RAPID METHODS FOR THE IDENTIFICATION OF CLOSTRIDIA[1, 2]

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Methods presently in use for the identification of clostridia are time consuming and sometimes unreliable. Our studies have resulted in the development of rapid and apparently reliable methods for the detection of carbohydrate fermentation and for the simultaneous detection of gelatin hydrolysis and indole production.

MATERIALS AND METHODS

Preparation of inocula. In one procedure, a cell suspension was made by centrifuging a 10 ml 15- to 18-hr culture in brain heart infusion broth (Difco) that had been grown in a hydrogen jar, and resuspending the cells in 3 ml of the supernatant. If the suspension was to be used in fermentation studies, one drop of sterile 0.04 per cent aqueous phenol red and enough sterile N NaOH to produce neutrality were added. In a second procedure, colonies were cut from a liver veal agar (Difco) plate that had been capped with a layer of the same medium, and transferred to a tube containing 2.5 ml of distilled water, 2 to 6 colonies being used, depending upon the size of the colonies. The tube was shaken to separate the agar cap from the base and to wash off the organisms to form a suspension.

Procedure for fermentation studies. Two-tenths ml of the cell suspension was placed in a tube (10 by 75 mm). One ml of test medium was added to the tube and it was incubated in a 37 C water bath. Cystine trypticase agar (BBL) plus 0.1 per cent sodium thioglycolate, adjusted to pH 7.5 and sterilized, was used as the basic test medium. When the medium was to be used, filter-sterilized carbohydrate was added to a concentration of 2 per cent. The medium was steamed for 15 min, and used as soon as it had cooled sufficiently. The cultures were examined for acid production at frequent intervals for 10 to 12 hr and a final observation was made after 24 hr.

Procedure for gelatin hydrolysis and indole formation studies. One ml of medium containing 1.0 per cent gelatin, 1.0 per cent agar, and 0.1 per cent sodium thioglycolate was placed in a tube (10 by 75 mm). The medium was inoculated, after solidification, by placing 0.2 ml of the cell suspension on the surface. The inoculum was then capped with 0.5 ml of pH 7.4 agar containing 0.06 per cent tryptophan and 0.1 per cent sodium thioglycolate, and the culture was incubated in a 37 C water bath. Cultures were tested after various incubation periods. For practical purposes, testing duplicate cultures after 4 and 24 hr seemed to be satisfactory.

A single test reagent to detect both the presence of indole and the hydrolysis of gelatin was obtained by mixing one part of Kovacs' (1928) reagent, made with butyl alcohol, with three parts of Frazier's (1926) reagent. Frazier's reagent may be kept at room temperature for a considerable period; Kovacs' reagent is stable for at least 1 month in a brown bottle in a refrigerator. These reagents should be mixed fresh just before use or stored in a refrigerator for not over 1 week in a tightly stoppered container protected from light.

To perform the test, a capillary pipette containing the test reagent was inserted through the cap and sufficient test reagent was released to force the cap upward approximately 5 mm. The appearance, within 5 min, of a clear zone in the upper part of the gelatin-agar column shows hydrolysis of gelatin. Indole formation is shown by a pink color in the butyl alcohol globules stratified on the surface of the test solution or caught in the cap medium. A pink color in the aqueous phase of the test solution is not indicative of indole formation. It is thought to be due to an unidentified product of tryptophan de-

