificity of the Illumigene assay

The analytic sensitivity of the Illumigene assay in comparison with that of IC (GDH - toxins A/B) and duplex PCR is reported in table 1. The detection limit was 5 cfu/g of faeces for the Illumigene assay, 1000 cfu/g of faeces for IC assay both for GDH and toxins A/B and 100 cfu/g of faeces for duplex PCR.
The Illumigene assay was negative when the DNA of enteropathogenic bacteria other than *C. difficile* as well as intestinal protozoa DNA was used as the template. Moreover, the detection of the DNA of *C. difficile* was not affected when it was present in faecal samples also containing the DNA of other bacteria and/or parasites. Finally, Illumigene gave a negative result when the DNA from the non toxigenic *C. difficile* strain ATCC 70057/ToxA- Tox B- was used as the template, and gave a positive result when the DNA from the toxigenic *C. difficile* strain ribotype 078/toxinotype V was used as the template.

Comparative evaluation of the results of Illumigene, TC, and IC

The results of the comparative evaluation of Illumigene, IC for toxins A/B and TC on the 306 analysed faecal samples are reported in table 2.
A total of 88 out of the 306 stool samples analysed were positive both by Illumigene and the combination of TC and IC with a concordance of 100%. Forty-one (46.6%) of the 88 samples were positive for all the assays whilst 47 (53.4%) were positive only for 2 of the compared assays, always including Illumigene and only TC (19) or IC (28), alternatively.
The 88 patients with positive samples were 82 Italians, and 6 foreigners from developing coun-

TABLE 1 - *Comparative analytical sensitivity of the different assays used in this study.*

Concentration of C. difficile strain ATCC VPI 10463	*Illumigene assay*	*IC for GDH*	*IC for toxin A/B*	*Duplex PCR for toxin A/B*
1.5×10^7 c.f.u./g	+	+	+	+
1000 c.f.u./g	+	+/-	+/-	+
100 c.f.u./g	+	-	-	+
10 c.f.u./g	+	-	-	-
5 c.f.u./g	+	-	-	N.P.
1 c.f.u./g	-	-	-	N.P.

ATCC VPI10463: *C. difficile* positive control ToxA+ Tox B+, wild type, toxinotype 0; +: Positive; -: Negative; +/-: Weakly positive; N.P.: Not performed; c.f.u./g : colony forming units per gram of faeces.

TABLE 2 - *Comparative results of Illumigene, IC assay for TOX A/B and TC.*

No. of specimens	*Illumigene*	*IC for Tox A/B*	*TC*
204	Negative	Negative	Negative
41	Positive	Positive	Positive
28	Positive	Positive	Negative
19	Positive	Negative	Positive
14	Positive	Negative	Negative

tries with a mean age of 73.4 years. Two-hundred four of the 218 negative samples by the combination of TC and IC were negative also by Illumigene with a concordance of 93.57%.
The fourteen samples with discordant results (negative by the combination IC/TC and positive by Illumigene) gave positive and negative results in 9 and 5 cases, respectively, when analysed by duplex PCR for *tcdA*/*tcdB* genes.
On the basis of the results of the comparative evaluation, the following algorithm was developed: to apply the Illumigene assay as a primary test on all the samples with a suspicion of CDI and, only on the Illumigene-positive samples, to proceed by IC for toxins A/B and TC.
The results of the application of this algorithm on 718 faecal samples are reported in table 3.
Five hundred thirty-nine out of the 718 stool samples analysed were negative while the remaining 179 samples were positive by Illumigene assay. All these 179 positive samples were positive also for the combination of TC and IC. In particular, eighty-seven (48.6%) of the 179 samples were positive for both assays whilst 92 (51.4%) were positive alternatively by TC (33) or IC (59).
The 179 patients with positive samples were 173 Italians and 6 foreigners from developing countries with a mean age of 76.8 years.

TABLE 3 - *Results of the application of the new algorithm for the diagnosis of CDI.*

No. of specimens	*Illumigene*	*IC for Tox A/B*	*TC*
539	Negative	N.P.	N.P.
87	Positive	Positive	Positive
59	Positive	Positive	Negative
33	Positive	Negative	Positive

