Diagnosis of *Clostridium difficile*-associated disease: examination of multiple algorithms using toxin EIA, glutamate dehydrogenase EIA and loop-mediated isothermal amplification

A. I. BAMBER*, K. FITZSIMMONS*, J. G. CUNNIFFE*, C. C. BEASOR*, C. A. MACKINTOSH* and G. HOBBS†

Medical Microbiology, Wirral University Teaching Hospitals, Clatterbridge Hospital, Bebington, Wirral; and †Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, UK

Accepted: 19 June 2012

ABSTRACT

The laboratory diagnosis of *Clostridium difficile* infection (CDI) needs to be accurate and timely to ensure optimal patient management, infection control and reliable surveillance. Three methods are evaluated using 810 consecutive stool samples against toxigenic culture: CDT TOX A/B Premier enzyme immunoassay (EIA) kit (Meridian Bioscience, Europe), Premier EIA for *C. difficile* glutamate dehydrogenase (GDH) (Meridian Bioscience, Europe) and the Illumigene kit (Meridian Bioscience, Europe), both individually and within combined testing algorithms. The study revealed that the CDT TOX A/B Premier EIA gave rise to false-positive and false-negative results and demonstrated poor sensitivity (56.47%), compared to Premier EIA for *C. difficile* GDH (97.65%), suggesting this GDH EIA can be a useful negative screening method. Results for the Illumigene assay alone showed sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) of 91.57%, 98.07%, 99.03% and 84.44%, respectively. A two-stage algorithm using Premier EIA for *C. difficile* GDH/Illumigene assay yielded superior results compared with other testing algorithms (91.57%, 98.07%, 99.03% and 84.44%, respectively), mirroring the Illumigene performance. However, Illumigene is approximately half the cost of current polymerase chain reaction (PCR) methods, has a rapid turnaround time and requires no specialised skill base, making it an attractive alternative to assays such as the Xpert *C. difficile* assay (Cepheid, Sunnyvale, CA). A three-stage algorithm offered no improvement and would hamper workflow.

KEY WORDS: Clostridium difficile.
Glutamate dehydrogenase.
Illumigene.
Loop-mediated isothermal amplification

Introduction

Clostridium difficile is a major nosocomial pathogen resulting in high rates of morbidity and mortality.[1–3] Exposure to the organism may lead to asymptomatic carriage in some patients,[4] yet in others symptoms can be wide ranging from mild watery diarrhoea to life-threatening pseudo-membranous colitis (PMC), and/or toxic megacolon.[5] Incidence and severity of *C. difficile* infection (CDI) has progressively increased worldwide,[6–8] and is associated with high rates of morbidity and mortality.[9–13] Risk factors include broad-spectrum antibiotic treatment,[14] proton pump inhibitors,[15] surgery and age over 65 years.[16,17] This increase may be due in part to the emergence of the hypervirulent 027 ribotype causing outbreaks in North America and Europe.[18–25]

The cost of patient care and management increases significantly with CDI,[5,11,26,27] as it is healthcare-associated whether due to direct exposure of a vulnerable patient to spores[28,29] or through eradication of normal gastrointestinal (GI) flora via antibiotics or surgical manipulation, enabling endogenous *C. difficile* to proliferate. Early and accurate diagnosis is therefore critical for the care of patients and others around them.

In order for disease to occur, toxin production by *C. difficile* is essential. Two major toxins, toxin A (tcdA) and toxin B (tcdB), are now recognised as *C. difficile* virulence factors.[30,31] Some *C. difficile* strains produce a third unrelated toxin (binary toxin CDT),[32] although its significance is unclear.[33]

Laboratory diagnosis of *C. difficile* has depended on the demonstration of tcdA or tcdB,[34] and there are various tests available.[35] Diagnosis balances accuracy, sensitivity and specificity with cost. The optimal screening system is now the subject of considerable debate. The technically demanding stool-cytotoxin test assays have been replaced by less labour-intensive, faster toxin enzyme immunoassays (EIA) as tissue culture assay is time-consuming, requires expertise and dedicated facilities.[3,11,36] However, studies have demonstrated reduced sensitivity in low-incidence test populations and poor sensitivity (65–85%) and specificity (95–100%),[37–39] giving rise to false-positive and false-negative results.[40] Repeat testing may be required to establish a diagnosis of CDI.[16,40–42]

Correspondence to Katie Fitzsimmons
Email: kfitzsimmons@nhs.net

Table 1. Specimens tested for *Clostridium difficile*.

