

NATIONAL STANDARD METHOD

PROCESSING OF FAECES FOR *CLOSTRIDIUM DIFFICILE*

BSOP 10

Issued by Standards Unit, Department for Evaluations, Standards and Training
Centre for Infections



Association of Medical Microbiologists



PROCESSING OF FAECES FOR *CLOSTRIDIUM DIFFICILE*

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AMENDMENT PROCEDURE

Controlled document reference	BSOP 10
Controlled document title	Processing of faeces for <i>Clostridium difficile</i>

Each National Standard Method has an individual record of amendments. The current amendments are listed on this page. The amendment history is available from standards@hpa.org.uk.

On issue of revised or new pages each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Issue no. Discarded	Insert Issue no.	Page	Section(s) involved	Amendment
3/ 29.09.08	1.2	1.3	All 2 7	All Status page 2.1	Department name changed Taxonomy sentence inserted. 'of' replaced by 'for' in subheading

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PROCESSING OF FAECES FOR *CLOSTRIDIUM DIFFICILE*

Type of specimen: Faeces

SCOPE OF DOCUMENT

This document describes the culture and identification of *Clostridium difficile* from faeces. It advises that all samples selected for investigation should be tested in the first instance with a toxin detection kit or cell cytoxin assay. The laboratory can then either store toxin positive faeces for culture at a later time if required or, if from a laboratory in England, they can request access to the *C. difficile* Ribotyping Network for England (CDRNE) through the Regional Microbiologists. Isolates may be referred to the Anaerobe Reference Laboratory from elsewhere in the UK (except Scotland) for outbreak investigation in conjunction with the Regional HPA laboratories under the DH/HPA surveillance scheme or to the HPA Centre for Infection (Laboratory of HealthCare Associated Infection). In Scotland isolates may be submitted to the Scottish *C. difficile* Reference Service according to criteria developed in conjunction with Health Protection Scotland (HPS) as part of the mandatory surveillance programme:

(<http://www.documents.hps.scot.nhs.uk/hai/sshaip/guidelines/clostridium-difficile/cdiff-protocol-v2-2007-10.pdf>)

[BSOP 30 - Investigation of Faeces Specimens for Bacterial Pathogens](#) and [BSOPID 8 - Identification of Clostridium Species](#) are recommended for additional background information.

INTRODUCTION

***Clostridium difficile* infection (CDI) and antibiotic associated diarrhoea (AAD)**

C. difficile is a Gram-positive, spore forming, strictly anaerobic rod, so named because of the difficulty in original culture and characterisation². Toxigenic strains produce large protein toxins A and B, both being major virulence factors³. Most disease associated with *C. difficile* is intestinal^{4,5} though *C. difficile* may be isolated from blood^{6,7} or tissues⁸.

Changes in the gut flora associated with broad spectrum antibiotics and chemotherapeutic agents can result in colonisation by *C. difficile*⁹. It is the commonest identifiable cause of AAD. Almost all drugs with an antibacterial spectrum of activity have been implicated causally in AAD. The most frequently implicated drugs are those which have a marked effect on the microflora of the colon. These include broad spectrum beta lactams, cephalosporins, clindamycin and fluoroquinolones¹⁰. The incidence of *C. difficile* infection has been shown to decrease once antibiotic therapy is controlled.

The production of two toxins A (enterotoxin) and B (cytotoxin) causes the characteristic mucosal damage consisting of plaque-like lesions leading to the formation of a pseudomembrane. Not all strains of *C. difficile* produce toxin and therefore not all can cause illness.

The spectrum of disease ranges from a self-limiting mild diarrhoea to the advanced and severe illness characteristic of pseudomembranous colitis. The most accurate diagnosis of pseudomembranous colitis is effected by endoscopic detection of colonic pseudomembranes or microabscesses in antibiotic-treated patients who are suffering from diarrhoea and who have *C. difficile* and its toxins in their stools.

The organism has been associated with outbreaks in hospitals and in extended care facilities for the elderly¹¹. It represents an important cause of hospital-acquired infection. *C. difficile* can be isolated from soil, hospital environments and both human and animal faeces¹². It is rarely found in the flora of

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normal adults but up to 50% of infants may be colonised in the first few months, although disease is rarely present at this age^{13,14}. *C. difficile* infection is more common in the elderly. The reasons for this are not clear although there is some evidence to suggest that these patients have a less effective natural barrier to infection¹⁵. The importance of age can be demonstrated by figures from CDSC which show that 81% of cases (in which age was reported) were from patients >65 years old¹⁶.

