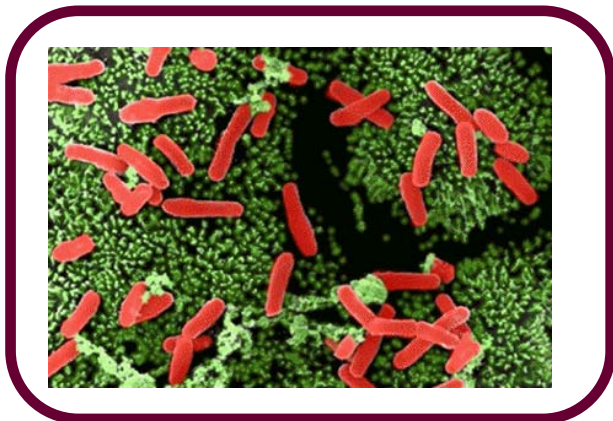


## A New Approach to Sensitive *C. difficile* Cytotoxin Testing

### Introduction

With over 100,000 hospital acquired-infections per year with a cost of over £1,000 million and the potentially fatal consequences for patient care, nosocomial infections are a big challenge for the UK National Health Service (NHS).

In recent years, *Clostridium difficile* has emerged as the primary cause of nosocomial infection in the United Kingdom. A Gram-positive, anaerobic and sporing bacillus, which was appropriately named *Bacillus difficilis* (1) in 1935 by Hall and O'Toole due to the difficulties they encountered in isolating the organism.



Picture 1. *Clostridium difficile*

Originally the cause of sporadic cases of *pseudomembranous colitis* and Antibiotic Associated Diarrhoea, in recent years there has been a shift and now *C. difficile* is a major cause of cross-infection. The organism is found regularly in the hospital environment and can be isolated from clothing and room fixtures. The transmission route is via diarrhoea from infected

patients and can be transmitted by hospital employees. Once in a hospital environment, the organism can persist for months due to the spores it produces. Therefore it is vital that infected patients are diagnosed accurately and rapidly to ensure they are properly isolated to reduce cross-infection and to ensure provision of correct treatment. Although *C. difficile* may be part of the normal flora in 0-3% of healthy adults, the carriage rate in hospitalised patients increases to 20% (2), due to infection acquired following disruption of the normal intestinal micro-flora due to the administration of antibacterial or chemotherapeutic agents which reduce microbial competition in the gut, allows *C. difficile* spores to germinate and multiply. Interestingly, up to 70% (2) of infants in a hospital nursery can carry toxigenic and non-toxicogenic *C. difficile* within their intestinal tract asymptotically, reflecting the environment in which they are born. The reason why infants do not develop the disease is not fully understood but it is obvious they are also a reason why the organism maybe present in a hospital environment.

The challenge for microbiology laboratories is to provide rapid, accurate and cost-effective detection of toxigenic *C. difficile* infection so patients' can be properly isolated and receive the correct treatment. Currently there is no clear testing algorithm, with microbiology laboratories using a variety of methods and assays for diagnosis, making it difficult to compare results between laboratories.

## Testing Methods

As discussed in Microlab #23 the diagnosis of *C. difficile* infection can be performed by using various *in vitro* assays; bacterial stool culture, conventional cytotoxin cell culture, enzyme immuno-assay for toxins, toxin assays in immunochromatographic formats or glutamate dehydrogenase (GDH) detection.

Opinions vary on the value of bacteriological stool culture, as it does not differentiate between toxigenic and non-toxigenic strains so therefore cannot be used alone as a diagnostic tool. Although the use of bacteriological stool culture does not differentiate between toxigenic and non-toxigenic strains, it has been reported 90% of strains carry the toxigenic gene (2) so have the potential to produce toxins and cause disease. It is therefore suggested by some that culture should be used in conjunction with clinical symptoms and/or toxin detection assays. In addition, a major benefit of culture is it provides isolates for characterisation, epidemiology and antimicrobial susceptibility.

The gold standard tissue culture method for cytotoxin is still used extensively due to its excellent specificity and sensitivity; 1 pg of toxin B is sufficient to cause rounding of the cells (3). The disadvantages of conventional cytotoxin tissue culture methods include the maintenance of the cell lines, specialised equipment & training as well as a turn-around time of up to 4 days. The use of different cell lines by different laboratories also makes it difficult to standardise and compare results, but if the disadvantages were overcome this could be the most valuable diagnostic tool.

