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Rapid Confirmation of *Clostridium perfringens* by Using Chromogenic and Fluorogenic Substrates

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The use of 4-methylumbelliferyl phosphate (MUP) and *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) for the identification of *Clostridium perfringens* was investigated. A liquid assay containing both MUP and ONPG was a highly specific alternative method for *C. perfringens* confirmation, reducing incubation time from 48 to only 4 h. The assay solution is easy to prepare, does not require anaerobic conditions for use, and has an extended shelf life.

The sulfite-reducing clostridium group, including *Clostridium perfringens*, has been shown to be of value in assessing fecal pollution of surface and ground water (9) and estuarine (7) and (10) ocean environments. *C. perfringens* is an anaerobic, gram-positive, spore-forming bacillus that is capable of surviving in soil and water for extended periods of time. Its presence in the absence of other fecal indicators may reflect remote or intermittent contamination in the distant past (9). *C. perfringens* spores have been demonstrated to be useful surrogate indicators for monitoring water treatment processes for the removal of viruses (11), *Giardia* cysts, and *Cryptosporidium* oocysts (11, 14). Their spores are similar in size to *Cryptosporidium* oocysts and have similar chlorine resistance properties (11, 14). Although primary isolation on tryptose sulfite cycloserine (TSC) agar takes 24 h, the standard method of confirmation for presumptive *C. perfringens* can take up to 72 h (1, 13). TSC agar incorporates sodium metabisulfite and ferric ammonium citrate as an indicator for sulfite reduction. Presumptive sulfite-reducing clostridia, including *C. perfringens*, produce black colonies. Isolates are subcultured onto blood agar (BA) for aerotolerance testing, purity check, and Gram staining before inoculation into nitrate motility medium (NMM) to detect nitrate reduction and motility and into lactose gelatin medium (LGM) to detect liquefaction of gelatin and lactose fermentation. Confirmation of isolates is labor intensive, requires significant anaerobic workspace, and is prone to misreporting of results due to the selection of mixed cultures upon subculturing from TSC agar.

The detection of acid phosphatase has been shown to be a useful diagnostic tool for identifying *C. perfringens* (4, 12). *C. perfringens* can metabolize 4-methylumbelliferyl phosphate (MUP) using the enzyme acid phosphatase to produce 4-methylumbelliferone, which fluoresces when placed under long-wavelength (365-nm) ultraviolet light. Additionally, *C. perfringens* ferments lactose to acid and gas, utilizing β-galactosidase in the process. β-Galactosidase activity has been used successfully for the confirmation of coliforms by detecting hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), which yields the chromogenic product *ortho*-nitrophenol (6). We describe the successful incorporation of MUP and ONPG into a liquid assay for confirmation of *C. perfringens*, which we term the MUP-ONPG assay. Compared to the standard technique, the MUP-ONPG assay demonstrated superior sensitivity and specificity for the rapid confirmation of *C. perfringens*.

Preparation of samples. Water samples were collected for processing from river, sewage effluent, and surface water storage locations. Selection for sulfite-reducing clostridia including *C. perfringens* spores was undertaken by heat treatment of water samples at 70°C for 20 min to kill vegetative cells. Heat-treated samples were then filtered through a 0.45-μm-pore-size cellulose-acetate membrane filter (Pall Gelman Corporation., Ann Arbor, Mich.). Membranes were then transferred onto freshly prepared TSC or TSC-MUP agar plates and incubated at 35°C for 24 h in an anaerobic environment.