Elderly medical patients and those undergoing general surgery¹⁷, oncology patients^{18,19} and those with chronic renal disease²⁰ are at particular risk of infection by *C. difficile*.

Clostridium difficile toxins and toxin detection

Demonstration of toxins of *C. difficile* in diarrhoeal stools is generally regarded as suggestive of CDI in the absence of any other recognised cause for gastrointestinal disturbance. In outbreaks it is suggested that primary culture of the organism is undertaken in tandem with toxin detection²¹. The culture of toxin negative faeces followed by toxin testing of the isolate may increase the number of patients diagnosed²².

Although considered by some to be the “gold standard”, use of tissue culture for the detection of *C. difficile* toxins by virtue of its cytopathic effect (neutralisable with *C. sordellii* antitoxin) requires technical expertise, and involves usually a 24 (up to 48) hour delay for the final result²³. Tissue culture, especially with Vero cells²⁴, may detect other faecal cytotoxins that are associated with diarrhoea e.g. *C. perfringens* enterotoxin. Cytopathic effect (CPE) that is not neutralised by *C. sordellii* antitoxin may indicate that another pathogen is present.

There are numerous commercially available EIA tests intended to detect the toxins of *C. difficile*. Some detect Toxin A, others A and B, although the sensitivity and specificity of these are variable²⁵⁻²⁹. Commercial EIAs that detect both toxins A and B are considered more appropriate than those which detect A alone³⁰, because infection due to A- B+ strains has been recorded.

Latex agglutination kits are available, but are not as accurate as EIA due to poor sensitivity³¹. Detection by counter immuno-electrophoresis (CIE) has been suggested, but this method lacks sensitivity and specificity^{32,33} and is not recommended.

These and other testing procedures are reviewed in a recent report presented to the Department of Health³⁴.

Typing of *C. difficile*

Typing of isolates of *C. difficile* is sometimes useful in the investigation of multiple cases of infection. Typing methods that have been used include bacteriophage/bacteriocin typing³⁵ and serotyping³⁶. PCR ribotyping is gaining acceptance as an internationally recognised method³⁷ and within England a PCR ribotyping network (CDRNE) has been established by the HPA for use when there is an increase in frequency of CDI, or increased severity, complication, recurrence or death rate associated with CDI. The Anaerobe Reference Laboratory in Cardiff currently provides the same service for Wales and the rest of the UK (except Scotland) and performs the typing for the DH/HPA surveillance scheme in England. In Scotland this service is provided by the Scottish *C. difficile* Reference Service, which is based at the Scottish *Salmonella* Reference Laboratory in Glasgow. Ribotyping and other more refined, molecular methods of strain differentiation are performed at the Centre for Infection. Other methods include cell surface protein profiles³⁸ and other DNA-based methods of analysis^{39,40}.

Other organisms associated with AAD

In addition to *C. difficile*, infection with *C. perfringens*, *Staphylococcus aureus*, *Klebsiella oxytoca*, *Candida* species and *Salmonella* species have been implicated with AAD^{41,42}.

TECHNICAL INFORMATION/LIMITATIONS

Interpretation of toxin results in children less than 2 years old should be treated with caution.

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1 SAFETY CONSIDERATIONS⁴³⁻⁵⁴

1.1 SPECIMEN COLLECTION

N/A

1.2 SPECIMEN TRANSPORT AND STORAGE

Leakproof container in a sealed plastic bag.

1.3 SPECIMEN PROCESSING

Containment Level 2 for routine work, or Containment Level 3 if the following organisms are suspected from clinical information or laboratory findings:

Salmonella typhi, *S. paratyphi* A, B and C, vero cytotoxin producing *E. coli* O157 (VTEC), and *Shigella dysenteriae*.

Note: Under normal circumstances culture for *C. difficile* would not be requested on patients suspected of having any of the above organisms. However, if clinical details or routine culture indicate any of the above then all specimen preparation and culture for *C. difficile* should be performed in the cabinet in CL 3.

Laboratory procedures that give rise to infectious aerosols must be conducted in a microbiological safety cabinet.

UV protective safety goggles must be worn when examining cultures with the fluorescent lamp.

The above guidance should be supplemented with local COSHH and risk assessments.

Compliance with postal and transport regulations is essential.

2 SPECIMEN COLLECTION

2.1 OPTIMAL TIME FOR SPECIMEN COLLECTION

As soon as possible after onset of symptoms.

2.2 CORRECT SPECIMEN TYPE AND METHOD OF COLLECTION

Specimen may be passed into a clean, dry, disposable bedpan or similar container and transferred into a leakproof container. The specimen is unsatisfactory if any residual soap, detergent or disinfectant remains in the pan.

