A Toxigenic Culture in 24 Hours for the Diagnosis of *Clostridium difficile* Infection

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Introduction

Since the end of the seventies, *Clostridium difficile* has emerged as a major nosocomial pathogen. The main virulence factors are two high molecular weight exotoxins, namely toxins A and B, that both exhibit cytotoxic and enterotoxic activities.

Several strategies for laboratory diagnosis have been proposed. All of them aim at decreasing the time to result and improving patient management. In case of a positive result, immediate treatment of the patient will improve their condition and limit the risk of room contamination. In addition, the rapid implementation of hygiene measures will prevent further spread of the disease.

Since October 2011, the diagnostic scheme for *Clostridium difficile infection (CDI)* in our laboratory (Fig.1a) is based on an algorithm including glutamate-dehydrogenase (GDH) and toxins (Tox A&B) detection by enzyme immunoassays (EIA) on all samples followed by a PCR on GDH + / Toxins - samples. Toxigenic Culture (TC) is performed on all stool samples as a reference method (Fig 1b). In addition, culture of faeces on selective medium (ChromID® *C.difficile* (bioMérieux, Lyon, France) overnight anaerobic incubation) and detection of toxin production on colonies by enzyme immunoassay (EIA) is performed and demonstrated a much better sensitivity than EIA on stools alone and a better specificity than culture alone (Delmée et al. 2005). Toxigenic culture (TC) is well established as a gold standard for diagnosis but is very slow as compared with toxin immuno- or molecular- assays.

In most strains toxin can be detected by EIA after 48 hour growth. Here we evaluated a new chemiluminescent fully automated immunoassay (CLIA) for the detection of *Clostridium difficile* toxins A&B on 24 hours colonies in combination with a new culture medium (*C.difficile* Colorex™ bioTRADING Mijdrecht, The Nederlands). Results were compared to toxigenic culture on MRC-5 cells after 48 hours culture.

Diagnostic algorithm

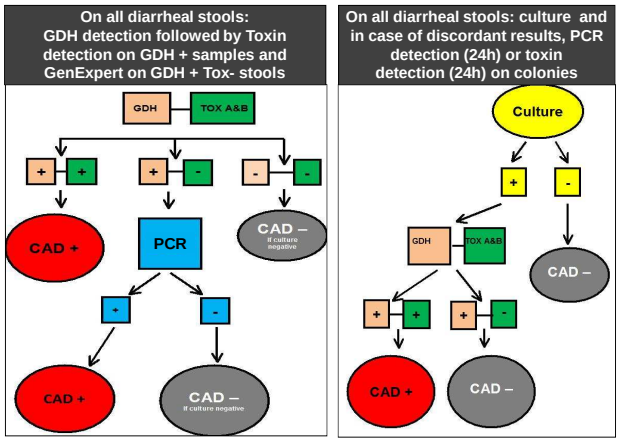

Fig.1a Fig. 1b

Materials and methods

Strains: National surveillance of *Clostridium difficile* infection (CDI) is pursued in Belgian hospitals since 2007 by the Institute of Public Health and the *C. difficile* National Reference Center (NRC). Every six months each hospital laboratory is invited to send to the NRC the first five strains isolated in the routine bacteriology laboratory. All strains are ribotyped since 2009. From 2009 to 2013, we received 2594 strains in the frame of our national surveillance. Among these isolates, a total of 433 different ribotypes were identified but 335 of them were only seen once. 89 ribotypes (85 toxigenic and 4 non-toxigenic strains) represent 90 % off all strains found in Belgium up to now. One strain belonging to each of these 89 ribotypes was investigated in this study.

Cultures: For this study the *C.difficile* Colorex™ medium has been manufactered ready prepared by bioTRADING prod.nr. K623P090KP (Mijdrecht, The Nederlands) After 24 h anaerobic incubation at 35°C the cultures were read with a binocular stereomicroscope, with the lightbeam through the Petridish under a certain angle.

Columbia Blood Agar 5% (Becton Dickinson, Franklin Lakes NJ,USA).

Identification: MALDI-Tof MS biotyper (Bruker Daltonik GmbH, Bremen, Germany) was used to confirm the *C. difficile* colonies.

Ribotyping: DNA were extracted with chelex and 16S - 23S rRNA intergenic spacer regions were amplified using primers as described by Barbut et al. (1). Amplicon size were analysed by capillary electrophoresis using an automatic sequencer (ABI 3100 Automated Capillary DNA Sequencer) and GeneMapper Analysis (Applied Biosystems, Inc.). A 35–500 bp ROX ladder (ABI) was used as internal marker.

Toxigenic culture (TC): 48 hours culture colonies supernatants were tested by cell cytotoxicity (CTA) on MRC-5 cells.

Liaison® *C.difficile* GDH and Toxins A&B assay: (Diasorin, Stillwater,MN USA). The test was performed according to the manufacturer's instructions.

Liaison principle

Liaison is a chemiluminescent fully automated immunoassay (CLIA) for the detection of *Clostridium difficile* GDH and TOX A&B (Diasorin). The Liaison automate offers a good traceable, reproducible, flexible and linkable automated random access instrument. The Liaison ® *C. difficile* Toxins A&B or GDH assay is a modified two-step, two-site sandwich assay for the detection of both Toxin A and Toxin B or GDH. The assay uses one monoclonal antibody for capture and one polyclonal for detection of the Toxin A molecule, and one polyclonal antibody for both capture and detection of the Toxin B molecule or a monoclonal antibody for capture and one polyclonal for detection of GDH. The assay uses 200µL of sample consisting of a mixture of sample diluent and stool extracted Toxins A&B or GDH which is incubated with isoluminol conjugated antibodies for Tox A and Toxin B or GDH.