Bristol Stool Chart criteria types 6 and 7 (liquid, unformed or mucoid, in the absence of laxative treatment) PLUS one or more of the criteria on the right:	• Requested by GP/ward/infection control
	• Specimens from post-operative patients
	• Specimens from patients previously treated with antibiotics
	• Visibly bloodstained faeces
	• Microscopic presence of pus cells and culture-negative (*Salmonella* sp. *Shigella* sp, *Campylobacter* sp. *E. coli* O157)
	• Specimens from patients with Crohn's disease or other inflammatory bowel disease
	• Specimens from patients 65 years and over

The NHS Centre for Evidence-based Purchasing (CEP) accompanied these findings with recommendation that such assays should not be used in isolation.[43] Current recommendations advise a two-step algorithm.[43–45]

Recently, there has been much interest in glutamate dehydrogenase (GDH) assays and molecular tests.[45–47] The former is a good negative screening method for *C. difficile*.[40] Shetty *et al*.[38] undertook a meta-analysis of 13 GDH studies, concluding that it should be used as part of a diagnostic algorithm. Swindles *et al*.[47] proposed the use of the GDH component of C. DIFF Quik Check Complete (Techlab, Blacksburg, VA) together with Gene Xpert (Cepheid, Sunnyvale, CA) as a confirmatory test based on an evaluation of 150 samples. A similar conclusion was reached by Goldenberg *et al*.[22] when examining a two-step GDH antigen real-time polymerase chain reaction (PCR) assay for detection of toxigenic *C. difficile*.

Use of PCR methods for detection of *tcdA* and *tcdB* genes offer improved sensitivity (92–97%) and specificity (100%) compared with tissue culture assay.[3] Although rapid and sensitive,[48] their application has been limited.[49] Goldenberg *et al*.[22] recommended BD GeneOhm *C. difficile* real-time PCR (BD Diagnostics, San Diego, CA) in combination with C. Diff Check Complete (Techlab, Blacksburg, VA) as a two-step algorithm. The Cepheid Xpert *C. difficile* assay (Xpert *C. difficile*) is a PCR method developed for ease-of use. Novak-Weekley *et al*.[46] demonstrated that this system yielded the highest sensitivity compared to multiple test algorithms evaluated in the study. Cost prohibits PCR as a primary test in most UK clinical laboratories and its value as a screening test is questionable.[22]

Non-PCR-based gene amplification techniques have been developed, including loop-mediated isothermal amplification (LAMP).[49–51,53] This method amplifies DNA under isothermal conditions with high sensitivity, specificity and simplicity.[48] The Meridian Illumigene system (Meridian Bioscience, Europe) has been developed for the detection of *C. difficile* toxins tcdA and tcdB. The assay detects the pathogenicity locus (PaLoc) of toxigenic *C. difficile*. The procedure is simple and rapid, enabling amplification to take place within one hour. Cost is significantly less than some available PCR methods.

There is a clear need for more sensitive and specific tests for the diagnosis of CDI. There is also a requirement for a sensitive screening method and a specific confirmatory test that is both easy to undertake and affordable.

The aim of the present study is to compare the sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV) and accuracy of three methods (toxin EIA, GDH EIA and Illumigene assay) for their ability to detect *C. difficile* toxins or gene promotors in the faeces of patients with diarrhoea, both individually and within specific testing algorithms, using toxigenic culture as the 'gold standard' for a positive specimen. The main objective is to develop a new diagnostic algorithm for the diagnosis of CDI, evaluating a new GDH EIA developed by Meridian Bioscience Inc (Cincinnati, OH) and Illumigene LAMP technology.

Materials and methods

Study population and sample collection

This was a prospective study conducted at Wirral University Teaching Hospital (WUTH), Department of Medical Microbiology. Predetermined inclusion criteria according to departmental standard practices were used to identify faecal samples requiring *C. difficile* testing (Table 1). The EIA and GDH EIA were performed on 811 consecutive samples meeting the above criteria. Duplicate specimens from the same patient and from patients under the age of two were excluded.

Any specimens positive for *C. difficile* by EIA and GDH EIA were further tested by the Illumigene system. Toxigenic culture (TC; culture and isolation of *C. difficile* from faeces followed by toxin testing of the actual isolate)[38] was performed on faecal specimens positive by any of the three aforementioned methods. All faecal specimens were routinely cultured for the presence of *C. difficile*.

Enzyme immunoassay

Toxin testing was performed using the Premier Toxin A&B microwell EIA assay (Meridian Bioscience, Cincinnati, OH). The assay was performed according to the manufacturer's instructions. Positive results for Premier Toxins A&B are indicated by values of ≥0.1 at 450–630 nm. All faecal specimens requiring *C. difficile* testing were tested by this method.

