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Clostridium difficile the Case for Revisiting Old Strategies

Introduction

The study of *Clostridium difficile* over the last few decades has focussed on the role of its two toxins, toxin A and toxin B, their role in the disease process, and the detection of these toxins as an aid to the diagnosis of *C. difficile* associated diarrhoea (CDAD). More recently the recognition of important variants, deficient in toxin A and/or toxin B yet still pathogenic has posed a problems to researchers and testing laboratories.

Although testing for toxin A and/or B has established itself as the standard method for *C. difficile* testing in most laboratories, the detection of these toxin deficient variants is now challenging this approach. The decision facing laboratories now is whether to continue using toxin A and B testing on its own, or introduce molecular methods which are both expensive and may not produce definitive results, or re introduce the original culture methods previously employed before toxin testing became available. The reintroduction of culture, which is a sensitive procedure, would allow the detection of both non toxigenic strains and the toxin variant strains.

Further complicating this discussion is the recognition of hyper-toxin producing epidemic strains, community and hospital binary toxin producing strains and metronidazole and vancomycin resistant strains of *C. difficile*.

Toxin A and B Testing

The production of cytotoxin (toxin B) was the first non culture method employed for the diagnosis of CDAD by laboratories. The detection of this toxin was achieved using tissue culture cell lines (Figure 1.) and a cytotoxin neutralisation method (1). Although this method was highly sensitive and extremely specific (2), it was limited in that not all laboratories possessed tissue culture capabilities It was expensive and lacked standardisation.

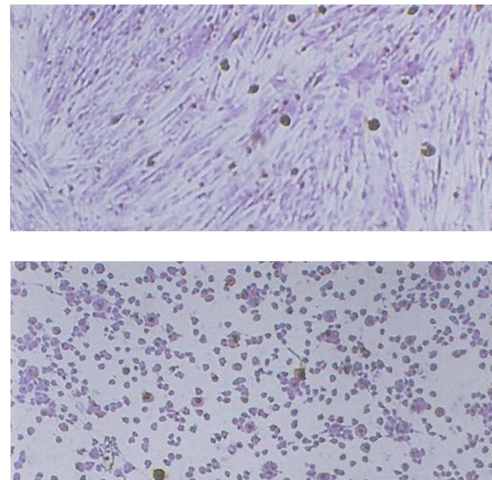

Figure 1. Normal Fibroblasts in Tissue Culture (Top). Fibroblasts demonstrating Cytopathic Effect after exposure to *C. difficile* Toxin B (Bottom).

The production of a second distinct toxin (enterotoxin, toxin A) was first identified in 1981 (3). The discovery of this second toxin resulted in the commercial development of a range of immunoassays capable of providing rapid toxin detection direct from patient samples. Due to their relatively low cost, speed and ease of use, these assays rapidly replaced tissue culture neutralisation as the method of choice in laboratories. Initially, these assays allowed the performance of Toxin A assays only however, over the last few years combo assays using rapid Enzyme Immunoassays or simple immunochromatographic techniques have allowed the simultaneous detection of both toxin A and toxin B.

Conventional Culture Methods

Over the years, opinions have varied as to the value of *C. difficile* stool culture as a diagnostic tool for CDAD. A major consideration in determining the value of culture is the argument that culture does not differentiate between toxigenic and non-toxigenic strains and on this basis, culture alone should not be considered as diagnostic. As the cause of CDAD is the toxins produced by this organism, it is believed that toxin testing should be performed both in the presence or absence of culture. The argument being that if diagnosis is based on culture results alone, patients yielding toxin negative cultures may be treated unnecessarily.

It has been established that over 90% of strains of *C. difficile* carry the toxin genes (4) and therefore have the potential to produce toxins with the resultant development of CDAD. On this basis, the alternative point of view is that the presence of the organism combined with an appropriate clinical presentation is a suitable indication of CDAD and basis for treatment.

The current consensus is that culture is best achieved using either the CCFA agar described by George et.al. (5) or a blood supplemented medium (6) containing Cefoxitin (15mg/ L) and Cycloserine (500mg/ L). The detection of *C. difficile* can be further enhanced by the addition of a sporulating agent, sodium taurocholate (1000mg/ L) or the use of ethanol or heat shock (7).

Identification of *C. difficile*

The accurate identification of *C. difficile* has proven difficult for laboratories primarily due to its poor and slow identification using commercially available anaerobic biochemical identification systems.

On agar plate media, colonies of *C. difficile* grow with fairly characteristic colonial appearance i.e. on the blood containing medium colonies appear as 4-6mm diameter irregular, raised, opaque and grey-white in colour (Figure 2.). In addition, under UV light they produce a characteristic fluorescence.