Following incubation, paramagnetic particles coated with capture antibodies for Toxin A and Toxin B or GDH are added to the reaction and are incubated. After the second incubation, the unbound material is removed with a wash cycle. The starter reagents are the added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units and is proportional to the concentration of Toxin A and Toxin B present in the calibrators, controls or samples.

After 24h growth on Colorex™ medium, a Mcfarland 2 suspension was used for testing. All 89 toxigenic isolates were correctly recognised as toxigenic or not by the Liaison® *C.difficile* Toxins A&B assay. For each of the six most frequent ribotypes, five additional strains were tested by Liaison, which correctly detected the production of toxin in all cases (see table1).

Results and Discussion

A Do all *Clostridium difficile* grow in the same way?

Starting with a 24 hours culture on Columbia Blood agar, we made a ten-fold serial dilution from a Mcfarland 2 suspension and plated 200µL of each dilution on *C.difficile* Colorex™ plates. Colonies were enumerated after 24h anaerobic incubation. We tested six most frequent ribotypes in triplicate. No difference in grow was observed and the average colony forming unit for 1 Mcf was 5 10^6 (1 10^6 - 1 10^7).

B Is the amount of toxine production in colonies of a same ribotype reproducible?

We tested six different ribotypes in triplicate: a two-fold serial dilution from a Mcf 2 suspension (after 48h anaerobic incubation on Columbia Blood agar) on MRC-5 cells. Cell cytotoxicity was read after 48 hours. No difference was observed between the triplicates. The experience was repeated using two different strains for each ribotype. There was no difference observed between strains belonging to the same ribotype. We also tested toxin production on *C.difficile* Colorex™ plates. The cell cytotoxicity was about one two-fold dilution lower than on Columbia Blood agar.

Mcf in physiological serum	suspension quantity		Liaison buffer	N=85 Tox pos strains	%
0,5	100µL	+	1000µL	54	64
0,5	200µL	+	1000µL	60	71
0,5	400µL	+	1000µL	65	77
2	200µL	+	1000µL	75	89
2	400µL	+	1000µL	78	92
2	1000µL	+	1000µL	78	92

Mcf in Liaison buffer	Liaison buffer	N=85 Tox pos strains	%
2	1000µL	85	100

Mcf in Liaison buffer	Liaison buffer	N=4 Tox neg strains	%
2	1000µL	4	100

Table 1 Liaison results on colony suspensions

Conclusion

The combination of the bigger colonies on the new *C.difficile* Colorex™(24 h anaerobic incubation at 35°C) and the higher sensitivity of the Liaison® *C.difficile* Toxins A&B assay allows to perform TC within 24 hours.

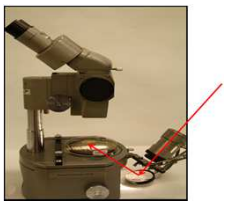

Fig.2 Binocular stereomicroscope

Fig.3 *Clostridium difficile* on CHROMagar™ *C.difficile*

Fig.4 Liaison (Diasorin)

Fig.5 Xpert *C. difficile*

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Abstract

Abstract

Clostridium difficile infection (CDI) is a global health problem. Toxigenic culture (TC) is well established as a gold standard for diagnosis but is very slow as compared with toxin immuno- or molecular- assays. In most strains toxin can be detected after only 48 hour growth. Here we evaluated a new chemiluminescent fully automated immunoassay (CLIA) for the detection of *Clostridium difficile* toxins A&B on 24 hours colonies. We tested 89 different ribotypes (from a collection of 370 different ribotypes found in Belgium since 2007), covering 90% of all strains. Intravariability of toxin production in the six most frequent ribotypes was studied.

Methods

The Liaison® *C.difficile* Toxins A&B assay (Diasorin, Stillwater,MN USA) was performed after 24 hour growth and confirmed by cell-cytotoxicity assay after 48 hour growth, using MRC-5 cells. All strains were cultured on *C.difficile* Colorex™ medium(bioTRADING, Mijdrecht, The Nederlands) for 24h at 35°C in anaerobic condition. For ribotyping, DNA were extracted with chelex and 16S - 23S rRNA intergenic spacer regions were amplified using primers as described by O'Neill, G. et al (Anaerobe 1996). Amplicon size were analysed by capillary electrophoresis using an automatic sequencer (ABI 3100 Automated Capillary DNA Sequencer) and GeneMapper Analysis (Applied Biosystems, Inc.). A 35–500 bp ROX ladder (ABI) was used as internal marker. Profiles were analyzed by comparison with those of reference strains from the European collection (Brazier classification) and with our own database.

Results

A selection of 89 isolates representing the most frequent ribotypes identified in the Belgian national *C.difficile* reference center was evaluated. These ribotypes represent 90% of all strains found in Belgium since 2007 in the national surveillance program. 85 were toxigenic strains and 4 non-toxigenic ones. After 24h growth on Colorex™ medium, a Mcfarland 2 suspension was used for testing. All 85 toxigenic isolates were correctly recognised as toxigenic or not by the Liaison® *C.difficile* Toxins A&B assay. For each of the six most frequent ribotypes, five additional strains were tested by Liaison, which correctly detected the production of toxin in all cases.

Conclusion

The combination of the new *C.difficile* Colorex™(24 h anaerobic incubation at 35°C) and the Liaison® *C.difficile* Toxins A&B assay on colonies allows to perform TC within 24 hours.