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TABLE 1

Fermentation studies with rapid method and Reed and Orr (1941) procedure

Clostridium Species	Glucose			Maltose			Lactose			Sucrose			Mannitol			Glycerol		
	a*	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
C. bifermentans	1†	2	2	2	2	2	0	0	0	0	0	0	0	0	0	2	2	5
C. bifermentans (C. sordelli)	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	1	2	5
C. botulinum type B	1	4	5	1	4	5	0	0	0	0	0	0	0	0	0	1	4	5
C. botulinum type C	2	2	2	2	2	2	5‡	0	0	5‡	0	0	0	0	0	2	2	5
C. capitovale	1	4	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. carnis	2	2	4	3	2	4	0	0	0	3	2	4	0	0	0	5‡	0	0
C. difficile	2	4	4	0	0	0	0	0	0	0	0	0	5	3	0	0	5	0
C. histolyticum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. parabotulinum type A	1	3	3	1	3	3	0	0	0	0	0	0	0	0	0	1	3	5
C. perfringens	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1
C. septicum	2	2	4	2	3	4	2‡	0	4	0	0	0	0	0	0	5‡	0	5
C. sporogenes	1	3	4	1	2	4	0	0	0	0	0	0	0	0	0	0	0	0
C. tetani	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. tetanomorphum	2	2	4	2	2	4	4	4‡	5	0	0	0	4‡	0	5	0	0	0

* a = Rapid test; cell suspensions from broth cultures; b = rapid test; cell suspensions from colonies; and c = Reed and Orr procedure.

† 1 = fermentation in 3 hr or less; 2 = fermentation in 6 hr; 3 = fermentation in 9 hr; 4 = fermentation in 12 hr; 5 = fermentatation in 24 hr; and 0 = no fermentation in 24 hr.

‡ Fermentation variable in repeated tests.

composition. It is not indolepropionic acid, indoleacetic acid, or skatole since they are more soluble in the alcohol phase than in the aqueous phase.

RESULTS

Typical results of fermentation studies using our rapid method as described with the two types of cell suspensions and the Reed and Orr (1941) procedure are given in table 1. Except as indicated, the results have been repeatable in several trials. Our procedure gave results considerably more rapidly than did the Reed and Orr procedure, in a few cases showing fermentation within 1.5 hr. Fermentations were slightly slower with the less dense suspensions from the colonies. The final results with the three procedures were identical except with a few reactions that have been described in the literature as variable. Our procedure with the more dense suspensions from the broth cultures appeared to be the most sensitive since it gave positive results most frequently with the organisms that gave variable reactions.

TABLE 2

Studies with rapid method and Reed and Orr (1941) procedures

Clostridium Species	Gelatin Hydrolysis		Indole Production	
	Rapid test procedure	Reed and Orr procedure	Rapid test procedure	Reed and Orr procedure
C. bifermentans	3.5*	11	0.25	4.5
C. bifermentans (*C. sordelli*)	2.0	10	0.25	5.0
C. botulinum type A	0.75	9	—	—
C. botulinum type B	0.1	2.5	—	—
C. botulinum (nontoxic)	0.75	5	—	—
C. capitovale	—†	—	0.25	24
C. histolyticum	0.25	8	—	—
C. perfringens	3.5	10	—	—
C. sporogenes	1.5	11	—	—
C. sporogenes	3.0	18	—	—
C. tetani	21	48	22	24
C. tetani	21	60	22	24

* Hours required for positive test.

† — = negative after 24 hr.

Typical results of a comparison of our procedure for the detection of gelatin hydrolysis and indole formation with the procedures of Reed and Orr (1941) are shown in table 2. Except for the strains of *Clostridium tetani*, gelatin hydrolysis was demonstrated by our procedure in from 5 min to 3.5 hr. and indole production within 25 min. The slowness of the reactions with the *C. tetani* may be diagnostic.

In addition to giving reliable results with stock cultures, as shown in tables 1 and 2, our rapid methods have proved to be useful in the identification of cultures of *Clostridium perfringens* and *Clostridium sporogenes* from primary isolation colonies. The reactions with the newly isolated strains have been at least as rapid as those with the stock cultures.

SUMMARY

Rapid methods for the detection of carbohydrate fermentation and for the simultaneous detection of gelatin hydrolysis and indole formation by clostridia have been described. The methods have the advantages of being easily performed, using small amounts of material, and giving rapid, reliable results.

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