Media for Confirming *Clostridium perfringens* from Food and Feces

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**ABSTRACT**

Several media recommended for confirming isolates of *Clostridium perfringens* from selective plating media were evaluated. Media for testing motility, reduction of nitrate to nitrite, fermentation of lactose, and liquefaction of gelatin were found to be the most useful. A modified motility-nitrate medium, developed during the study, and lactose-gelatin medium were the most satisfactory for doing these tests. Fermentation of salicin and raffinose in peptone-yeast extract medium was also useful for differentiating atypically reacting strains of *C. perfringens* from a variety of culturally similar clostridia.

Because several *Clostridium* species are able to grow in the selective plating media used for enumerating *Clostridium perfringens* in foods and feces, a minimum number of confirming tests should be done to assure that the colonies counted actually are *C. perfringens* (1). The inadequacy of procedures commonly used during the past decade to distinguish this organism from a variety of culturally similar clostridia (5) was in part due to the poor performance of the media used and to a misunderstanding of the adequacy of the tests to differentiate *C. perfringens* from other *Clostridium* species. The tests recommended by Angelotti et al. have been used extensively in the United States and elsewhere (7). The antitoxin half-plate method, rarely used in the United States, has also been used for this purpose, primarily in Great Britain (12). Results obtained by Nakamura et al. suggest that this method would yield false-positive results with *Clostridium paraperfringens* and possibly with *Clostridium absonum* because lecinthinas of these species react with *C. perfringens* antiserum (8). Angelotti et al. believed that grampositive, obligately anaerobic spore-formers which reduced sulfite, were non-motile, and reduced nitrate to nitrite were confirmed as *C. perfringens*. However, the results of recent studies show that these criteria are inadequate to differentiate *C. perfringens* from culturally similar clostridia (5,7,8). The culture media recommended were also difficult to use routinely but are more practical since they have been modified (6,11).

Recognition of the culturally similar species, *C. paraperfringens*, *C. absonum*, and *Clostridium celatum*, which like *C. perfringens* are non-motile and reduce nitrate to nitrite, has resulted in efforts to modify confirmatory tests for *C. perfringens* to prevent false results. Suggested additional or alternative criteria include fermentation tests for lactose, salicin, and raffinose, and a test for liquefaction of gelatin (5,6).

The purpose of this study was to determine which of these tests are the most useful and to investigate the factors that affect test results obtained with both the original and the modified confirmatory media.

**MATERIALS AND METHODS**

**Cultures**

All *C. perfringens* cultures and many of the culturally similar clostridia used were from the Food and Drug Administration (FDA) stock culture collection. The type strains of *C. absonum* ATCC 27555 and *C. paraperfringens* ATCC 27639 were obtained from the American Type Culture Collection, Rockville, MD. Additional strains of these species and five atypical *C. perfringens* strains isolated from spices and other foods in our laboratory were classified by Lillian V. Holdeman at the Anaerobe Laboratory of VPI and SU, Blacksburg, VA. Ms. Holdeman also supplied authenticated strains of *C. absonum* VPI 6003, *C. paraperfringens* 4446, and *Clostridium sordellii* strains VPI 2975 and VPI 2984 for use in the study. The *C. celatum* GD-1 culture was supplied by A. H. W. Hauschild, Health and Welfare, Canada. Cultures were maintained at room temperature in a medium prepared from fresh cooked beef. Test cultures were propagated in the same medium or in fluid thiglycollate medium (4).

**Motility-nitrate media**

Indole-nitrite (IN) medium was obtained from BBL as the complete dehydrated medium and was prepared according to directions. All of the other media were prepared from ingredients as specified by the original authors. Modifications of the various media were made as indicated in the formulas given below: Motility-nitrate (MN) medium consisted of 0.3% beef extract, 0.5% peptone (Difco), 0.3% agar, and 0.1% potassium nitrate (1). Buffered supplemented motility-nitrate (SMN) medium was prepared from MN medium by adding 0.5% each of D-galactose and glycerol (6). Buffered supplemented motility-nitrate (BSMN) medium was prepared in the same manner as SMN medium except that 0.25% disodium phosphate was added and the pH was adjusted to 7.3 ± 0.1. To prepare buffered motility-nitrate (BMN) medium, the potassium nitrate level in BSMN was increased from 0.1% to 0.5% (5). Tryptose-phosphate-nitrate (TPN) medium consisted of 1% tryptose (Difco), 0.25% NaHPO₄, 0.5% potassium nitrate, and 0.3% agar.
Ingredients were dissolved in distilled water and the pH was adjusted to 7.3. All motility-nitrate media were sterilized by autoclaving for 15 min at 121 C.

