Evaluation of a new 24 h culture medium for the isolation of *Clostridium difficile* from stool samples

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24rd ECCMID
Barcelona 2014
Poster P0731

Introduction

Since the end of the seventies, *Clostridium difficile (Cdif)* 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.
During the first years of the 21st century, the epidemiology of *C. difficile* infections (CDI) dramatically changed in North America and Europe. A significant increase of incidence as well as of severity of CDI were reported on both sides of the Atlantic ocean. The rapid emergence and spread of a specific clone of *C. difficile* was rapidly demonstrated. The increased virulence of this clone is associated with the overproduction of toxins A and B and the production of binary toxin. Primarily detected in North America, *C. difficile* « 027 » was rapidly identified in outbreaks that occurred in several european countries (UK, The Netherlands, Belgium and France).
All strategies should aim at a same-day diagnosis in case of suspicion of CDI. In case of a positive result, the immediate treatment of the patient will improve his condition and limit the risk of room contamination. And the rapid implementation of hygiene measures will prevent further spread of the disease. With such a goal and such implications however, the accuracy of the laboratory diagnosis is of crucial importance.
False positive results may induce inadequate treatment and increase cost due to isolation procedures and false negative results may lead to outbreaks.
In evaluation studies for diagnostic tests Toxigenic Culture is more and more accepted as the standard reference method.
Nevertheless there is culture and culture. Looking for a good commercial culture medium to be used in a automated inoculation system, we compared two commercial media Chrom ID (bioMérieux, Lyon,France) and CHROMagar™ *C.difficile* (CHROMagar™, Paris, France).

Materials and methods

Stools: from inpatients (>2y) suffering from antimicrobial- or chemotherapy-associated diarrhea. Between Jul 2013 and Dec 2013 retrospectively 95 positive stools (kept at -80°C) and prospectively 161 stools were tested.

Inoculations: The prospective 161 stools were inoculated manually using a 10 µL loop. The retrospective 95 positive stools were inoculated using a 30 µl loop on BD™Innova. To obtain liquid stools they were prediluted minimally with physiologic serum.

Cultures: Chrom ID (bioMérieux, Lyon, France), (24 h anaerobic incubation at 35°C). For this study the CHROMagar *C.difficile* medium has been manufactered ready prepared by bioTRADING (prod.nr. K623P090KP) as *C.difficile* Colorex™.

Colorex™ is a registered brand name of CHROMagar™, Paris France, when supplied ready prepared (24 h anaerobic incubation at 35°C).

Reading: All cultures were read with a binocular stereomicroscope, with the lightbeam through the Petridish under a certain angle.

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. Profiles were analyzed by comparison with those of reference strains from the European collection (Brazier classification, BR…) and with our own database, (UCL…).

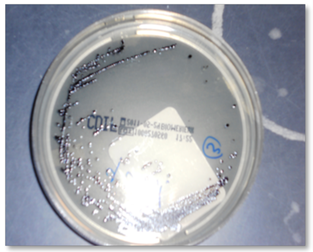

Fig.1 *Clostridium difficile* on ChromID agar (bioMérieux)

Fig.4 BD™Innova

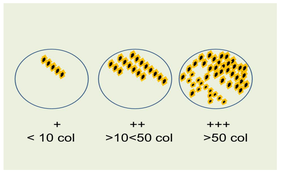

Fig 5 Culture reading

Results

Between Jul 2013 and Dec 2013, we inoculated retrospectively 95 positive stool samples and all of them grew on both chromogenic plates. Prospectively we analysed 161 routine samples. We isolated *Cdiff* in 51(31.7%) samples on chromID and 54 (33.5%) on CHROMagar.

All isolated *Cdiff* colonies on the CHROMagar™ plate were fluorescent and larger than on chromID. We detected 44 different ribotypes and some ribotypes (BR023, BR020, BR014 and UCL 20a, UCL 412) stayed colorless but with the typical colony-shape for *Cdiff* on chromID plates. Other colonies were black but were not confirmed as *Cdiff.*

All suspicious colonies were analysed with the MALDI-TOF MS (Bruker). Two *C. Hathewayi* were black and looked very much like *C. difficile*, but could be recognised with a binocular. Some ribotypes (BR014, BR020) of *C. difficile*, stayed uncolored after 24 h but became coloured sometimes after 48 h. Other colonies were black but did not looked like *C. difficile.*

We tested at least one strain from the 23 most frequent ribotypes in Belgium in 2012. All strains gave fluorescence under ultraviolet illumination. Although the fluorescence diffuses rather quickly in the agar plate that we used.

