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 factor of this pathogen is the production of toxins A and B, weight exotoxins, namely toxins A and B, that both exhibit cytotoxic and antimicrobial 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 nosocomial infection. In addition, the rapid implementation of appropriate measures will prevent further spreading 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 immunoassay (EIA) on all samples followed by a PCR on GDH + Toxins (sensitive and specific toxigenic Culture (TC) is performed as a reference method (Fig 1b)). In addition, culture of faeces on selective medium (Columbia Blood Agar) 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 (D'Alme et al., 2005). Toxigenic culture (TC) is well established as a gold standard for diagnosis but is very slow as compared to toxin immuno- or molecular assays.

In most strains toxigen can be detected by EIA after 18 hour growth. Here we evaluated a new chemiluminescent immunoassay which allows to perform TC within 24 hours.

Materials and methods

Samples: 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 from fresh faecal samples that are submitted to the laboratory. All strains are ribotyped since 2009. From 2009 to 2013, we received 254 strains in the frame of our national surveillance. Among these isolates, 89% were toxigenic which is in total of 43 different ribotypes were identified but 335 of them were only once seen. 89 ribotypes (85 toxigenic 4 non-toxigenic strains) represent 90 % of 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 manufactured newly prepared by BioTRADING products (BioTRADING, Mijdrecht, the Netherlands). After 24 hour anaerobic incubation at 35°C the colonies were read with a binocular stereomicroscope, with the lightbeam through the Petri dish under a certain angle.

- Columbia Blood Agar 5% (Becton Dickson, Franklin Lakes, NJ, USA)
- Chromagar C. difficile (CHROMagar)

Identification: MALDI-TOF MS biopsy (Bruker Daltonk, Bremen, Germany) was used to confirm the C. difficile colours.

Ribotyping: DNA were extracted with a kit 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 automated sequencer (ABI 3100 Automated Capillary DNA Sequencer, Applied Biosystems) and GeneMapper Analysis (Applied Biosystems, Inc.). 35-500 bp ROX ladder (ABI) was used as internal marker.

Toxicigenic culture (TC): 48 hours culture on Columbia Blood Agar, the colonies were cultured on a selective medium (Cdifficile Colorex™) and identified by BioTRADING Mijdrecht, the Netherlands. The combination of the biggest colonies on the new C.difficile Colorex™ medium, staining with chromogenic stain, and the higher sensitivity of the Liaison C. difficile Toxins A&B assay allows to perform TC within 24 hours.

Diagnostic algorithm

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 Mcf 2 suspension and plated 200µl of each dilution on C.difficile Colorex™ plates. Colonies were enumerated after 24 hour anaerobic incubation. We tested six most frequent ribotypes in triplicate. No difference in growth was observed and the average colony forming unit for 1 Mcf was 5.10² ± 10⁻² in triplicate.

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 experiment was repeated using two different strains for each ribotype. Toxicity was determined and compared between the same ribotype. We also tested toxigenic production on C.difficile Colorex™ plates. The cell cytotoxicity was about one two-fold dilution lower than on Columbia Blood agar.

Results and discussion

The combination of the biggest colonies on the new C.difficile Colorex™ medium and the higher sensitivity of the Liaison C. difficile Toxins A&B assay allows to perform TC within 24 hours.

Conclusion

A new scheme for the diagnosis of Clostridium difficile infection (CDI) is used in our laboratory. This scheme offers a good traceable, reproducible and automated detection of toxigenic cultures and provides a result within 24 hours. This permits a rapid decrease in the time to result and an improvement in patient management.

Refrences


Orendi J. et al. (2011), “A two-stage algorithm for the detection of Clostridium difficile including PCR, can we replace the tox EIA?” Journal of Hospital Infection (2011) 1-3.

Materials and methods

Nucleic acid extraction: DNA were extracted with a kit 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 automated sequencer (ABI 3100 Automated Capillary DNA Sequencer, Applied Biosystems) and GeneMapper Analysis (Applied Biosystems, Inc.). 35-500 bp ROX ladder (ABI) was used as internal marker.

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Liaison principle

Liaison is a chemiluminescent fully automated immunoassay (CLIA) for the detection of Clostridium difficile GDH and Tox A&B (DiaSorin). The Liaison automates offers a good traceable, reproducible 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 bioluminescent conjugated antibodies for Tox A and Toxin B or GDH. Following incubation, paramagnetic particles coated with capture antibodies for Toxin A or 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 calibrator, controls or samples. After 24 hour growth on Colorex™ medium, a Mcf 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).