Glutamate dehydrogenase EIA

A new and unmarketed GDH EIA (Meridian Bioscience, Cincinnati, OH), supplied by Launch Diagnostics (Kent, England), was evaluated. Testing was performed according to the manufacturer's instructions using the Dynex DS2 automated EIA system (Dynex Technologies, West Sussex, England). Positive results for GDH antigen are indicated by values ≥0.1 at 450/630 nm. All faecal samples requiring *C. difficile* testing were tested by this method.

Illumigene isothermal loop amplification
The Illumigene assay was used to confirm positive samples by any of the methods above. The Illumigene *C. difficile* assay based on LAMP methodology (Meridian Bioscience, Europe) was performed according to the manufacturer's instructions. Due to overall cost limitations, Illumigene was only undertaken on stool samples positive by any other method. An assumption was made that the Illumigene would be negative if all other tests were also negative. This did not examine the potential for the generation of false-positives from primary specimens, and would affect its reliability (specificity, NPV, PPV).

Culture of **C. difficile**
Bacterial cultures were performed routinely and blinded to other test results. All faecal specimens were inoculated into a Robertson's cooked meat broth overlaid with brain-heart infusion broth (E+O Laboratories, Perth UK). The broths were incubated anaerobically at 36°C for 24 h. Broths were then subcultured to Brazier's *Clostridium difficile* selective agar medium (PB1055A, Oxoid, Basingstoke, UK) and incubated anaerobically at 36°C for 48 h. Isolates were identified as *C. difficile* by:

- colony morphology
- horse manure-like smell
- yellow-green/chartreuse fluorescence under UV
- Gram-positive, spore-forming bacillus
- *C. difficile* latex agglutination test (Oxoid, Basingstoke, UK).

Isolates conforming to the aforementioned criteria were subcultured onto Fastidious Anaerobe Agar (FAA) supplemented with 7% (v/v) horse blood to obtain pure cultures for isolate storage on microbank beads (–70°C).

Toxigenic culture
Toxigenic culture was performed by taking three picks of single colonies (all manipulations took place in an anaerobic atmosphere). Isolates were inoculated into Robertson's cooked meat broth and incubated at 36°C for 48 h. The supernatant was then tested using the Premier Toxin A+B microwell EIA assay (Meridian Bioscience, Europe) according to the manufacturer's recommendations (as liquid sample volume). This technique was deemed to be the 'gold standard', as advocated by Schmidt and Gilligan.[54]

Analysis
A sample was considered to contain toxigenic *C. difficile* if toxigenic culture was positive. Performance characteristics were to be calculated for the Toxin A+B EIA, GDH EIA and Illumigene assay relative to the toxigenic culture. Accuracy is defined as the percentage overall agreement between the two tests or algorithms being compared (i.e., the total number of test positives divided by the number of gold-standard positives x100).

Performance was assessed by combining the results of GDH EIA and Toxin A+B EIA versus toxigenic culture; GDH EIA and Illumigene versus toxigenic culture; and Toxin A+B EIA and Illumigene versus toxigenic culture. As previously discussed, test results for Illumigene were assumed to be negative if all other tests yielded a negative result.

Statistical methods
Sensitivity and specificity were calculated, together with percentage false negatives and percentage false positives compared to the gold standard. To analyse the different methods/algorithms, the χ^2 test was used.

Results

Table 2 shows the statistical results. For combinations, the first test listed is the screen, and the second is the confirmatory test. In all cases, individual tests or combinations were compared to toxigenic culture

Table 3 summarises the results of the χ^2 (P value) test calculated for the different testing algorithms. A three-step algorithm (Premier Toxin EIA/ Premier GDH EIA/Illumigene) yielded P values that were statistically significant for specificity, accuracy and PPV. There was no statistical significance for the sensitivity results of GDH EIA/Illumigene (P=0.08), which was surprising given the large gap in the sensitivity values obtained for Premier Toxin EIA /Illumigene and Premier GDH EIA/Premier Toxin EIA algorithms, although this may be due to the relatively small number of positive samples in the study.

Table 2. Tests compared to toxigenic culture.

	Launch Premier Toxin A+B EIA	Launch Premier GDH EIA	Illumigene	Launch Premier GDH EIA + Launch Premier Toxin A+B EIA	Launch Premier Toxin A+B EIA + Illumigene	Launch Premier GDH EIA + Illumigene	Launch Premier GDH EIA + Illumigene + Launch Premier Toxin A+B EIA
	1	2	3	4	5	6	7
Number of specimens	810	810	808	810	808	808	808
Sensitivity	56.47	97.65	91.57	56.47	55.42	91.57	93.98
Specificity	95.45	94.90	98.07	99.17	99.59	98.07	97.66
Accuracy	91.36	95.19	97.40	94.69	95.05	97.40	97.28
NPV	94.92	99.71	99.03	95.11	95.13	99.03	99.30
PPV	59.26	69.17	84.44	88.89	93.98	84.44	82.11

Table 3. Results of χ^2 testing (*P* values).

