Diagnostic Microbiology and Infectious Disease 75 (2013) 42–45

Contents lists available at SciVerse ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Comparison of a frozen human foreskin fibroblast cell assay to an enzyme immunoassay and toxigenic culture for the detection of toxigenic *Clostridium difficile*☆,☆☆,★

Alastair J. Strachan [a,*], Natalie E. Evans [a], O. Martin Williams [b], Robert C. Spencer [b], Rosemary Greenwood [c], Chris J. Probert [d]

[a] *Department of Clinical Sciences, University of Bristol, Bristol, UK*
[b] *Health Protection Agency Microbiology Services, Bristol, Bristol, UK*
[c] *Research Design Service–South West, University Hospital Bristol NHS Foundation Trust, Bristol, UK*
[d] *Department of Gastroenterology, University of Liverpool, Liverpool, UK*

ARTICLE INFO

Article history:
Received 19 June 2012
Received in revised form 12 September 2012
Accepted 20 September 2012
Available online 26 October 2012

Keywords:
Clostridium difficile
Enzyme immunoassay
Toxigenic culture
Cytotoxin

ABSTRACT

This study set out to validate the Hs27 ReadyCell assay (RCCNA) as an alternative CCNA method compared against a commonly used commercial enzyme immunoassay (EIA) method and toxigenic culture (TC) reference standard. A total of 860 samples were identified from those submitted to the Health Protection Agency microbiology laboratories over a 30-week period. RCCNA performed much better than EIA when using TC as a gold standard, with sensitivities of 90.8% versus 78.6% and positive predictive value of 87.3% to 81.9%, respectively. The Hs27 Human Foreskin Fibroblast ReadyCells are an easy-to-use and a sensitive CCNA method for the detection of toxigenic *Clostridium difficile* directly from stool. A turnaround time of up to 48 h for a negative result and possible need for repeat testing make it an unsuitable method to be used in most clinical laboratory setting.

1. Introduction

Clostridium difficile is the most common cause of nosocomial colitis, with symptoms ranging from asymptomatic carriage to severe diarrhoea, pseudomembranous colitis, toxic megacolon, and death (Williams and Spencer, 2009). These symptoms are the result of the toxins excreted by the bacteria; non–toxin-producing strains of the bacteria are nonpathogenic (Williams and Spencer, 2009). A fast and accurate method for the diagnosis of the infection is required to improve patient care and reduce the risk of transmission. Since 2007, the prevalence of *C. difficile* infection (CDI) has decreased in the UK (Health Protection Agency, 2011), although it is still rising in other countries (Crobach et al., 2009).

The choice of laboratory test can have a significant impact on the accuracy of a *C. difficile* diagnosis (Crobach et al., 2009; Carroll, 2011; Planche and Wilcox, 2011). Cell cytotoxin neutralisation assays (CCNA) are recommended as the gold standard (GS) for detecting *C. difficile* toxin B in a laboratory environment (Crobach et al., 2009; Carroll, 2011; Planche and Wilcox, 2011), but the drawbacks of using this method including the 48-h turnaround time, cell line maintenance, and technical expertise have led to many laboratories choosing enzyme immunoassays (EIA) as their diagnostic method; EIA have a shorter turnaround times and cost less than CCNA. EIAs are commonly used to detect toxins A and B, but it has been reported that their ability to accurately diagnose a toxigenic *C. difficile* infection can be substandard (Carroll, 2011).

A new commercial method of cytotoxin testing using Hs27 Human Foreskin Fibroblast (HFF) ReadyCells (Diagnostic Hybrids, Athens, OH, USA) and requiring no cell line maintenance was recently introduced to overcome the problems of the EIA and previous CCNA testing methods. These cells are an alternative to the commonly used Vero cells whose performance has been well documented (Eastwood et al., 2009; Novak Weekley et al., 2010). Although the merits of CCNA testing for *C. difficile* diagnosis are also known, there is little published experience of the new method. A recent review highlighted the availability of commercially available frozen HFF cells but noted their use requires validation (Planche and Wilcox, 2011).