Nitrile test reagents.

Solution A for the nitrile test was prepared by dissolving 1 g of sulfanilic acid in 125 ml of 5 N acetic acid. Solution B consisted of either 1 g of α-naphthol or N-(1-naphthyl) ethylene diamine dihydrochloride in 200 ml of 5 N acetic acid. To test for nitrile, 0.5 ml of solution A and 0.2 ml of solution B were added to the culture.

Nitrile destruction

Destruction of nitrile by C. perfringens was determined by adding filter-sterilized NaNO₂ to IN base without nitrile to obtain 200 and 400 μg of nitrile/ml of medium. A 0.5-ml inoculum of fresh fluid thioglycollate culture was used per 11 ml of IN base containing nitrile and the culture was incubated at 35 C. At 30-min intervals, 0.5 ml of culture was diluted to 3 ml with distilled water, and 0.25 ml each of sulfuric acid and α-naphthylamine solution was added to detect nitrile (I). When color intensity was greatest, usually within 5 min, percent absorbance was measured at 540 nm with a Cary model 118 spectrophotometer. The approximate nitrile level was determined by comparing the result with a standard curve derived with basal medium containing various concentrations of nitrile.

Gelatin media

Lactose-gelatin was prepared as specified by Hauschild and Hilshemer (6). The salicin-gelatin and raffinose-gelatin media were prepared by adding 1% salicin or raffinose, 12% gelatin, 0.5% Na₂HPO₄, and 0.005% phenol red to polypropylene yeast extract (PY) medium (8). The pH was adjusted to 7.4 and the medium was autoclaved for 15 min at 121 C. The basal PY medium consisted of 2% polypropylene (BBL), 0.5% yeast extract, and 0.5% NaCl. Thioglycollate-gelatin (thio-gel) medium was obtained from Difco. This medium and the basal medium of Lactose-gelatin used for preliminary experiments were prepared with 1% salicin or raffinose substituted for lactose.

Fermentation tests

Salicin and raffinose fermentations were determined in PY medium as specified in the AOAC method (5). Production of acid from salicin was checked after 24 h of incubation and again after 3 days. PY-raffinose was checked for acid after 3 days. The spot plate method with phenol red indicator was used during the early part of the study, but for subsequent testing, 15.0-cm Whatman No. 31 filter paper disks were saturated with a 0.2% bromthymol blue solution made slightly alkaline with ammonium hydroxide. The disks were air dried and stored for later use. A 2-mm platinum loop was used to transfer 0.04 ml of culture to the indicator disk. The precision of the color change was checked with a variety of cultures ranging in pH from 5.8 to 7.2. These determinations were confirmed with a Fisher Accumet 420 electronic digital pH meter equipped with a microprobe combination electrode. Salicin and raffinose fermentations in PY-gelatin media were observed daily for acid and gas production.

Antitoxin half-plate

The lactose egg yolk medium was prepared as indicated by Willis and Hobbs (72) and plated in 15 × 100-mm culture dishes. Test cultures were grown overnight in fluid thioglycollate medium. Test plates were prepared by the even application of 0.05 ml of Clostridium welchii (perfringens) type A diagnostic serum obtained from Burroughs-Wellcome (r.l.) and Co., Greenville, NC) to one-half of the medium. The medium was inoculated by streaking once across the plate with a 4-mm loopful of culture from the untreated side. After anaerobic incubation for 24 h, plates were checked for lecithinase activity and neutralization of this enzyme by the antiserum. Lactose fermentation was also determined by observing a color change of the neutral red from red to yellow and then to fuchsia after holding the plates at room temperature for about 4 h.

