Comparison of Methods for Isolation and Confirmation of Clostridium perfringens from Spices and Herbs

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ABSTRACT

Rapid Perfringens Medium (RPM), Perfringens Enrichment Medium (PEM) and Tryptose Sulfite Cycloserine agar medium (TSC) were compared for determination of Clostridium perfringens in spices and herbs. Of 147 samples, 62 (42%) contained C. perfringens when RPM was used; lower percentages of positive isolations were found with PEM (23%), TSC surface plate (19%) and TSC pour plates (26%). Heat-treatment of sample suspensions yielded additional isolates. C. perfringens was isolated by one or more of the techniques from 43 (80%) of 54 different kinds of spices and herbs and from 86 (59%) of 147 samples. Replacing glucose in RPM with raffinose and polymyxin and neomycin with cycloserine did not improve the efficacy of this medium. A good correlation was found between conventional confirmation tests for C. perfringens and tests for acid phosphatase, lecithinase, reverse CAMP test and an antiserum test.

Many foods contain small numbers of Clostridium perfringens in the presence of many other bacteria. Foods associated with food poisoning outbreaks caused by C. perfringens usually contain large numbers of this organism. Because a rapid loss of C. perfringens cells occurs in foods if they are frozen or held under prolonged refrigeration, it may be difficult to isolate C. perfringens from these foods.

Several methods are available for quantitation of moderate to high numbers of C. perfringens in foods, whereas only a limited number of enrichment media designed for isolation of low numbers of C. perfringens have been described. The Tryptose-Sulfite-Cycloserine agar pour plate (TSC) is superior to selective enrichment in a liquid sulfite medium, containing polymyxin B sulfate, for isolation of small numbers of C. perfringens from frozen ground beef (2). Erickson and Deibel (3) developed and evaluated Rapid Perfringens Medium (RPM), consisting of fortified litmus milk broth with selectivity based on the antibiotics polymyxin B sulfate and neomycin sulfate. Of 774 naturally contaminated food samples, 546 (71%) contained C. perfringens when RPM was used, whereas only 168 (22%) of the samples were positive when plated on Sulfite-Polymyxin-Sulfadiazine agar. All tubes of RPM demonstrating stormy fermentation were confirmed as containing C. perfringens. Debevere (1) developed a method (PEM) for isolation and determination of small numbers of C. perfringens cells based on enrichment under anaerobic conditions in a fluid thioglycollate medium without glucose, containing 400 μg of D-cycloserine/ml.

In the present study, RPM and PEM were compared for determination of C. perfringens in spices and herbs. TSC agar medium (5), both as a pour plate and as a surface plate, was included in the comparison. For identification of C. perfringens, suspect colonies from selective media are usually confirmed by doing a gram stain (gram-positive rods) and tests for nitrate reduction (+), motility (−), lactose fermentation (+) and gelatin liquefaction (+). The antitoxin half-plate method (12) is also used for confirmation of C. perfringens, although some culturally similar clostridia can produce false-positive results (7).

Another test proposed for rapid identification of C. perfringens is that for acid phosphatase (11). Mead et al. (8) suggested that strains from C. perfringens isolation media should be screened for acid phosphatase activity at the purification stage and only positive strains should be subjected to further tests.

The synergistic reaction between alpha-toxin-producing C. perfringens and CAMP-factor-producing Streptococcus agalactiae has been tested with a large number of C. perfringens strains and other clostridia. Of 123 C. perfringens strains 119 (97%) gave a positive reaction in the reverse CAMP-test, whereas of 45 non-C. perfringens strains all were negative in this test (4). All these identification tests, together with an antiserum test used in our laboratory for presumptive identification of C. perfringens, were compared with clostridial isolates from spices and herbs.

MATERIALS AND METHODS

Samples
A total of 147 samples of 54 different kinds of spices and herbs were purchased from three retail suppliers.

Isolation media
Rapid Perfringens Medium (RPM) was prepared as described by
The medium consists of two solutions (A and B), which were mixed in tubes. Solution A: 140 g of litmus milk powder (Difco) in 1 L of distilled water and sterilized in an autoclave for 5 min at 120°C and cooled. To this solution 150 mg of neomycin sulfate (Sigma) and 25 mg of polymyxin B sulfate (Sigma) were added. Solution B: fluid thioglycollate medium (Difco), 60 g; gelatin, 120 g; peptone, 10 g; glucose, 10 g; K2HPO4, 10 g; yeast extract, 6 g; NaCl, 3 g; ferrous sulfate, 1 g and distilled water, 1 liter. This preparation was boiled gently to dissolve the gelatin, and 5-ml amounts were dispensed in tubes and autoclaved for 5 min at 120°C.