	Sensitivity	Specificity	Accuracy	PPV	NPV
1 *vs.* 2	<0.0001	0.6241	0.0021	0.1481	<0.0001
1 *vs.* 3	<0.0001	0.0048	<0.0001	0.0002	<0.0001
1 *vs.* 4	1	<0.0001	0.0085	0.0002	0.8725
1 *vs.* 5	0.8911	<0.0001	0.0032	0.0001	0.8588
1 *vs.* 6	<0.0001	0.0048	<0.0001	0.0002	<0.0001
1 *vs.* 7	<0.0001	0.0213	<0.0001	0.0008	<0.0001
4 *vs.* 6	<0.0001	0.0717	0.0131	0.1049	<0.0001

The CDT TOX A/B Premier EIA kit yielded 26 positive results that were negative by all other tests, and 35 toxin-negative results that were positive by all other tests. The number of reported positives during this study would therefore have increased by nine (1.1%). This finding supports reported observations regarding the reduced sensitivity and specificity of toxin EIA tests.[39,41,43]

Discussion

Data on CDI are often collated and publicised as a surrogate marker of hospital cleanliness and infection control performance. Nationally, targets are set and trusts must keep within these or face criticism and financial penalty.[53] Performance may affect litigation premiums. Whichever testing approach is chosen, the optimum detection of CDI must be balanced against cost. While EIA toxin testing is widely used, a Centre for Evidence- based Purchasing (CEP) evaluation of kits recommended that these kits should not be used in isolation.[41] More recently, a UK multicentre CDI diagnosis trial has recommended a multistep approach.[43] Whichever diagnostic algorithm is chosen in a diagnostic laboratory, all CDI toxin-positive results must be reported to the mandatory surveillance system.[43]

An accurate diagnosis has several clinical implications; if CDI is missed, patients will not be treated appropriately, and despite universal precautions for patients with diarrhoea, it is likely that these patients will disseminate more spores into the environment, putting other patients at risk.[55] If a false-positive diagnosis of CDI is made, patients will undergo unnecessary and inappropriate antibiotic therapy, which is costly and may place them at higher risk of CDI in the future.[1]

The results of CEP evaluation are consistent with those of a recently published review of other studies that have examined the performance of *C. difficile* toxin detection kits.[56] It is apparent from the available published data on the performance of commercial toxin detection kits that there are significant differences in the sensitivity and specificity of these kits. In the CEP evaluation, no single assay was superior in terms of both sensitivity and specificity. However, five assays (Remel Xpect, Techlab Tox A/B Quik Chek, Premier Toxin A + B, Vidas *C. difficile* Toxin A & B and Techlab Toxin A/B II) appear to be superior to the other four. However, more sensitive tests tend to be less specific, and vice versa.[45] Current recommendations advise a two-step algorithm.[43] Good screening tests should have a high sensitivity and NPV, particularly when there is a low prevalence of the target pathogen, but not at the expense of creating too many false-positive results.

In the present study, 12.5% of samples were culture-positive and 10.5% were toxigenic culture-positive. These prevalence figures are similar, but the test population was drawn from patients who were suspected of having disease (risk factors, symptoms, unformed/liquid stools, no prior laxatives, and were selected by the criteria laid down in Table 1). Therefore, with a sensitivity of 56.5%, CDT EIA, the existing diagnostic test at the time of the study, makes an inappropriate primary screen.

The GDH EIA had a 97.6% NPV and 99.7% sensitivity, making it an ideal screening test. Specificity is potentially compromised as GDH is not linked to toxin or toxin genes. Compared to the Toxin A+B EIA test, GDH EIA had significantly better sensitivity ($P<0.001$), NPV ($P<0.001$) and accuracy ($P=0.0019$), supporting the recommendation by Shetty *et al.* that it should be used as part of a diagnostic algorithm.[38]

Confirmatory tests should have high specificity and PPV. In isolation, Illumigene was significantly better than Toxin EIA when measured against any parameter ($P<0.001$–0.0048), and with high sensitivity and specificity subject to the design limitations described. It could be used as a single-stage diagnostic test if cost was not an issue.