RESULTS AND DISCUSSION

Motility-nitrate media

Figure 1 shows that the rate of nitrile accumulation after 18 h of incubation was greater in supplemented motility-nitrate (SMN) medium and in buffered motility-nitrate (BMN) medium than in the indole-nitrate (IN) and tryptose-phosphate-nitrate (TPN) media. The latter two media have been used extensively in FDA laboratories for making motility and nitrile reduction determinations with C. perfringens. The higher nitrile levels in SMN and BMN media appeared to be due to better growth and probably to other factors which resulted from the addition of galactose and glycerol. Buffering the SMN medium with Na₂HPO₄ also resulted in increased nitrile levels as shown in Table 1. The reason for the lower levels of nitrile in the IN and TPN media (Fig. 1) was not determined, but further testing of the IN medium suggested that it was due to rapid destruction of nitrile. When nitrile was added to IN basal medium without nitrile at 200 and 400 μg/ml, it decreased steadily and was undetectable in many C. perfringens cultures after 4 h of incubation. The rapidity of nitrile destruction in this medium appeared to be related to addition of glucose to encourage better anaerobe growth.

Because of these effects it was concluded that modification of SMN to the medium referred to as BMN was desirable to obtain consistently positive results with C. perfringens when the safe α-naphthol test reagent was used as recommended by the AOAC (5). Although this reagent is preferable from a safety standpoint, it requires a higher level of nitrile in the medium to obtain a positive
organism

\begin{table}
\centering
\caption{Effects of potassium nitrate concentration and buffering with disodium hydrogen phosphate on nitrite levels produced by Clostridium perfringens and 7 other clostridia in supplemented motility-nitrate medium.}
\begin{tabular}{lccc}
\hline
Organism & strain & Nitrite level obtained \( \text{g}\/ml \) & \\
 & & SMN & SMN & BMN \( \text{KNO}_3 \) \( \text{Na}_2 \text{HPO}_4 \) \% \\
\hline
\text{C. perfringens} & FD-1 & ± & + & ++++ \\
& FD-2 & + & ++ & ++++ \\
& S-40 & ++ & +++ & ++++ \\
& S-45 & + & ++ & ++++ \\
& S-80 & + & ++ & ++++ \\
& IU-686 & + & ++ & ++++ \\
& IU-3344 & ± & ± & ++++ \\
& CDC-3131 & + & + & ++++ \\
& 79392 & ± & ± & ++ \\
& NCTC 8238 & + & + & ++ \\
& NCTC 8798 & ++ & +++ & ++++ \\
& VPI 10240 & + & ++ & ++++ \\
& C. absonum & VPI 6903 & + & ± & + \\
& C. bifermentans & VPI 6903 & + & ± & + \\
& C. butyricum & VPI 6903 & + & ± & + \\
& C. celatum & VPI 6903 & + & ± & + \\
& C. paraperfringens & ATCC 27639 & + & ++ & ++ \\
& C. sordellii & VPI 2984 & - & - & - \\
& C. sporogenes & E-17 & - & - & - \\
\hline
\end{tabular}
\end{table}

\^As measured with the a-naphthol test reagent after 24 h incubation at 35 \(^\circ\)C.
\^aSame as SMN except 0.25\% \text{Na}_2 \text{HPO}_4 \text{ was added.}
\^cSymbols: Maximum nitrite concentration > 50 \text{mg/ml} = ++++; strong nitrite test equivalent to 25 \text{mg/ml} = +++; positive test equivalent to 6 \text{mg/ml} = ++; weak positive test equivalent to 3 \text{mg/ml} = +; trace, limit of the test = ±; no detectable nitrite, i.e. <1 \text{mg/ml} = -. 

test as shown in Table 1. Comparative testing also showed that the performance of SMN medium was improved by the addition of 0.25\% \text{Na}_2 \text{HPO}_4 \text{ (BSMN) and that satisfactory results were obtained in the modified medium referred to as BSMN when N-(1-naphthyl) ethylene diamine dihydrochloride was used as the test reagent instead of a-naphthol. Both of these reagents have been recommended as suitable substitutes for a-naphthylamine, a carcinogen which could represent a hazard to laboratory personnel (3,5).