☆ The authors have no conflicts of interest and have made a significant contribution to the production of this manuscript.

☆☆ Alastair Strachan and Natalie Evans contributed to the design and drafting of the manuscript. Rosemary Greenwood contributed to the statistical analysis. Martin Williams and Chris Probert contributed to the study design and were involved in reviewing the manuscript. Robert Spencer was involved in the reviewing of the manuscript.

★ Address where research was conducted: Health Protection Agency Microbiology Services, Level 8, Bristol Royal Infirmary, Marlborough Street, Bristol, BS2 8HW.

* Corresponding author. Tel.: +44-117-3422514; fax: +44-117-3311833.
E-mail address: ally.strachan@bristol.ac.uk (A.J. Strachan).

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http://dx.doi.org/10.1016/j.diagmicrobio.2012.09.013

The aim of this study was to assess Hs27 ReadyCell assay (RCCNA) as an alternative CCNA method and to compare their diagnostic capability for toxigenic *C. difficile* against a commonly used commercial EIA method and toxigenic culture (TC) reference standard.

2. Materials and methods

Routine clinical samples sent to the laboratory were tested for *C. difficile* if they matched stool form types 5 to 7 on the Bristol Stool Scale (Lewis and Heaton, 1997) and met any of the following patient criteria: aged ≥65 years, taking or had recently taken antibiotics, a hospital inpatient, immunosuppressed, requested by the patient's clinician. From those who met these criteria, samples were selected that were fresh (<24 h since being collected), >5 mL in volume, from patients aged ≥18 years old who had diarrhoea for >24 h.

2.1. Enzyme immunoassay

The Premier *C. difficile* Toxin A & B microwell EIA (Meridian Bioscience, Cincinnati, OH, USA) was used in accordance with the Health Protection Agency (HPA) standard operating procedures on the DS2 analyser (Launch Diagnostics, Kent, UK) by HPA staff. Optical densities (OD) were determined using the manufacturer's guidelines at 450 and 630 nm; a positive result was determined by an OD ≥0.1 and a negative result by an OD <0.1.

2.2. Cell cytotoxin neutralization assay

Human foreskin fibroblast Hs27 ReadyCells (Diagnostic Hybrids) were used for the CCNA. One millilitre of stool was frozen on receipt and testing performed in batches. Samples were defrosted and added to 3 mL of specimen diluent (dilution 1:4) and centrifuged at 3500 × *g* for 10 min. The supernatant was removed and passed through a 0.45-micron sterile syringe filter (Whatman, Dassel, Germany). Two sterile 1.5-mL Eppendorf tubes were prepared for each sample, 1 containing 0.2 mL of specimen diluent, the other 0.2 mL of antitoxin control, with 0.2 mL specimen filtrate added to both (dilution 1:8) and left to incubate at room temperature for 30 min. The HFF ReadyCells were removed from storage at −70 °C and defrosted in the ReadyCell heat block (Diagnostic Hybrids) for 4 min. The cells' maintenance media was removed and 0.8 mL of fresh Refeed Medium (Diagnostic Hybrids, Athens, OH, USA) added to each cell vial. With a sterile pipette tip, 0.2 mL of the specimen filtrate and 0.2 mL of the antitoxin control solution were added to separate RCCNA vials. All vials were incubated at 37 °C with 5% CO_2 for a maximum of 48 h. Cell lines were examined at 24 and 48 h of incubation using an inverted microscope, ×10 magnification, for signs of cytopathic effect (CPE). A positive result was defined by ≥50% cell lysis with no evidence of cytotoxicity in the relevant antitoxin control vial.

2.2.1. Repeat samples

Samples where no clear result could be determined (both specimen and control vial displaying CPE, destruction of cell monolayer) were repeated with titrations of 1:8, 1:16, and 1:32 added to separate RCCNA vials and incubated as above.