Combined testing algorithms

With a sensitivity of 56.5%, Launch Premier Toxin A+B CDT EIA falls short of the requirement for a reliable screening test in any combined testing algorithm. When Illumigene was used to confirm CDT, this yielded 99.2% specificity and 94.7% PPV, but a sensitivity of just 57.8% (i.e., many positives would be missed, but those that are confirmed are likely to be true positives). If CDT EIA was combined with GDH, a significant number of positive samples would not be confirmed, and the combined sensitivity (56.5%) falls far below that of GDH in isolation (97.7%).

Novak-Weekley *et al.*[46] concluded that applying GDH EIA as a screening test supported by a PCR confirmatory test produced a robust, cost-effective two-stage algorithm. Swindles *et al.* proposed the use of the GDH component of C. DIFF Quik Check Complete, together with Xpert as a confirmatory test.[47] However, the Cepheid GeneXpert has significant cost associated for the instrument and test cartridges. Illumigene costs are approximately half those of GeneXpert and performance is similar in this evaluation (Illumigene/Xpert sensitivity 91.6/94.4%, specificity 98.1/96.3%, PPV 84.4/84%, NPV 99.0/98.8%). Illumigene failed internal control on just two out of 153 assays (1.3%),

giving it a slight advantage over the GeneXpert. When used in combination with GDH EIA as a screening test as part of a two-stage algorithm, results were also comparable (Premier GDH+Illumigene/Techlab GDH+Xpert sensitivity 91.6/86.1%, specificity 98.1/97.8%, PPV 84.4/88.6%, NPV 99.0/97.2%).[46] Illimigene assay kits are half the price of GeneXpert kits and the equipment is significantly less. However, *C. difficile* assay is currently the only test for this platform and therefore does not offer the versatility of GeneXpert or other systems.

Using Launch Premier GDH EIA as a screen and confirming with Illumigene offered improvement in sensitivity, accuracy and NPV over a combination of Launch Premier GDH EIA and Toxin A+B EIA.

Introducing a three-stage algorithm using Launch Premier GDH EIA as a screen and confirming with Illumigene and Launch Premier Toxin A+B EIA does not increase the PPV and would add more time and expense to diagnosis.

Limitations of this study

Cost prohibited the testing of all samples using Illumigene, therefore an assumption was made that if culture and all other methods were negative then Illumigene would be negative. This assumption would affect reliability (specificity, PPV, NPV). This meant that false-positive tests that might have occurred were not detected, potentially skewing the data.

Limitations of combined algorithm

A non-specific clostridial product, GHD is not linked to toxin production. Illumigene detects the toxin B gene and does not quantify toxin. Therefore, it could be argued that it confirms only the potential for disease. However, when compared to toxigenic culture, the combination figures above show good correlation. Inflammatory markers such as lactoferrin have been used as part of a more complex algorithm to diagnose disease, but additional tests add further cost and ultimately CDAD must be a clinical diagnosis. Identifying patients with toxigenic *C. difficile* may, however, prevent spread to other vulnerable patients.

Clinical review

The aforementioned lack of sensitivity with the Toxin A+B EIA resulted in false-negative test results on 35 samples from 31 patients over the study period. A brief review was undertaken of these cases:

- nine patients had evidence of colitis
- seven patients had repeated stool samples submitted
- nine patients died within two months of the false-negative result (four patients within five days)
- five patients were in 'high risk' clinical areas (critical care/haematology/oncology)
- five patients had a previous positive CDT on record
- three patients subsequently had a positive result reported via the standard CDT Toxin A+B testing protocol.

These findings need to be interpreted with caution; a full case note review would be more robust but there was insufficient resource. However, it does at least provide some evidence that the laboratory protocol of using Toxin A+B EIA has a detrimental clinical impact.

Clinical considerations

Launch Premier Toxin A+B EIA yielded 26 positive samples that were negative by all other test, and 35 toxin-negative samples positive by all other tests. The number of reported positives during this study would have increased by nine (1.1%), which might cause concern in relation to the aforementioned target figures. However, the correct patients would have been identified for treatment and isolation, and 26 would not have received inappropriate therapy. Moreover, by correctly identifying patients with disease, a short-term rise in reporting rates of CDI can be offset with the application of better control and prevention measures, limiting the potential of spread within healthcare units and, theoretically, reducing CDI in the long term.[57] Testing costs must be balanced against the overall financial burden of CDI.[58]

GeneXpert is noted for its ease of use.[46] Illumigene yielded a confirmatory result within one hour. The preparation and hands-on time was less than 5 min per sample. Assay users require no special skills, knowledge or experience, and can be trained on a single run, making this test applicable for laboratory support staff to undertake.