Formed stools are unsuitable for investigation for *C. difficile*. These should be rejected with the appropriate comment appended to the report.

2.3 ADEQUATE QUANTITY AND APPROPRIATE NUMBER OF SPECIMENS

A liquid specimen of 1-2mL is sufficient for culture and toxin detection. Repeat testing of samples is not indicated within a 28 day period. This applies to repeat testing of positives. On the contrary, a negative test if symptoms persist should be retested as it is known that a one-off negative can occur.

In suspected outbreaks, samples should be stored at 4°C or frozen at -20°C for culture. Isolates confirmed as *C. difficile* may be referred for ribotyping in accordance with the Anaerobe Reference Laboratory (ARL: guidelines for typing investigations listed on-line at: www.hpa.org.uk/cfi/arl) or the relevant Scottish guidance: (<http://www.documents.hps.scot.nhs.uk/hai/sshaip/guidelines/clostridium-difficile/cdiff-protocol-v2-2007-10.pdf>)

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In general sending 10 isolates from each outbreak should be enough.

3 SPECIMEN TRANSPORT AND STORAGE

3.1 TIME BETWEEN SPECIMEN COLLECTION AND PROCESSING

Specimens of faeces should be transported to the laboratory and processed as soon as possible.

3.2 SPECIAL CONSIDERATIONS TO MINIMISE DETERIORATION

Refrigerate for up to 2 days if unable to process within 2 hours. Freeze at -20°C or below if unable to process within 2 days of collection⁵⁵.

All *C. difficile* toxin positive faecal samples should be kept refrigerated or frozen so that culture can be performed to recover isolates for typing⁵⁶. (It is not necessary to keep the whole specimen an aliquot in a small eppendorf would suffice). The duration of storage needs to be determined locally but should allow appropriate outbreak investigation.

4 SPECIMEN PROCESSING

4.1 TEST SELECTION

If clinically indicated, patients who fulfil any of the following criteria should be screened: antibiotic-associated diarrhoea (everyone over the age of 2⁵⁷); pseudomembranous colitis; and post antibiotic treatment on all patients over 65 years old (in Scotland all patients with diarrhoea over 65 years old).

Manufacturers instructions must be followed when using toxin detection kits manufacturers instructions must be followed. It is recommended that a kit that is capable of detecting both A and B toxins is used. In order to ensure that strain type monitoring can be carried out in the event of an outbreak, samples of all positive stool samples should be at 4°C or -20°C for later culture.⁵⁸

The culture and identification of *C. difficile* from faeces is intended to be followed in outbreaks or as part of enhanced surveillance. This method is described in section 4.4 Culture and Investigation.

4.2 APPEARANCE

N/A

4.3 MICROSCOPY

N/A

4.4 CULTURE AND INVESTIGATION

Alcohol shock method

The advantage of using alcohol shock for selection of *C. difficile* is that only spores should survive this process and it eliminates the growth of other non sporing faecal organisms. The selective agents are usually based on cefoxitin and cyloserine (although others have been described), and these are usually inhibitory to most other clostridial species. The resulting growth from an active case of infection is often a pure culture of *C. difficile*.

It should be noted that the same medium from different suppliers may give different colonial appearances and the descriptions given here are not absolute.

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1. Make an approximate 1:1 suspension of stool sample; methylated spirit / absolute alcohol in a screw capped glass bijou.
2. Mix by vortexing and leave to settle at room temperature for 30min.
3. With a disposable pastette inoculate two drops (approx 50 -75 µL) of the deposit to cefoxitin-cycloserine egg yolk agar* (CCEY) selective agar and streak for single colonies. At the same time culture the control organisms on CCEY from their spore suspension and incubate as outlined below.
4. Incubate anaerobically at 35°C - 37°C for 48 - 72 hrs. Cultures may be examined after overnight incubation, but should not be removed from the anaerobic cabinet because sporulation is inhibited on selective media and young cultures may die on exposure to air. If using anaerobic jars, cultures must not be examined before 48 hours incubation.

* Egg-yolk supplement is optional, blood agar can also be used.