2.3. Toxigenic culture

All stool samples were processed for TC according to a protocol modified from the one set out by Eastwood et al. (2009). 0.5 mL of stool was added to 0.5 mL of industrial methylated spirit or at a ratio of 1:1 and left to 'shock' at room temperature for 30 min. One loop full of the shock was inoculated onto Brazier's agar (Oxoid, Cambridge, UK) and incubated in anaerobic conditions for 48 h. Suspected *C. difficile* colonies were inoculated onto fastidious anaerobic agar (Oxoid) and reincubated for a further 48 h. Positive *C. difficile* culture was determined by meeting all the following: yellow/green colonies under UV fluorescence, a positive latex agglutination (Microgen Bioproducts, Camberley, UK), and the characteristic horse barn odour. Positive *C. difficile* cultures were run on the DS2 analyser for CDT EIA testing as outlined above.

2.4. Statistical analysis

Statistical analysis was performed using the IBM SPSS 19 software package (SPSS, Chicago, IL, USA) to provide kappa values and 95% confidence intervals. Sensitivities, specificities, positive predictive values (PPVs), and negative predictive values (NPVs) were also derived for each test method.

3. Results

A total of 860 samples were identified from those submitted to the HPA microbiology laboratories from 2 hospital trusts (University Hospital Bristol NHS Foundation Trust and Royal United Hospital Bath) over a 30-week period. The prevalence of *C. difficile* amongst these cases of diarrhoea was 10.9% with EIA, 11.4% with TC, and 11.9% with CCNA.

The results for comparing the 3 toxigenic CDI diagnosis methods are presented in Table 1. The EIA and RCCNA were compared against one another using the TC as the gold standard (GS) reference method to compare and evaluate their results. RCCNA performed much better than EIA when using TC as a GS, with sensitivities of 90.8% (CI 83.3–95.7) and 78.6% (CI 69.1–81.2), respectively. There was only a slight difference in specificity with 98.3% (CI 97.1–99.0) for the RCCNA, only slightly higher than 97.8% (CI 96.5–98.7). RCCNA was better at identifying a true-negative result with a NPV of 98.8% (CI 97.8–99.5). The false-negative rate was 9.2% (4.3–16.7) for RCCNA, compared to a much higher 21.4% [13.8-30.9] for EIA. The EIA false-positive rate of 2.2% (1.3–3.5) was only 0.5% higher than the recorded level of 1.7% (0.9–2.9) for the RCCNA. PPVs were higher for the RCCNA (87.3% [79.2–93.0]) than for the EIA (81.9% [72.6–89.0]).

Kappa values for EIA and RCCNA were 0.777 and 0.876, respectively, showing a higher level of repeatability for the RCCNA when TC is used as GS. When the reliability of each method was evaluated, no statistical significance could be found between EIA and TC ($P = 0.627$), EIA and RCCNA (0.200), and RCCNA against TC (0.523).

A total of 3.3% (28/860) of RCCNA samples included in the study were repeated. Eight-two percent (23/28) were repeated due to 'bunching' of the cell monolayer, and a further 18% (5/28) showed CPE in both vials. Samples which needed repeating incurred a further cost of approx £27.00 per sample.

Fifteen percent of positive *C. difficile* cultures were negative on the EIA component of the TC algorithm and therefore resulted as negative for TC. It is possible that these cultures contained nontoxigenic *C. difficile* strains or that the Toxin A & B EIA result was a false negative.

4. Discussion

There is still much debate regarding the best method for the diagnosis of toxigenic *C. difficile*, with the need for an accurate and relevant diagnosis often compromised by the requirement of a rapid result. A key advantage of the CCNA test method has been its sensitivity to detect small quantities (1 pg) of *C. difficile* toxin (Lyerly et al., 1988). Due to factors such as reductions in available technical expertise, costs, and cell line maintenance, CCNA methods have not been adopted by most clinical laboratories for the diagnosis of the disease. A recent survey found that only 3.6% of English National Health Service laboratories used the CCNA method for the detection of CDI (Goldenberg and French, 2011).

The RCCNA is a new commercially available method of cytotoxin testing, designed to test directly from stool and overcome the