RIBOTYPE	% STRAINS in Belgium 2012 (N=638)	chromID	CHROMAGAR
BR014	10,5	++**	++
BR020	8,8	++**	++
BR002	8,2	++	++
BR078	7,5	++	++
BR027	5	++	++
UCL 46	2,7	++	++
UCL 16b	2,4	++	++
BR023	2,4	++*	++
UCL 16L	2,2	++	++
BROO1	2,2	++	++
UCL 33	2	++	++
UCL 23f	1,9	++	++
UCL 044	1,7	++	++
UCL 5a	1,7	++	++
UCL 47	1,6	++	++
UCL 32*	1,6	++	++
UCL 16r	1,1	++	++
BR015	1,1	++	++
UCL 20a	1,1	++*	++
UCL 48d	1,1	++	++
UCL 49	1,1	++	++
BR087	<1	++	++
BR012	<1	++	++

** colonies stay sometimes uncoloured after 24 h
* colonies stay always uncoloured

Table 1 Most frequent ribotypes, In Belgium tested

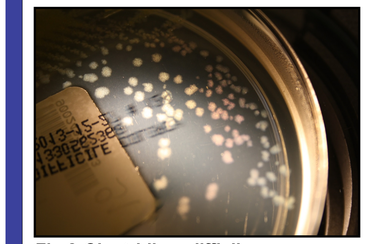

Fig.2 *Clostridium difficile* on CHROMagar™ *C.difficile*

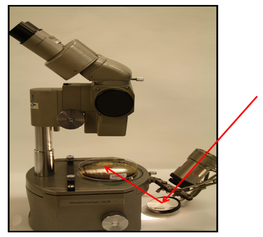

Fig.3 Binocular stereomicroscope

Discussion

When aiming at a quicker diagnosis of CDI to reduce the incubation time is crucial. Both Chrom ID (bioMérieux, Lyon, France) and CHROMagar™ *C.difficile* (CHROMagar™, Paris France) allow isolation of *Clostridium difficile* from stools after 24 h anaerobic incubation at 35°C. In both media endogen flora is reduced to a minimum. Manually inoculation is still superior, since the stool sample is not prediluted, but predilution homogenise the stool sample making comparison more rigourous. The medium allows a major reduction of the incubation period (24h). Another point of attention is that, after 24h incubation, the toxigenic status of the strain must be determined by a molecular biology method instead of an immunoassay. On the Chrom ID medium *Clostridium difficile* grows as very small colonies which are coloured black. The colonies of *Clostridium difficile* are bigger after 24h on the CHROMagar™.The Chrom ID medium has to be used with precaution, since some ribotypes stay uncoloured on the plate while other bacteria coloured black and were not identified as *Clostridium difficile*. (binocular reading can solve this problem)*Clostridium difficile* can more easily be recognised on the CHROMagar™ medium by an unexperienced eye. In case of doubt ultraviolet illumination can solve the problem. The CHROMagar™ is an excellent new 24h *Clostridium difficile* detection medium.

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Abstract

Objective. Toxigenic culture remains one of the most sensitive diagnostic method for *Clostridium difficile* (*Cdiff*) infection and is usually considered as a gold standard in diagnostic method evaluations. However, it is very slow as compared with other rapid tests like immunoassays or molecular biology. Here we evaluated two different chromogenic media allowing *Cdiff* isolation after only 24h incubation using the BD™Innova which is an automated specimen processor.

Methods. Two commercial media, the CHROMagar™ *C.difficile* (CHROMagar™, Paris France) and chromID *C.difficile* (bioMérieux, Lyon,France), were compared in a retrospective and prospective study. A suspension of diarrheal stools was inoculated manually with 10µl loops or was processed with a 30µl loop on BD™Innova. All media were incubated in anaerobic conditions at 35°C. Plates were read after 24h incubation. Colonies of *Cdiff* are black on chromID, whereas they are fluorescent under UV light (365nm) on CHROMagar. Identification was confirmed by MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany).

Results. Between Jul 2013 and Dec 2013, we inoculated retrospectively 95 positive stool samples and all of them grew on both chromogenic plates. Prospectively we analysed 161 routine samples. We isolated *Cdiff* in 51(31.7%) samples on chromID and 54 (33.5%) on CHROMagar. All isolated *Cdiff* colonies on the CHROMagar™ plate were fluorescent and larger than on chromID. We detected 44 different ribotypes and some ribotypes (023, 020, 014 and UCL 20a, UCL 412) stayed colorless but with the typical colony-shape for *Cdiff* on chromID plates. Other colonies were black but were not confirmed as *Cdiff.*

Conclusion. The new fluorescent CHROMagar™ *C.difficile* is an excellent medium for the detection of *C.difficile* in stool samples after 24h. Larger colonies make identification easier. Even after automated inoculation CHROMagar™ demonstrated to be the most sensitive and allows a major reduction of the incubation period. On chromID, black colonies were not always confirmed as *C. difficile* and colonies of certain ribotypes were colorless but nevertheless easily recognised by their typical appearance using a binocular.