Like Gene Xpert, Illumigene provides accurate results, yet in the UK cost is likely to exclude its use as a single-algorithm diagnostic test. The GDH EIA test costs are comparable to toxin EIA although users may pay a slight premium for this new technology. The increase in cost of this two-stage algorithm is therefore the price of the additional Illumigene test. This must be offset against the benefits in prompt diagnosis, treatment, prevention and control, as previously discussed. However, combining GHD with Illumigene does little to reduce the overall performance, while making screening/confirmation more cost-effective than using Illumigene in isolation. A three-stage algorithm offered no benefit and increased cost.

Conclusions

Laboratory diagnosis of CDI depends on the detection of the toxins tcdA and/or tcdB, for which there are numerous diagnostic tests available. Current recommendations advise a two-step algorithm. An appropriate algorithm should combine a screening test with high sensitivity and NPV, followed by a confirmatory test with high specificity, while not adversely affecting the sensitivity of the primary test. An evaluation of three methods for the diagnosis of CDI, both as standalone tests and in combined algorithms, was conducted in this study.

The results confirm the CEP guidance; testing based solely on the detection of *C. difficile* toxin by an EIA would result in a significant number of false negatives. Additionally, toxin EIA makes an inappropriate primary screen in a two-stage algorithm. However, GDH EIA makes an excellent screening test when used as part of a diagnostic algorithm to improve specificity.

When deciding on the best combination, factors to consider are performance, labour, turnaround times, prevalence of *C. difficile* in the patient population, and cost. The Illumigene is approximately half the cost of some current PCR methods, has a rapid turnaround time and requires no specialised skill base, making it an attractive alternative to PCR assays such as the Xpert *C. difficile* assay.

The statistical parameters for Illumigene were identical to those of Illumigene/GDH combined, yet GDH EIA is a fraction of the cost of Illumigene, reaffirming it as a cost-effective screen. The combination of GDH EIA (screen) and Illumigene (confirmatory test) as a two-stage algorithm yielded sensitive and specific results that were comparable to GDH EIA-PCR algorithms. This combination will still produce false positives but it gives less discrepant results between the negative screen and confirmatory test if an EIA toxin detection method is used as confirmation. These results are also achievable within a reasonable time frame.

Detecting the presence of *C. difficile* or production of *C.difficile* toxin is not the same as confirming disease. There is no definitive laboratory test to confirm CDI. Whatever combination of tests is applied, the users must be aware of the limitations of the algorithm in use and must always interpret the results in the context of clinical presentation. In particular, appropriateness of testing, infective markers and other underlying causes of diarrhoea must be taken into account. □

None of the authors has any interests/conflicts of interests relating to commercial products named is this article. There was no incentive to produce this report.

References

1 Bartlett JG. Narrative review: the new epidemic of *Clostridium difficile*-associated enteric disease. *Ann Intern Med* 2006; **45** (10): 758–64.

2 Cookson B. Hypervirulent strains of *Clostridium difficile*. *Postgrad Med J* 2007; **83** (979): 291–5.

3 Poutanen SM, Simor AE. *Clostridium difficile*-associated diarrhea in adults. *CMAJ* 2004; **171** (1): 51–8.

4 Kyne L, Warny M, Qamar A, Kelly CP. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. *N Engl J Med* 2000; **342** (6): 390–7.

5 Wilcox MH. Gastrointestinal disorders and the critically ill. *Clostridium difficile* infection and pseudomembranous colitis. *Best Pract Res Clin Gastroenterol* 2003; **17** (3): 475–93.

6 Debast SB, Vaessen N, Choudry A, Wiegers LEAJ, Van den Berg RJ, Kuijper EJ. Successful combat of an outbreak due to *Clostridium difficile* PCR ribotype 027 and recognition of specific risk factors. *Clin Microbiol Infect* 2009; **15** (5): 427–34.

7 Jamal W, Rotimi VO, Brazier J, Duerden BI. Analysis of prevalence, risk factors and molecular epidemiology of *Clostridium difficile* infection in Kuwait over a 3-year period. *Anaerobe* 2010; **16** (6): 560–5.

8 Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. *Clostridium difficile* in retail ground meat, Canada. *Emerg Infect Dis* 2007; **13** (3): 485–7.

9 Dawson LF, Valiente E, Wren BW. *Clostridium difficile* – a continually evolving and problematic pathogen. *Infect Genet Evol* 2009; **9** (6): 1410–7.

10 McFarland LV, Beneda HW, Clarridge JE, Raugi GJ. Implications of the changing face of *Clostridium difficile* disease for health care practitioners. *Am J Infect Control* 2007; **35** (4): 237–53.