The final medium was prepared by aseptically adding 5 ml of A to each tube of B. The tubes were tightly capped. After adding 1 ml of 1:10 dilutions of the samples in peptone physiological saline solution to RPM tubes, incubation took place at 46°C for 20 h. From RPM tubes showing stormy fermentation, a loopful was streaked on TSC agar plates containing egg yolk.

In an attempt to improve selectivity of RPM for C. perfringens, the glucose in this medium was replaced with raffinose; also neomycin and polymyxin were replaced by D-cycloserine (400 μg/ml). RPM and modifications of this medium were compared for isolation of C. perfringens from 75 samples of spices and herbs. TSC agar medium was inoculated from each enrichment tube. In addition, plates of TSP agar medium (cycloserine in TSC replaced by polymyxin) were inoculated from RPM and from RPM tubes containing cycloserine.

Perfringens Enrichment Medium (PEM) consisted of fluid thioglycollate medium without glucose, containing 400 μg of D-cycloserine/ml (1). Inoculation, incubation and isolation were done as described for RPM, except that PEM was incubated anaerobically by using the GasPak system (BBL).

Tryptose Sulfite Cycloserine (TSC) agar medium was prepared as described by Harmon et al. (5). The TSC surface plates contained egg yolk, whereas the TSC pour plates were used without egg yolk. On the TSC surface plates, 0.1 ml of a 1:10 dilution of the samples was spread, whereas 1.0 ml of this suspension was used in the TSC pour plates. After inoculation, the TSC agar in the plates was overlayed with about 10 ml of sterile TSC agar (without egg yolk). The plates were incubated anaerobically.

**Confirmation tests**

Tests for lactose fermentation, gelatin liquefaction, motility and nitrate reduction were carried out as described by Harmon (6). The antitoxin half-plate method used was based on that described by Harmon and Kautter (7). Petri dishes containing nutrient agar (Oxoid) with 10% egg yolk were used. C. perfringens type A diagnostic serum (Wellcome) was spread over one-half of the medium by using a cotton swab. Test cultures were first grown overnight on neomycin sheep blood agar. Then antitoxin half-plates were inoculated by streaking the test culture in a straight line across the plate from the untreated side. After anaerobic incubation for 20-24 h at 37°C, plates were checked for lecithinase activity and neutralization of this enzyme by the antiserum.

To detect presence of acid phosphatase, the method described by Schallneh and Brandis (10) was used. The test reagent was prepared by dissolving 0.2 g of α-naphthyl-(1)-phosphate (Merck) and 0.4 g of diazonium-iodanesidion (Merck) in 10 ml of 0.2 M acetate buffer (pH 4.5). Colonies from 24-h-old cultures on neomycin sheep blood agar plates were spread on a filter paper and a few drops of test reagent were added. Appearance of a purple or brown-red color within 3 min. was recorded as positive.

The reverse CAMP test (RCT) described by Hansen and Elliott (4) was used (Fig. 1). The culture to be tested was streaked in a straight line on a sheep blood agar plate. An overnight culture of S. agalactiae was then streaked at a right angle to the test culture streak. The streaks did not touch, but were separated by a distance of about 1-2 mm. The plates were incubated anaerobically at 37°C for 20 h. A positive RCT was indicated by formation of an "arrowhead" between the streaks of the test culture and S. agalactiae.

To make the antiserum test (Fig. 2), isolates were streaked in a straight line on a sheep blood agar plate. A strip of filter paper containing a few drops of C. perfringens type A diagnostic serum (Wellcome) was placed across the test culture streaks. After incubation for 24 h at 37°C, an interruption of the zone of hemolysis, where culture and paper strip crossed, indicated the presence of C. perfringens (Fig. 2).

**RESULTS AND DISCUSSION**

**Comparison of media**

C. perfringens was isolated with one or more of the methods from 43 (80%) of 54 different kinds of spices and herbs and from 86 (59%) of 147 total spice and herb samples examined. The numbers of C. perfringens present in the samples were estimated by plating in TSC agar medium. Most of the positive samples contained between 10 and 500 C. perfringens cells per gram. Numbers ex-
ceeding 1000 per gram were found in only three samples, viz. mixed spices (1300/g), ginger (2900/g) and savory (2000/g). Our results indicate a much higher incidence in spices than those reported by Powers et al. (9), who found that the incidence of C. perfringens in spices amounted to 15% (17/114) and numbers ranged from 50-2850/g. With such high incidences of contamination as we found, foods may easily become contaminated with C. perfringens by addition of spices and herbs. If proper time-temperature conditions for heating, cooling, and holding of foods are not applied, a risk of food poisoning by C. perfringens may be created.