Media. All chemicals were obtained from Sigma Chemical Company (Sigma-Aldrich, St. Louis, Mo.) or BDH Laboratory Supplies Pty. Ltd. (Poole, Dorset, England). TSC agar and BA were prepared following the manufacturer's recommendations (Oxoid Australia Pty. Ltd., Heidelberg, Australia). NMM and LGM were prepared following recommended instructions (1, 13). TSC-MUP agar was prepared by adding MUP to molten (55°C) TSC agar to a final concentration of 85 mg/liter. The MUP-ONPG assay mixture was prepared by adding to 1 liter of distilled water 5 g of ammonium sulfate, 10 g of sodium chloride, 5.3 g of HEPES buffer sodium salt, 6.9 g of HEPES buffer organic acid, 1 g of ONPG, and 0.15 g of MUP; the final pH was 7.3 ± 0.1. Following filtration through a 0.2-μm-pore-size cellulose-acetate membrane filter (Pall Gelman), 100-μl aliquots were dispensed into sterile reaction tubes.

Inoculation and incubation of test media. Anaerobic, sulfite-reducing, gram-positive bacilli (presumptive *C. perfringens*) were purified on BA prior to inoculation into confirmatory media. All culture media were incubated at 35°C in an anaerobic workstation (Don Whitley Scientific Pty. Ltd., Shipley, West Yorkshire, England) with a gas mixture of 10% hydrogen in 90% nitrogen. TSC agar, TSC-MUP agar, and BA plates were incubated for 24 h; NMM and LGM inoculated culture media were incubated for 48 h. Isolates were inoculated into the MUP-ONPG mixture by rolling a sterile toothpick over the entire surface of an isolated colony and then emulsifying it in the mixture, ensuring that there was sufficient inoculum to

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produce visible turbidity. The suspension was then overlaid with sterile mineral oil to prevent evaporation. Tubes were incubated in a heating block at 35°C for up to 24 h.

Biochemical tests. Gram staining was performed following standard procedures (5). Presumptive *C. perfringens* isolates, which produced discrepant results between the standard and test confirmation methods, were identified using Vitek anaerobic identification (ANI) cards following the manufacturer's recommendations (Bio-Merieux, Marcy l'Etoile, France). Briefly, ANI cards were inoculated with 1.8 ml of a 3 McFarland equivalence turbidity standard of the test organism suspended in saline solution (sodium chloride, 4.5 g/liter) and then incubated for 4 h at 35°C prior to reading of the results. These cards utilize 28 enzymatic tests, a Gram stain reaction, and an indole test to identify anaerobic bacteria using a specially adapted database.

Reference cultures. *C. perfringens* ATCC 13124 and *C. perfringens* NCTC 8237 give positive results for all *C. perfringens* confirmatory tests. *Clostridium sporogenes* ATCC 19404 gives positive results for sulfite reduction but is negative for the confirmation of *C. perfringens*.

TSC-MUP agar. Initially we investigated the incorporation of MUP into TSC agar as a possible way of combining primary isolation and secondary confirmation of *C. perfringens*. In this trial, 30 river and surface water samples taken from locations known to be impacted by fecal contamination were assessed. Samples were heat treated and allowed to cool, and following filtration, membranes were placed onto TSC-MUP agar. Plates were incubated anaerobically at 35°C for 24 h. Fluorescence on TSC-MUP agar was difficult to interpret under UV due to diffusion of 4-methyl-umbelliferone, especially when colonies were clustered together or when they were present in large numbers. In some cases fluorescence appeared to be masked by particulate matter. Subculturing of MUP-positive, presumptive *C. perfringens* isolates back onto TSC-MUP agar demonstrated that some isolates failed to fluoresce when retested. Two such isolates were identified as *Clostridium bifermentans* and *Clostridium subterminale* using Vitek confirmation.

Due to the limitations of TSC-MUP agar for direct confirmation of *C. perfringens* we tested the agar as an indirect confirmation medium, whereby sulfite-reducing colonies arising on TSC agar were confirmed by spot inoculation and incubation of TSC-MUP agar. A total of 224 presumptive *C. perfringens* isolates obtained from 37 river and wastewater samples were transferred to TSC-MUP agar; 212 of 224 isolates fluoresced on TSC-MUP agar, whereas 169 of 224 were confirmed by the standard confirmation technique. The data confirmed that although TSC-MUP agar is sensitive (100%), it also has a high false-positive rate (25.4%) when confirming *C. perfringens*, with *C. subterminale* being the most common MUP-positive non-*C. perfringens* microorganism identified. An interesting observation at this point was that the majority of *C. perfringens* isolates were positive for UV fluorescence within 4 to 6 h of TSC-MUP agar being inoculated.