11 Monaghan T, Boswell T, Mahida YR. Recent advances in *Clostridium difficile*-associated disease. *Gut* 2008; **57** (6): 850–60.

12 Owens RC. *Clostridium difficile*-associated disease: an emerging threat to patient safety: insights from the Society of Infectious Diseases Pharmacists. *Pharmacotherapy* 2006; **26** (3): 299–311.

13 Planche T, Arnold A. *Clostridium difficile. Medicine* 2009; **37** (12): 641–3.

14 O'Connor KA, Kingston M, O'Donovan M, Cryan B, Twomey C, O'Mahony D. Antibiotic prescribing policy and *Clostridium difficile* diarrhoea. *QJM* 2004; **97** (7): 423–9.

15 Kim JW, Lee KL, Jeong JB *et al*. Proton pump inhibitors as a risk factor for recurrence of *Clostridium difficile*-associated diarrhea. *World J Gastroenterol* 2010; **16** (28): 3573–7.

16 Department of Health and Health Protection Agency. Clostridium difficile *infection: how to deal with the problem.* London: DH/HPA, 2008 (www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_093220).

17 Poxton IR. *Clostridium difficile. J Med Microbiol* 2008; **57** (Pt 6): 683–4.

18 Bauer MP, Notermans DW, Benthem BHB *et al*.; ECDIS Study Group. *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* 2011; **377** (9759): 63–73.

19 de Boer ED, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ. Prevalence of *Clostridium difficile* in retailed meat in The Netherlands. *Int J Food Microbiol* 2011; **144** (3): 561–4.

20 Dubberke E, Reske K, Noble-Wang J *et al.* Prevalence of *Clostridium difficile* environmental contamination and strain variability in multiple health care facilities. *Am J Infect Control* 2007; **35** (5): 315–8.

21 Garey KW, Jiang ZD, Yadav Y, Mullins B, Wong K, Dupont HL. Peripartum *Clostridium difficile*: case series and review of literature. *Am J Obstet Gynecol* 2008; **199** (4): 332–7.

22 Goldenberg SD, Cliff PR, Smith S, Milner M, French GL. Two-step glutamate dehydrogenase antigen real-time polymerase chain reaction assay for detection of toxigenic *Clostridium difficile. J Hosp Infect* 2010; **74** (1): 48–54.

23 Keel MK, Songer JG. The comparative pathology of *Clostridium difficile*-associated disease. *Vet Pathol* 2006; **43** (3): 225–40.

24 MacCannell DR, Lowie TJ, Gregson DB *et al*. Molecular analysis of *C. difficile* PCR ribotype 027 isolates from Eastern and Western Canada. *J Clin Microbiol* 2006; **44** (6): 2147–52.

25 Vinh DC. Strains and toxins of *Clostridium*. *CMAJ* 2005; **172** (3): 312–3.

26 Gilligan PH. Is a two-step glutamate dehyrogenase antigen-cytotoxicity neutralization assay algorithm superior to the Premier toxin A and B enzyme immunoassay for laboratory detection of *Clostridium difficile*? *J Clin Microbiol* 2008; **46** (4): 1523–5.

27 Johnson AP. New antibiotics for selective treatment of gastrointestinal infection caused by *Clostridium difficile*. *Expert Opin Ther Pat* 2010; **20** (10): 1389–99.

28 Bobulsky G, Al-Nassir W, Riggs M, Sethi A, Donskey C. *Clostridium difficile* skin contamination in patients with *C. difficile*-associated disease. *Clin Infect Dis* 2008; **46** (3): 447–50.

29 Burns DA, Heap JT, Minton NP. *Clostridium difficile* spore germination: an update. *Res Microbiol* 2010; **161** (9): 730–4.

30 Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* 2010; **467** (7316): 711–3.

31 Voth DE, Ballard JD. *Clostridium difficile* toxins: mechanism of action and role in disease. *Clin Microbiol Rev* 2005; **18** (2): 247–63.

32 Rupnik M. Heterogeneity of large clostridial toxins: importance of *Clostridium difficile* toxinotypes. *FEMS Microbiol Rev* 2008; **32** (3): 541–55.

33 Pelleschi ME. *Clostridium difficile*-associated disease: diagnosis, prevention, treatment, and nursing care. *Crit Care Nurse* 2008; **28** (1): 27–35.

34 Kelly CP, Pothoulakis C, LaMont JT. *Clostridium difficile* colitis. *N Engl J Med* 1994; **330** (4): 257–62.

35 Simor AE. Diagnosis, management, and prevention of *Clostridium difficile* infection in long-term care facilities: a review. *J Am Geriatr Soc* 2010; **58** (8): 1556–64.