The results of the comparative tests were substantiated by further testing SMN and the two buffered modifications with a total of 108 \text{C. perfringens} isolates and 21 strains of culturally similar clostridia. Results of these tests indicated that the BMN and BSMN media were more suitable for conducting the motility and nitrate reduction tests with \text{C. perfringens} because of the better growth response and higher nitrite levels produced by many of the strains.

Results of these tests also showed that like \text{C. perfringens}, all of the \text{C. absonum}, \text{C. celatum}, and \text{C. paraperfringens} cultures were non-motile and reduced nitrate to nitrite; thus, they could not be distinguished from \text{C. perfringens} without doing additional tests. However, these two tests were important for distinguishing the \text{C. sordellii} strains which were weakly motile and usually produced only a small amount of nitrite.

Carbohydrate fermentation and gelatin liquefaction

The data presented in Table 2 show the response of 43 strains of \text{C. perfringens} and 21 strains of other clostridia in lactose-gelatin (6) and in a salicin-gelatin medium which was developed during this study. Lactose was fermented by all of the test organisms except \text{Clostridium bifermentans} and \text{Clostridium sporogenes}, which were included as negative controls. Gelatin was liquefied within 48 h by all except two strains of \text{C. perfringens} but was usually not liquefied by the culturally similar clostridia. A few strains labeled \text{C. absonum} or \text{C. sordellii}, as well as six recent isolates characteristic of these species, liquefied gelatin slowly in lactose-gelatin medium. It was also noted that lactose-gelatin cultures of \text{C. perfringens} were densely turbid while those of culturally similar species were clear, with cells sedimented to the bottom. The phenol red indicator in lactose-gelatin cultures was often completely reduced, giving a false impression that acid was produced from lactose. Because we encountered difficulty in obtaining gelatin which retained a suitable viscosity after autoclaving the lactose-gelatin medium, we recommend expanded testing of isolates of special interest which react atypically in this medium.

An attempt was made to combine tests for fermentation of either salicin or raffinose with that for gelatin liquefaction. Raffinose was utilized too slowly to allow rapid liquefaction of gelatin. Peptone yeast extract (PY) medium supplemented with salicin and gelatin could be used for performing these tests at the same time. but sterile lactose had to be added to the culture after 24 h of incubation to encourage gelatin liquefaction. Because of this requirement, there appears to be little advantage in use of a salicin-gelatin medium. Similar results were also obtained when thio-gel or lactose-motility (LM) broth (11) was used as the basal medium.

However, rapid fermentation of salicin in PY medium was found to be useful for differentiating \text{C. perfringens} from culturally similar species (5). All of the culturally similar organisms tested were invariably positive, while only 13\% of 108 \text{C. perfringens} isolates produced acid from salicin and most of those were weakly positive. To test for acid, we preferred spot testing a 2-mm loopful of

\begin{table}
\centering
\caption{Reactions of 43 \text{C. perfringens} strains and 8 other clostridia in lactose-gelatin and salicin-gelatin media.}
\begin{tabular}{lcccc}
\hline
Organism & No. of strains & Lactose-gelatin & Salicin-gelatin & \\
& & Lactose & Gelatin & Salicin & \\
\hline
\text{C. perfringens} & 38 & + & + & + \\
& 1 & + & + & + \\
& 2 & + & - & - \\
& 3 & - & - & - \\
& 6 & + & - & - \\
& 3 & + & + & + \\
& 3 & - & - & - \\
\hline
\text{C. sporogenes} & 2 & + & - & - \\
\hline
\end{tabular}
\end{table}

\^aAfter 48 h of incubation at 35 \(^\circ\)C.
\^bSterile lactose was added after 24 h of incubation to encourage gelatin liquefaction.
\^cMeans carbohydrate was fermented or gelatin was liquefied.
\^dMeans carbohydrate was not fermented or gelatin was not liquefied.
culture on filter paper saturated with a 0.2% solution of bromthymol blue. Production of acid from raffinose in PY medium was even more consistent with the C. perfringens strains. Only one C. perfringens strain failed to produce acid from raffinose. Of the culturally similar clostridia, one strain, C. paraperfringens 4446, produced acid from raffinose, but it also rapidly fermented salicin. Thus, it was concluded that fermentation tests in PY medium were useful for differentiating C. perfringens from culturally similar clostridia when the tests were used in combination with BMN medium and lactose-gelatin.

