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Identification of *Clostridium difficile*: evaluation of genotypic, phenotypic and proteomic methods

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Clostridium difficile infection (CDI) is an important cause of nosocomial diarrhoea. Stool culture, a sensitive method for the detection of *C. difficile*, is necessary for epidemiological investigation, for monitoring antibiotic resistance, and for providing a reference standard against which to validate assays. It is, however, expensive, time-consuming, requires toxin testing and also technical expertise.[1]

The clinical significance of culture remains unclear as carriage may occur.[2] Although the UK national standard operating procedure (SOP)[3] provides guidance for culture and identification of *C. difficile*, wide variations in methods of identification were reported in a survey of eight European countries.[4] The aim of this study is to compare phenotypic identification using the UK national SOP[3] with identification by 16S ribosomal DNA (rDNA) sequencing and matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS).

The local research ethics committee confirmed that, as the study represented a service evaluation of recognised diagnostic methodologies performed on anonymised excess diagnostic materials, further formal ethical approval was not required. Consecutive anonymised faeces samples from patients aged ≥18 years submitted to pathology laboratories at St George's Hospital, London, for faecal occult blood (FOB) or *Helicobacter pylori* antigen detection between 4 January 2010 and 9 February 2010 were analysed. Additionally, cytotoxin enzyme immunoassay (EIA)-positive faeces samples from patients suspected of CDI, and stored *C. difficile*-positive samples (collected between August and December 2009) were also included. Repeat samples received ≤ 28 days after the first sample were excluded. Samples (stored at 4°C) were analysed within five days of collection.

The samples were cultured for *C. difficile* after alcohol shock[3] for spore selection. The alcohol-faeces suspension (50 µL) was streaked on cycloserine cefoxitin fructose agar (CCFA; Oxoid, Basingstoke, UK) and Brazier's cycloserine cefoxitin egg yolk plate (CCEY; E&O Laboratories, Bonnybridge, Scotland). Plates were incubated anaerobically at 37°C for 48 h and examined by two readers (blinded). Positive controls for both selective media were used daily.

The colonies were identified by typical morphology (CCFA: ~2–4 mm in diameter, non-haemolytic, grey/white with rhizoid edge; CCEY: ~1.5–3 mm in size, grey, flat growth, no opacity around colonies). Suspected colonies were identified further after anaerobic subculture for up to

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Table1. Differential tests for recognition of *C. difficile* colonies.[3]

	C. bifermentans	*C. sordellii*	*C. glycolicum*	*C. innocuum*	*C. difficile*
Fluorescence at 365 nm	No	No	No	Yes	Yes
Lecithinase on Brazier's CCEY medium	Yes	Yes	No	No	No
Latex agglutination	Yes	Yes	Yes	No	Yes

48 h on Columbia blood agar (CBA; Table 1). As CCFA medium lacks egg yolk, lecithinase production was not observed. The growth of *C. difficile* and other bacteria were quantified as scanty (<10 colonies), light (present in the first streak), moderate (present in the second streak) and heavy (present in the last streak).

The *C. difficile* isolates were tested for cytotoxin production by a cell cytotoxicity assay (CCTA) using a previously described method[5] and were saved in cooked meat medium (bioMerieux, UK) at room temperature.

Presumptive saved *C. difficile* isolates were identified by 16S rDNA sequencing and MALDI-TOF MS after anaerobic incubation at 37°C for 48 h on CBA. A boil extraction method was used to extract DNA from colonies and the supernatant was added to the polymerase chain reaction (PCR) tube. Primers previously described[6] were used to amplify a 1380 bp region of the 16S ribosomal RNA (rRNA) gene, and the products were quantified and then sequenced (Source Bioscience, Nottingham, UK).

The MALDI-TOF MS method was performed using a Bruker Microflex LT (Bruker Daltonik, Bremen, Germany). An *in situ* formic acid lysis method was used, which represented a minor modification of the direct analysis method.[7] For samples with no reliable identification (scores <1.7), a full ethanol/formic acid extraction method was performed following the manufacturer's instructions. A culture (≤48 h) of *Escherichia coli* (ACTC 25922) was used as a control.

A total of 163 samples were processed (120 *H. pylori* samples, 24 FOB samples, six cytotoxin EIA-positive samples, and 13 stored *C. difficile*-positive samples). *C. difficile*, phenotypically identified in 11.6% (19/163) samples, was confirmed both by sequencing and MALDI-TOF MS in 84.2% (16/19) isolates with full consensus. All 16 isolates grew on both culture media and were positive for toxin production by CCTA. The remaining three were identified as *C. bifermentans* (2/3) and *C. butyricum* (1/3) by sequencing. The MALDI-TOF MS method gave no reliable identification for one (1/2) isolate of *C. bifermentans* and *C. butyricum* (1/1). *C. butyricum* (1/1) and *C. bifermentans* (1/2) grew on CCFA and CCYA; the other isolate of *C. bifermentans* (1/2) grew only on CCFA.

Sensitivity of CCFA and CCEY was 16/16 (100%, 95% confidence interval [CI] 79.4–100%) and specificity was 144/147 (98.0% [CI 94.2–99.6%]) and 145/147 (98.6% [95.2-99.8%]), respectively. *C. difficile* growth on CCFA was moderate or abundant in 93.7% (15/16) and 100% (16/16) on CCEY, mixed with other flora in 81.2% (13/16) samples and 56.2% (9/16) on CCFA and CCEY, respectively. There was consensus in the observations by both readers except for two samples, where a third reader was consulted.

Identification of *C. difficile* from 16/163 specimens (9.8%) reflects the method of sample selection. Patients in the FOB and *H. pylori* groups were likely to have low prevalence of *C. difficile* and hence provided a challenge to the sensitivity of different culture media. The specimens from patients with known or suspected *C. difficile* were chosen to provide a comparison of positive samples and to confirm the specificity of two different culture media.

Phenotypic methods may misidentify other *Clostridium* species as *C. difficile*, as illustrated above. Apart from colonial morphology, these methods rely on ultraviolet (UV) fluorescence, latex agglutination and lecithinase production (on CCEY; Table 1). Ultraviolet fluorescence, which varies with the type of medium and incubation conditions,[8] may be exhibited by other anaerobes. The latex agglutination is also not specific for *C. difficile* (Table 1). Although the CCEY medium was more selective, no difference was observed in the sensitivity of the two media.

As correlation between 16S rDNA sequencing and MALDI-TOF MS proved to be excellent, MALDI-TOF MS can be considered a useful, rapid method to confirm phenotypic identification. However, larger studies would be required to confirm some of these findings. □

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