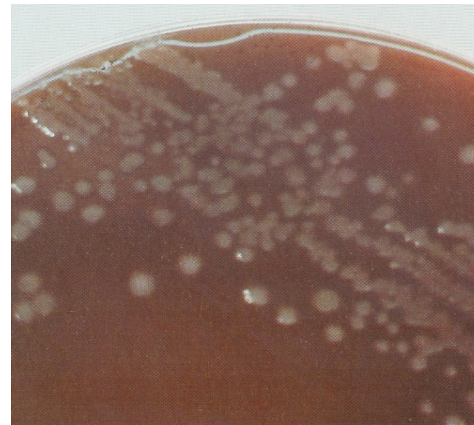

Figure 2. Typical C. difficile colonies on CCFA containing blood.

Isolated colonies may subsequently be transferred to cooked meat broth, grown and a sample of the broth examined for fatty acids using Gas Chromatographic methods. *C. difficile* produces a characteristic major isocaproic acid peak. This procedure generally requires in excess of 48 hours and the availability of expensive equipment not routinely found in clinical microbiology laboratories.

Microgen Bioproducts has addressed these identification issues by the development of a simple, rapid and inexpensive latex agglutination test for the identification of colonies of *C. difficile* isolated on any type of agar plate media.

The Microgen C. difficile Latex Agglutination Test (M41) consists of a purified polyclonal antibody cocktail coated onto latex particles. A suspect colony is emulsified in sterile isotonic saline on a reaction card to produce a smooth homogeneous suspension. A single drop of the

test latex is added to the suspension, the two are mixed briefly and the card gently rocked and observed for agglutination. The presence of an obvious agglutination within 2 minutes is confirmation of *C. difficile*. The use of this test provides a very easy, cost effective and rapid method of confirming the presence of *C. difficile* colonies on agar plate cultures without the need for training and expensive equipment.

C. difficile Toxin Variants

Toxin A+/ B+ Variants

Molecular studies have determined that toxin A is encoded by the 8.1 kb *tcdA* gene, whilst toxin B is encoded by the 7.9 kb *tcdB* gene. These genes forming part of a highly stable 19.6 kb pathogenicity locus, *PacLoc*. Toxigenic strains of *C. difficile* possess this *PacLoc*, whilst non-toxigenic strains lack *PacLoc* but have a 115 bp fragment.

The “normal” or most commonly encountered toxigenic strains are these variants.

Toxin A-/ B+ Variants

The existence of toxin A negative, toxin B positive variants was first described in 1992 (8,9). It has since been established that these strains lack a portion of the *tcdA* gene. Since these variants were first discovered, they have been reported from many countries worldwide. Although their incidence is generally low, 2.5 – 3% of all isolates (10), they can found to be present in as many as 7 – 12% of isolates (11).

Toxin A+/ B- Variants

At this point in time, the incidence of these variants appears to be extremely low with only a few cases being reported (12).

Based on current reports it appears that such strains may possibly evolve in cases in which patients are on prolonged treatment for CDAD.

Binary Toxin CDT

Currently, the role of Binary toxin CDT is not clear. However, it has been established that similar toxins produced by other species of Clostridia may cause necrotizing enteritis or diarrhoea. The Binary toxin CDT has been found to be encoded in a different region to the *PacLoc* and therefore has the potential to act in conjunction with toxins A and B or act alone in “non-toxigenic” strains.

It has been suggested that there is a direct correlation between the presence of this toxin and the severity of the disease and that this toxin is most likely to be encountered in community acquired diarrhoea (13).

Clinical Significance

The belief that toxin A production is necessary for disease is now being challenged. A number of confirmed reports of disease caused by Toxin A-/ B+ Variants have appeared in the literature (10,11). The detection of these variants should still be reported and they should still be considered as clinically significant as the presence of Binary toxin CDT cannot be discounted.

TESTING STRATEGIES – WHERE TO NOW?

The ready access to commercially available toxin A assays allows the rapid reporting of typical *C. difficile* infections. However, these assays on their own will not allow the detection of Toxin A-/ B+ variants or Toxin A-/ B- variants producing Binary toxin CDT. The presence of these variants, although low is sufficiently important enough to support the re introduction of culture and isolation of *C. difficile* as a standard routine procedure. Whilst there have only been a few reports of Toxin A+/ B- variants, laboratories performing cytotoxin assays only should also consider reintroducing culture. In addition, the recent reports of Binary toxin CDT production by *C. difficile* also supports the need to perform culture, the results of which should be interpreted in conjunction with the clinical presentation of the patient.

The availability of numerous selective culture media for *C. difficile* which are readily available from commercial suppliers, combined with the Microgen *C. difficile* Latex Agglutination test (M41) should simplify the culture process and allow its easy integration into routine laboratory testing.

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