4.5 IDENTIFICATION

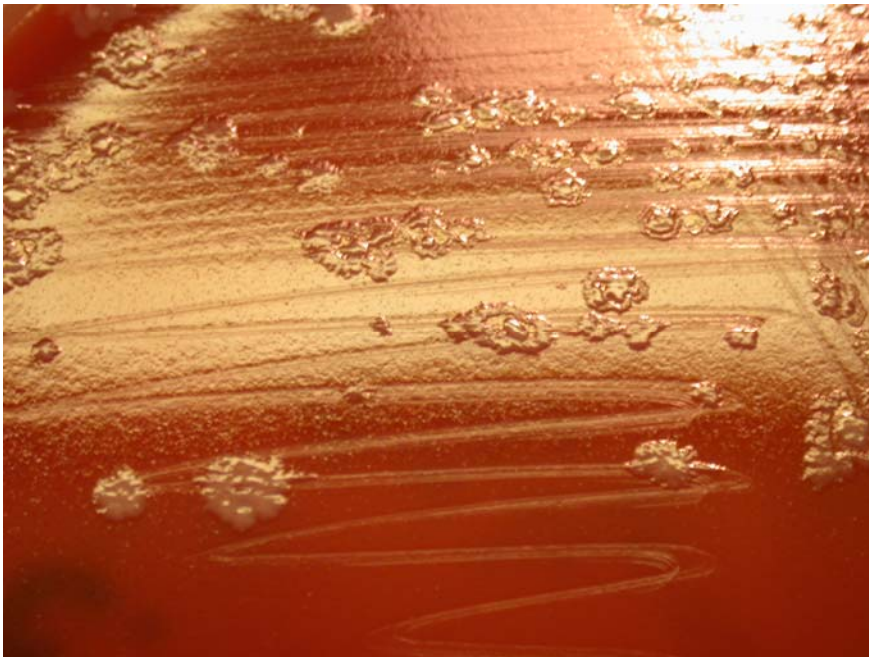
Colonies of *C. difficile* can be recognised by the following characteristics:

- If using egg-yolk based agar, a lack of opacity surrounding the colonies due to non-production of lecithinase (unlike *C. bifermentans*, *C. perfringens* or *C. sordellii*). Follow individual media manufacturer's guidelines on colonial morphology.
- Green-yellow fluorescence under long-wave UV light (see below).
- Agglutination with *C. difficile* latex reagent for somatic antigen (see below).

For ease of identification it is useful to sub-culture a putative *C. difficile* colony on FAA blood agar.

4.5.1 EXAMINATION OF PLATES

Colonies of *C. difficile* may be smooth or rough and may vary considerably in size. Typical colonies may be seen after sub-culture of suspect colonies from selective media on Fastidious Anaerobe Agar (See Plate 1):



Courtesy of Dr Jon Brazier of the Anaerobic Reference Laboratory

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Plate 1. Colonies of *C. difficile* on Fastidious Anaerobe Agar

Colonial Fluorescence

- Remove the test and control plates from the incubator and examine the colonies for fluorescence. Expose the colonies to long wave ultra-violet light (365 nm) in a darkened room or light box held closely to the UV source and view by reflection.

NB. UV protective goggles must be worn.

- Colonies of *C. difficile* may vary in the intensity of fluorescence, but this will appear as a green-yellow or chartreuse colour. Fluorescence is poorly developed on some agar bases and is strongest on FAA. It is important to compare fluorescence of the test colonies with that of the control organisms to clarify positive and negative results. The colonial fluorescence of cultures >48 hrs old on non-selective agars will diminish due to increased sporulation.
- Mark any suspect (fluorescent) colonies on the underside of the plate with a felt tip pen. Sub-culture to a Fastidious Anaerobe Blood Agar (FAA) plate and incubate anaerobically for 48hrs.

NB: Gram staining is rarely useful directly from selective agars, but from blood agar plates sub-terminal spores should be visible with most vegetative rods staining as Gram-positive with some Gram variable forms in common with many other clostridial species. Routine Gram staining is not recommended in this NSM.

Latex agglutination test for somatic antigen

Use *C. difficile* somatic antigen latex agglutination and follow the instructions in the kit insert.

Limitations of the test

Cross-reactions with this reagent are known to occur with *C. bifermentans*, *C. sordellii* and *C. glycolicum* (See Table 1).

Controls

Set up controls alongside test cultures and on each new batch of medium (see 4.4).

Control organisms required:

C. bifermentans
C. sordellii
C. difficile

Other clostridial species are commonly mistaken for *C. difficile*. These include *C. innocuum*, *C. glycolicum*, *C. bifermentans* and *C. sordellii*. However, these may be differentiated according to the criteria listed in Table 1.

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4.5.2 INTERPRETATION OF RESULTS

Table 1. Differential tests for recognition of colonies of *C. difficile*

	<i>C. difficile</i>	<i>C. bifermentans</i>	<i>C. sordellii</i>	<i>C. glycolicum</i>	<i>C. innocuum</i>
UV (Fluorescence) at 365 nm	+	-	-	-	+
Latex agglutination	+	+	+	+	-
Lecithinase on Brazier's CCEY medium	-	+	+	-	-

4.6 REFERRAL FOR OUTBREAK INVESTIGATIONS

4.6.1 ISOLATES

Isolates from outside England (except Scotland) from situations that warrant typing investigations should be referred to the Anaerobe Reference Laboratory (ARL) for PCR ribotyping as detailed below. Isolates should only be referred for ribotyping following discussion and agreement with the designated laboratory.