36 Cooke RP, Collins J, Galloway A, Holland D, Trigg G. Audit of the laboratory diagnosis of *Clostridium difficile* infection in the UK. *J Hosp Infect* 2010; **75** (4): 282–6.

37 Cardona DM, Rand KH. Evaluation of repeat *Clostridium difficile* enzyme immunoassay testing. *J Clin Microbiol* 2008; **46** (11): 3686–9.

38 Shetty N, Wren MWD, Coen PG. The role of glutamate dehydrogenase for the detection of *Clostridium difficile* in faecal samples: meta-analysis. *J Hosp Infect* 2010; **77** (1): 1–6.

39 Wilkins TD, Lyerly DM. *Clostridium difficile* testing: after 20 years, still challenging. *J Clin Microbiol* 2003; **41**: 531–4.

40 Wren MWD, Sivapalan M, Kinson R, Shetty NP. Laboratory diagnosis of *Clostridium difficile* infection. An evaluation of tests for faecal toxin, glutamate dehydrogenase, lactoferrin and toxigenic culture in the diagnostic laboratory. *Br J Biomed Sci* 2009; **66** (1): 1–5.

41 Eastwood K, Else P, Charlett A, Wilcox M. Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay to cytotoxin testing and cytotoxigenic culture methods. *J Clin Microbiol* 2009; **47** (10): 3211–7.

42 Pratt RJ, Pellowe CM, Wilson JA *et al.* epic2: National evidence-based guidelines for preventing healthcare-associated infections in NHS hospitals in England. *J Hosp Infect* 2007; **65** (Suppl 1): S1–64.

43 Department of Health. *Updated guidance on the diagnosis and reporting of* Clostridium difficile. London: DH, 2012 (www.dh.gov.uk//publications).

44 Arnold A, Pope C, Bray S *et al*. Prospective assessment of two-stage testing for *Clostridium difficile. J Hosp Infect* 2010; **76** (1): 18–22.

45 Wilcox MH, Coia J, Planche T; CDI Diagnosis Working Group. Questions and answers about the laboratory diagnosis of *Clostridium difficile* infection (CDI), 2009 (www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1238055363795).

46 Novak-Weekley SM, Marlowe EM, Miller JM *et al. Clostridium difficile* testing in the clinical laboratory by use of multiple testing algorithms. *J Clin Microbiol* 2010; **48** (3): 889–93.

47 Swindells J, Brenwald N, Reading N, Oppenheim B. Evaluation of diagnostic tests for *Clostridium difficile* infection. *J Clin Microbiol* 2010; **48** (2): 606–8.

48 Kato H, Yokoyama T, Kato H, Arakawa Y. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. *J Clin Microbiol* 2005; **43** (12): 6108–12.

49 Parida M, Sannarangaiah S, Dash KP, Rao PVL, Morita K. Loop-mediated isothermal amplification (LAMP): a new generation of innovative gene amplification techniques; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol* 2008; **18** (6): 407–21.

50 Boehme CC, Nabeta P, Henostroza G *et al.* Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *J Clin Microbiol* 2007; **45** (6): 1936–40.

51 Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies – a review. *Nucleosides, Nucleotides Nucleic Acids* 2008; **27** (3): 224–43.

52 Yamazaki W, Ishibashi M, Kawahara R, Inoue K. Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Vibrio parahaemolyticus. BMC Microbiol* 2008; **8**: 163.

53 Department of Health. The Health and Social Care Act 2008: Code of Practice for the NHS on the prevention and control of healthcare-associated infections and related guidance. London: DH, 2008 (www.dh.gov.uk/en/Publicationsandstatistics/Publications/PublicationsPolicyAndGuidance/DH_081927).

54 Schmidt ML, Gilligan PH. *Clostridium difficile* testing algorithms: what is practical and feasible? *Anaerobe* 2009; **15** (6): 270–3.

55 Calfee DP. *Clostridium difficile*: a reemerging pathogen. *Geriatrics* 2008; **63** (9): 10–21.

56 Planche T, Aghaizu A, Holliman R *et al*. Diagnosis of *Clostridium difficile* infection by toxin detection kits: a systematic review. *Lancet Infect Dis* 2008; **8** (12): 777–84.

57 Adalja AA, Kellum JA. *Clostridium difficile*: moving beyond antimicrobial therapy. *Crit Care* 2010; **14** (5): 320.

58 Wilcox MH, Cunniffe JG, Trundle C, Redpath C. Financial burden of hospital-acquired *Clostridium difficile* infection. *J Hosp Infect* 1996; **34** (1): 23–30.