Due to the many difficulties associated with cell culture, laboratories have adopted enzyme immuno-assays (EIA) for *C. difficile* toxins A and/or B which allowed batching of samples and fast turn-around time, but sensitivity reports of as low as 38% (5), raises questions on the value of EIAs for toxins as a stand-alone test.

Recently, assays for glutamate dehydrogenase (GDH) an enzyme produced by *C. difficile* strains have started to be evaluated and used by clinical laboratories. The problem with these assays is they do not differentiate between toxigenic and non-toxigenic strains and other bacteria may also produce this enzyme, resulting

in a low positive predictive value. GDH testing can be a valuable screening tool for eliminating negative samples but positive results will require the use of a confirmatory toxin detection assay.

Also confusing the issue are assays for the detection of faecal Lactoferrin which is a marker of intestinal inflammation, as damage to intestinal musosa by toxins A and B leads to a rapid influx of inflammatory cells. Inflammatory response plays a key role in how quickly *C. difficile* infection develops in to *pseudomembranous colitis* and may help define the severity of the disease but they can not be used as a diagnosis tool for *C. difficile* infection.

### **Is there a toxin EIA or membrane assay which is sensitive enough to be used as a stand alone diagnostic?**

The Health Protection Agency (HPA) produced an evaluation report in February 2009 on *C. difficile* toxin detection assays which tested 600 samples for the presence of *C. difficile* Toxin using nine commercially available toxin detection assays, against the gold standard cytotoxin culture (6). The nine commercial toxin kits included 5 well-type EIAs, 3 membrane assays and 1 automated immunoassay. The highest sensitivity when compared to the cytotoxin assay was 80.8% and the lowest was 60% respectively. The HPA concluded that the low sensitivity of all of the assays, in the context of widespread testing, raises doubts about their appropriateness when used as a single test for the laboratory detection of *C. difficile* toxins. The use of these commercially available toxin detection kits can result in a hidden cost due to the number of inaccurate results: a missed true positive may result in a cost far higher than the acquisition of the kits as it may result in the transmission of infection with additional cost of new infections and an increased length of stay for infected patients and the possibility of fatal consequences. The HPA report confirms that no testing algorithm which contains 1 diagnostic assay is sensitive or specific enough to meet the requirements of modern *C. difficile* diagnosis.

Johns Hopkins Hospital, Baltimore, Maryland, USA, evaluated a two-step algorithm for detecting toxigenic *C. difficile* which consisted of an EIA for GDH and then for GDH antigen-positive specimens, a cell culture cytotoxicity

neutralisation assay (CCNA) which was Human Foreskin Fibroblasts, simultaneously inoculated with two aliquots of one specimen, one of which had been incubated with anti-toxin B and then observed for cytopathic effect at 24 and 48 hours. The hospital was using a stand-alone EIA approach for detecting toxins A and B but it became apparent the EIA for detecting Toxin A and B had a sensitivity of 38% (5) which is much lower than the 66-94% reported in the Food and Drug Administration approved package insert so led to a costly and time-consuming practice of multiple tests for one patient.

In their study, out of a total of 366 samples, 266 were submitted for testing by EIA for GDH and CCNA. The 100 other specimens were EIA tested for GDH as well as Toxin A and B, 38 of the 100 were tested by CCNA. CCNA was performed on all 27 antigen-positive and 10 randomly selected GDH antigen/Toxin A and B negative specimens.

Out of the 266 samples, GDH EIA had a positive frequency of 18% and CCNA had a positive frequency of 9%, resulting in a positive predictive value of 49% and negative predictive value of 99.5% for GDH EIA. Out of the 100 samples, Toxin A and B EIA had a positive predictive value of 100% and a negative predictive value of 89% whilst GDH EIA had a positive predictive value of 59% and negative predictive value of 100%. The high GDH EIA sensitivity and the negative predictive value indicates that the GDH was a suitable first step-assay for detecting toxigenic *C. difficile* but it must be confirmed by a sensitive toxin detection assay.

The simplicity of the testing algorithm eliminates the possibility of false-positive EIA Toxin A and B results and allowed the hospital to provide sensitive *C. difficile* diagnosis with an adequate turn-around time, cost-effectively. The only disadvantage with this algorithm which may prevent other hospitals adopting it may be the problems associated with conventional cell culture, such as the maintenance of cell lines, specialised equipment required and the laborious protocol.