**TABLE 1.** Comparison of media for the isolation of Clostridium perfringens from 147 samples of spices and herbs.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Positive isolations</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>number</td>
</tr>
<tr>
<td>RPM</td>
<td>62</td>
</tr>
<tr>
<td>RPM (sample suspension 30 min, 70°C)</td>
<td>54</td>
</tr>
<tr>
<td>PEM</td>
<td>34</td>
</tr>
<tr>
<td>TSC surface plate</td>
<td>28</td>
</tr>
<tr>
<td>TSC pour plate</td>
<td>38</td>
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</tbody>
</table>

The results shown in Table 1 demonstrate that RPM was superior to PEM and TSC agar media for isolation of C. perfringens from spices and herbs. C. perfringens was isolated by using RPM without heat treatment of the sample suspensions, from 62 (42%) of the samples. A total of 41 samples were positive both with and without heat treatment of the sample suspensions. From 21 samples, C. perfringens was isolated only when no heat treatment was applied, whereas a positive isolation was obtained from 13 samples only after heat treatment.

More positive isolations were obtained with the TSC pour plate than with the TSC surface plate, which can be explained by the larger inoculum used in the pour plate. Because organisms other than C. perfringens can also produce black colonies on the TSC plates, it was necessary to confirm a rather large number of colonies from these plates. Other clostridia, such as C. paraperfringens (gelatin-negative), C. beijerinckii (nitrate-negative, motility-positive), C. sordelli and C. bifermaments (both lactose negative, motility-positive), as well as Lactobacillus and Bacteroides species were isolated from the TSC plates.

Table 2 shows that modifications of the RPM medium did not result in increased isolation of C. perfringens from the samples examined. RPM and RPM with raffinose did not give false-negative results, whereas RPM with cycloserine gave false-negative results with 5 samples (7%). In these experiments, RPM gave a higher number of false-positive results (9%) than RPM with raffinose (3%). Although the number of typical colonies on TSC and TSP agar media differed only slightly, the interference by other flora was much greater on the polymyxin-containing medium than on the medium with cycloserine.

From the data presented here, it can be concluded that RPM is a suitable medium for screening of C. perfringens in spices and herbs. RPM tubes showing stormy fermentation strongly indicate presence of C. perfringens. However, because false-positive results may occur, confirmation tests are necessary. A disadvantage of RPM is the rather laborious preparation of the medium, though large quantities can be made because the medium can be stored at 4°C for an indefinite period (3). An important advantage is that anaerobic incubation is not needed.

Additional research is needed to evaluate the usefulness of RPM for detection of C. perfringens in other foods, especially non-dry foods like meat, chicken, soups and milk products.

**Utility of confirmation tests**

There was a good correlation between conventional confirmation tests for C. perfringens (LGMN) and the acid phosphatase, lecithinase, reverse CAMP and antiserum tests (Table 3). No false-negative results were found with the latter tests and in only a few instances were apparently false-positive results obtained. All of the tests other than LGMN gave dubious results with a limited number of strains, especially with C. paraperfringens isolates, which gave false-positive results with the reverse CAMP and antiserum tests. Although good results were obtained with the antiserum and the lecithinase tests, the disadvantage of these tests is the need for C. perfringens - antiserum. Use of this serum is not necessary in the phosphatase and RCT-tests, and an additional advantage of the phosphatase-test is that overnight incubation is not necessary. Therefore the acid phosphatase and the reverse CAMP test can be recommended as alternative tests for presumptive identification of C. perfringens.

**TABLE 2.** Comparison of RPM and modifications of RPM for the isolation of Clostridium perfringens from 75 samples of spices and herbs.

<table>
<thead>
<tr>
<th></th>
<th>Number of samples</th>
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<tbody>
<tr>
<td></td>
<td>RPM + raffinose</td>
</tr>
<tr>
<td>RPM</td>
<td>41</td>
</tr>
<tr>
<td>RPM + raffinose</td>
<td>39</td>
</tr>
<tr>
<td>RPM + D-cycloserine</td>
<td>24</td>
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*aFrom the RPM tubes a loopful was streaked on TSC agar medium. Typical colonies were identified using tests for nitrate reduction, motility, lactose fermentation and gelatin liquefaction.
TABLE 3. Comparison of confirmation tests for Clostridium perfringens with 100 isolates from spices and herbs.

<table>
<thead>
<tr>
<th>Antiserum test</th>
<th>Acid phosphatase</th>
<th>Lechhimase test</th>
<th>Reverse-CAMP</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>x</td>
<td>x</td>
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LGMNb

- Black colonies on TSC agar plates.
- Lactose fermentation, gelatin liquefaction, motility, nitrate reduction.
- LGMN typical for C. perfringens.
- One or more tests of LGMN not typical for C. perfringens.

REFERENCES