N.P.: Not performed

DISCUSSION

Because of the increased awareness of the importance of CDI, the impact of the disease in terms of healthcare costs is nowadays more fully understood. *In vitro* diagnostic testing for *C. difficile* and its toxins has shown a great improvement in recent years, resulting in better healthcare for the patient. For the clinical laboratory, the question of whether the laboratory should perform *C. difficile* testing has instead become a question of the most appropriate combination of assays to be used for the accurate detection of toxigenic *C. difficile* in patients with a clinical suspicion of CDI (Wilkins *et al.,* 2003).
Recently, new molecular assays for the detection of *C. difficile* have been developed. Our study evaluated the performance of the new molecular assay Illumigene™ *C. difficile* in diagnostic practice for the detection of toxigenic *C. difficile* DNA in clinical samples compared to the combination of toxigenic *C. difficile* culture and the detection of toxins A/B by immunochromatographic assay, and its possible application as a primary test in the diagnosis of CDI.
In our experience, the Illumigene assay revealed a detection limit lower than that of the IC for GDH and toxins A/B. The Illumigene assay was also more sensitive than duplex PCR for the detection of *tcdA*/*tcdB* genes used in this study as an additional test to discriminate discordant results.
The Illumigene assay proved highly specific, being neither affected by the presence of the DNA of enteropathogenic bacteria other than *C. difficile* or parasites, nor influenced when *C. difficile* was simultaneously present in the sample together with different microrganisms.
Moreover, the Illumigene assay was able to recognize the strain ribotype 078/toxinotype V, one of the most virulent strains isolated in recent years. Among those strains, 027/NAP1/BI was not tested in our study because such a strain has not been isolated in Italy to date.
Among the 47 out of 88 samples found positive

both by the Illumigene assay and the combination of TC and IC in this comparative study, 28 were negative for TC and 19 for IC. In these cases, the negativity of TC was probably due to the known difficulties encountered in bacteria isolation based on the intrinsic complexity when using faeces as the matrix sample (Cohen *et al.*, 2010; Delmee *et al.,* 2005), while the negativity of the IC could be due to the concentration of toxins that could have been lower than the detection limit of the assay, or due to the degradation of these proteins, or an incorrect sample collection. The same aspects could also explain the discrepancy found in 14 samples between the results of the Illumigene assay (positive) and of IC and TC (both negative). In these 14 samples the positive result of the Illumigene assay was confirmed in 9 cases by the positive result of duplex PCR whilst the negativity of this assay in the remaining 5 cases could be due to the higher analytic sensitivity of the Illumigene assay. Moreover, in all the 14 discordant samples a positive result by GDH IC assay was obtained (data not shown). Taking into account that the GDH assay does not distinguish between toxigenic and non toxigenic strains, all these data taken together likely corroborate the hypothesis of false negative results by the conventional assays TC and IC for toxins A/B and emphasize that, for these samples, only the Illumigene assay led to an accurate diagnosis of CDI.

Interestingly, 2 out of the 204 concordant negative samples were positive by IC for GDH only (data not shown): in these cases it can be supposed that non toxigenic *C. difficile* strains were involved since the presence of GDH enzyme alone does not discriminate between toxigenic and non toxigenic strains. According to this hypothesis, a *C. difficile* non toxigenic strain was isolated from one of these samples.

Furthermore, 1 sample out of the 88 concordant positive samples was positive both by the Illumigene assay and TC but negative by IC, both for toxins A/B and GDH: in this case, the negative results of both IC assays *versus* the positivity of Illumigene and TC was probably due to the sensitivity of Illumigene that is higher than that of IC as also demonstrated in this study. According to this hypothesis, the duplex PCR, previously demonstrated to be less sensitive than Illumigene, was also found to be negative in this case (data not shown).

A rapid and accurate diagnosis of CDI is important since it may result in the prevention of nosocomial transmission and allows a specific antibiotic therapy to be promptly administered to patients. The combination of a quick turnaround time with a high performance might result in a better management of CDI and in a timely implementation of control measures.

From a practical point of view, as also reported by Doing and Hintz (2011), in our hands the Illumigene assay proved easy to perform, not requiring particular skills or facilities, and offered a noteworthy rapidity giving results within 1 hour. Based on this rapidity and on the demonstrated excellent sensitivity of the new diagnostic Illumigene assay, we conclud that it can be advantageously used in our setting as a primary assay for CDI diagnosis as a replacement for the IC assay for GDH, previously used in our laboratory for the same purpose though not distinguishing between toxigenic and non toxigenic strains. Therefore the algorithm we propose for CDI diagnosis is the following: to apply the Illumigene assay as a primary assay on all samples sent to the laboratory with a suspicion of CDI and, only on the Illumigene-positive samples (addressing patients with a negative Illumigene result to other investigations) proceed to IC for toxins A/B and TC to assess the viability of the strain and the production of toxins. The current application of this algorithm in the diagnostic practice of our laboratory on 719 patients allowed prompt and accurate diagnosis of CDI cases and administration of targeted therapy. Moreover, by using Illumigene, thanks to its excellent sensitivity, an accurate diagnosis of CDI was established requiring no more than one sample per patient. In our experience, after the introduction of the Illumigene assay, even if there was a small rise in the cost for the laboratory diagnosis of CDI (28 *versus* 26 euros), we consider that the better efficiency of the Illumigene assay as a primary test is advantageous, because subsequent investigations are required only for positive samples leading to less involvement of laboratory staff with a consequent general reduction of costs.

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