MUP-ONPG assay. As acid phosphatase activity alone was unreliable for the confirmation of *C. perfringens*, a liquid assay was developed which combined acid phosphatase detection with β-galactosidase activity in an attempt to improve specificity. MUP and ONPG substrates were incorporated in HEPES buffer in the presence or absence of 0.2% (wt/vol) sodium lauryl sulfate and 1% (wt/vol) sodium chloride, concentrations which have previously been reported to enhance β-galactosidase activity (3, 8).

TABLE 1. Comparison of the MUP-ONPG assay to a standard method[a] for confirmation of *C. perfringens*

Standard confirmation of *C. perfringens*	No. of isolates		
	MUP-ONPG positive	MUP-ONPG negative	Total
Positive	152	1	153
Negative	12	168	180
Total	164	169	333

[a] Analysis performed according to reference 2.

Presumptive *C. perfringens* isolates were emulsified in MUP-ONPG reaction mixtures and then overlaid with sterile mineral oil to eliminate evaporation. Reaction tubes including controls were placed in a heating block at 35°C and checked hourly over a 4-h period, with a final reading being taken at 24 h. The presence of any yellowing or fluorescence of the reaction mixture within the 4-h period was recorded as a positive result. Confirmation of *C. perfringens* using MUP-ONPG in HEPES buffer (pH 7.3) with 1% (wt/vol) sodium chloride in the absence of lauryl sulfate showed the best correlation to the standard confirmatory technique, with 11 of 11 environmental isolates and both *C. perfringens* control strains being positive at 4 h. Importantly, in this assay *C. perfringens* demonstrated acid phosphatase activity (UV fluorescence) within 1 h and β-galactosidase activity (yellow coloration) within 4 h.

The identities of 333 anaerobic, sulfite-reducing, gram-positive isolates arising on TSC agar, which were from 40 wastewater effluent, 10 river, and 18 surface water samples impacted by fecal contamination, were confirmed using the MUP-ONPG assay and the standard method (1, 13). The MUP-ONPG mixture confirmed 164 of 333 isolates as *C. perfringens*, compared to 153 of 333 using the standard method (Table 1). The 12 isolates negative by the standard method that were positive using the MUP-ONPG assay were identified as *C. perfringens* using Vitek analysis. One isolate, identified as *C. perfringens* by standard and Vitek analyses, demonstrated β-galactosidase activity but failed to give significant acid phosphatase activity within the 4-h period. The MUP-ONPG assay demonstrated a high level of sensitivity (99.3%), although specificity (93.3%) was adversely affected by the number of false-negative results (7.1%) obtained by the standard method of confirmation. When two-sided 99% critical values were applied using McNemar's test for agreement (2), no significant disagreement ($P > 0.01$) was detected.

Conclusion. Direct isolation and confirmation of *C. perfringens* on TSC-MUP agar following membrane filtration was unreliable due to diffusion of the UV fluorescent product 4-methylumbelliferone and interference from particulate matter. Transfer of colonies from TSC agar to TSC-MUP agar for confirmation gave a high false-positive rate, indicating that reliance on acid phosphatase activity alone for confirmation is unreliable. Combining detection of acid phosphatase and β-galactosidase activity using a MUP-ONPG assay improved confirmation to a level superior to that of the standard method, yielding results within 4 h. This test does not need to be

performed anaerobically, removing the need for prereduction of media and complex anaerobic manipulations. Aliquots of the MUP-ONPG mixture can be stored at −20°C for up to 3 months prior to use, without loss of sensitivity. The method provides a convenient, cost-effective, and rapid alternative to the standard method of confirmation for *C. perfringens*.

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