## The Alternate Solution

The use of Hs27 ReadyCells available in the UK from Microgen Bioproducts and manufactured by Diagnostic Hybrids, offers sensitive and convenient gold standard *C. difficile* cytotoxin testing which overcome the problems associated with traditional cytotoxin testing and the variability of Toxin A/B detection by EIA or membrane assay.

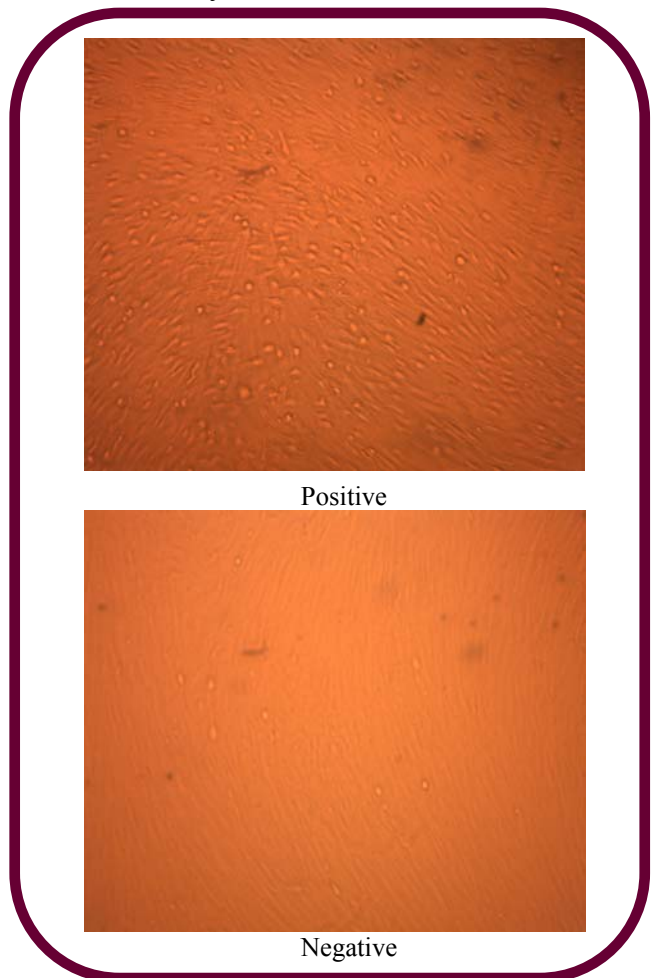


Figure 1. Positive & negative cytotoxin assay using Hs27.

Hs27 ReadyCells are Human Foreskin Fibroblast (HFF) cells, which require no cell line maintenance as they are stored as ready-to-use shell vials at  $-70^{\circ}\text{C}$  for up to 5 months, and can be thawed and inoculated within 4 minutes, eliminating wastage as well as the associated cost and quality issues of conventional cell culture. The use of Hs27 is also capable of determining the presence of cytotoxin in stool samples as early as 6 hours which can allow a same day result to be reported (see Table 1). The ReadyCell format also allows the cells to be easily integrated into any testing algorithm and is

	Hs27 ReadyCells Shell Vial				Vero Cells Shell Vial (Fresh)			
	6 hrs		24 hrs		6 hrs		24 hrs	
Toxin Dilutions	Tox/ Pt	Anti	Tox/ Pt	Anti	Tox/ Pt	Anti	Tox/ Pt	Anti
1:2	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
1:4	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
1:8	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
1:16	Neg	Neg	Pos	Neg	Neg	Neg	Pos	Neg
1:32	Neg	Neg	Pos	Neg	Neg	Neg	Pos	Neg
1:100	Neg	Neg	Pos	Neg	Neg	Neg	Pos	Neg
1:500	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1:1000	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Sample 1	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Sample 2	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Sample 3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Sample 5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Table 1. Comparison of Hs27 ReadyCells in Shell Vial format and Fresh Vero Cells for the detection of *C. difficile* cytotoxin with and without Toxin neutralisation (source: Diagnostic Hybrids).

an ideal confirmatory test for any screening assay, as it offers the sensitivity of a bioassay: 1 pg of toxin B causes rounding of the cells to indicate a positive reaction (3). Alternatively Hs27 FreshCells can be used to provide a sensitive screening assay. The use of Hs27 cells offers simple cytotoxin assay that can be integrated into any microbiology laboratory, regardless of whether they currently perform cell culture or have cell culture capabilities.

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Hs27 ReadyCells and Hs27 FreshCells are Trademarks of Diagnostic Hybrids, Athens, USA.

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