Antitoxin half-plate

Results in Table 3 show the representative strains of C. perfringens and the culturally similar Clostridium species on the antitoxin half-plate of Willis and Hobbs (12). The response of C. paraperfringens was almost identical to that of some C. perfringens strains. C. perfringens antiserum completely inhibited the lecithinase activity of these strains, thus yielding a false-positive test for C. perfringens. The lecithinase activity of

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of strains</th>
<th>Lactose fermentation</th>
<th>Leicithinase production</th>
<th>Lecithinase inhibition by C. perfringens antiserum (type A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. perfringens</td>
<td>40</td>
<td>+</td>
<td>+</td>
<td>complete</td>
</tr>
<tr>
<td>C. absonum</td>
<td>2</td>
<td>+</td>
<td></td>
<td>partial</td>
</tr>
<tr>
<td>C. bifermantans</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>partial</td>
</tr>
<tr>
<td>C. celatum</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>partial</td>
</tr>
<tr>
<td>C. paraperfringens</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>complete</td>
</tr>
<tr>
<td>C. sardiniensis</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
</tbody>
</table>

As determined by the method of Willis and Hobbs.

Not applicable.

strains labeled C. absonum and C. sardiniensis was only partially inhibited by C. perfringens antiserum but in other respects the strains were much the same as C. perfringens. The same was true for C. bifermantans although this species was lactose-negative. As noted by Nakamura and his colleagues, the partial inhibition of C. absonum was an indication that this species was not C. perfringens, and we found this to be true of strains designated as C. sardiniensis (10). The antitoxin half-plate was capable of differentiating C. celatum from C. perfringens because C. celatum invariably failed to produce lecithinase. However, this test would give false negative results with the lecithinase-negative C. perfringens strains. Some workers have referred to lecithinase-negative strains as C. plagurum (9).

When adequate controls were utilized, the antitoxin half-plate differentiated several culturally similar clostridia from C. perfringens; however, both false positive and false negative tests occurred with some culturally similar clostridia. In fact, the response of some of our cultures was so similar to C. perfringens that we suggest that supplementary tests be performed with isolates whenever it is at all likely that culturally similar species are present.

Application of the confirmatory tests

Results obtained in this study show that BMN medium and BSMN medium in combination with lactose-gelatin were the most satisfactory media for confirming isolates of C. perfringens from selective sulfite agars. Additional tests may be occasionally necessary to assure that isolates actually are C. perfringens because of the similarity of reactions obtained with other species such as C. absonum, C. celatum, and C. paraperfringens. These species were distinguished from C. perfringens only by their inability to liquefy gelatin within the 44-h incubation period specified for this test. In addition, the C. sardiniensis strains fermented lactose, and three of the six strains liquefied gelatin within 48 h. These strains reduced nitrate to nitrite and could only be distinguished from C. perfringens by a weakly positive motility test. For these reasons, it is suggested that supplementary tests for fermentation of salicin and raffinose be employed when doubtful results are obtained with either the motility-nitrate or lactose-gelatin media. Isolates which rapidly ferment salicin and fail to produce acid from raffinose in PY medium within 3 days may be assumed to be one of the culturally similar species.

However, when test results are clear-cut, gram-positive, obligately anaerobic non-motile bacteria which reduce nitrate to nitrite, ferment lactose, and liquefy gelatin within 48 h should be provisionally identified as C. perfringens. Only two of 108 C. perfringens isolates tested failed to give this result on repeated testing. These two strains failed to liquefy gelatin in the lactose-gelatin medium in fewer than 3 days and then did so feebly. Our results with these “atypical” C. perfringens strains were confirmed by Lillian V. Holdeman at the Anaerobe Laboratory, VPI and SU, Blacksburg, VA (personal communication). As more experience is gained with the newly defined species, other tests may prove to be more appropriate for confirming isolates as C. perfringens: however, at the present time the tests recommended as part of the AOAC and APHA methods appear to be adequate (2, 5).

REFERENCES