With a charcoal transport swab, swab all the growth from a 48 hour anaerobic culture of *C. difficile* on non-selective media such as FAA and replace swab in tube of transport medium.

NB: It is important to send growth from a 48 – 72 hour culture to ensure sporulation.

Anaerobe Reference Laboratory
NPHS Microbiology Cardiff
University Hospital of Wales
Cardiff
CF14 4XW

<http://www.hpa.org.uk/cfi/arl/>

Within Scotland, isolates from situations that warrant typing investigations should be referred to the Scottish *C.difficile* Reference Service for PCR ribotyping. Full contact details, referral criteria and isolate submission details are given on the website:

<http://www.ssrl.scot.nhs.uk/cdiffhome.asp>

Retention of isolates

When harvesting the growth from the FAA purity plate take a sweep of the growth and mix this in a fresh bijou of alcohol/saline (2:1) labelled appropriately. Store at -20°C. Alternatively, make a heavy suspension of the colonies in alcohol/saline (2:1) and store at -70°C.

4.6.2 FAECES

Within England there is now a *C. difficile* Ribotyping Network (CDRNE) consisting of six laboratories (in Leeds – reference centre, Birmingham, London, Manchester, Newcastle and Southampton) which is accessed in agreement with the relevant Regional Microbiologist. Access to this network should occur if a laboratory believes they have or could have a problem with an increased frequency or severity of cases of *C. difficile* infection, including increases in mortality, complications or recurrence rates. A standardised request form has been widely circulated in electronic format, which must be completed to access the service. Further details are available via the HPA www site.

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DH/HPA National *C. difficile* Surveillance Scheme

Referrals submitted as part of DH/HPA national *C. difficile* surveillance scheme ('designated week') should not use the Outbreak Investigation form but the separate one labelled "DH/HPA National *C. difficile* Surveillance Scheme, HPA Regional Laboratories will request a pre-determined number of toxin positive stool samples within a given week from hospitals in their region rotation.

4.7 ANTIMICROBIAL SUSCEPTIBILITY TESTING

The ARL is monitoring the antimicrobial susceptibilities of all isolates submitted under the DH/HPA surveillance scheme using the E test method for MIC determination to eight antibiotics. It is important that regular testing is done by all CDRNE laboratories to screen for any emerging resistance to the drugs of choice for treatment, namely metronidazole and vancomycin. Similar surveillance is performed in Scotland by the Scottish *C.difficile* Reference Service in conjunction with Health Protection Scotland (HPA).

5 REPORTING PROCEDURE

5.1 MICROSCOPY

N/A

5.2 CULTURE

Isolates of *C. difficile* submitted for typing investigations. Further report to follow.

5.3 ANTIMICROBIAL SUSCEPTIBILITY TESTING

N/A

5.4 TOXIN DETECTION

C. difficile toxin detected
C. difficile toxin not detected

6 REPORTING TO THE HPA⁵⁹ (LOCAL AND REGIONAL SERVICES AND CENTRE FOR INFECTIONS)

Refer to the following:

Health Protection Agency publications:

"Reporting to the CDR : A guide for diagnostic laboratories"

"Hospital infection control: Guidance on the control of infection in hospitals"

Department of Health letter 11th April 2007 from the Chief Medical Officer and the Chief Nursing Officer⁶⁰.

Health Protection Scotland publication

"Protocol for the Scottish Surveillance Programme for *Clostridium difficile* Associated Disease":

<http://www.documents.hps.scot.nhs.uk/hai/sshaip/guidelines/clostridium-difficile/cdiff-protocol-v2-2007-10.pdf>

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Refer to current guidance on CDSC and COSURV reporting

Local guidelines

7 ACKNOWLEDGEMENTS AND CONTACTS

This National Standard Method has been reviewed and revised by Dr Jon Brazier of the Anaerobe Reference Laboratory and the National Standard Methods Working Group for Clinical Bacteriology (http://www.hpa-standardmethods.org.uk/wg_bacteriology.asp). The contributions of many individuals in clinical bacteriology laboratories and specialist organisations who have provided information and comment during the development of this document, and final editing by the Medical Editor, are acknowledged.

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PROCESSING OF FAECES FOR *CLOSTRIDIUM DIFFICILE*

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