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New Presumptive Identification Test for *Clostridium perfringens*: Reverse CAMP Test

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The reverse CAMP test proved to be a highly sensitive test since 97.0% of all *Clostridium perfringens* cultures tested gave an easily discernible positive reaction.

Clostridium perfringens, although part of the normal intestinal flora of man, can cause a variety of diseases (1, 3, 5, 20). Detection of this organism in clinical specimens has been aided by improved techniques for achieving anaerobiosis and the development of several biochemical tests to differentiate it as to species from other clostridia (7, 8). One such test, the Nagler reaction, was devised for the identification of *C. perfringens* and other lecithinase-producing clostridia (17). Unfortunately, this test is not specific for *C. perfringens*, as the lecithinases of other clostridia also give a positive Nagler reaction (9, 16).

In the usual characterization tests, *C. perfringens* gives results similar to those of several other species (10, 12, 13). The complexity of easily differentiating *C. perfringens* from other sulfite-reducing, sporeforming anaerobes is a prime reason why an indicator system for fecal pollution of water using *C. perfringens* has not been widely adopted in the United States, although such systems have been used elsewhere (2, 4). Therefore, although improved identification tests for *C. perfringens* have been developed, none is totally reliable when used alone.

Gubash (11) noted synergistic hemolysis by alpha-toxin-producing *C. perfringens* and beta-hemolytic *Streptococcus agalactiae* on human blood agar. The characteristic arrowhead-shaped, synergistic hemolytic pattern between these two organisms provided a reliable test for the presumptive identification of group B streptococci. This observation prompted us to determine whether the synergistic action of the alpha-toxin of most *C. perfringens* strains and the CAMP factor of *S. agalactiae* is a reproducible reaction limited to *C. perfringens* among the clostridia.

The initial cultures of clostridia used in this work were isolated from water and sewage samples taken from the Barren River near the Bowling Green, Ky. sewage treatment plant. Sulfite-reducing bacteria were isolated in egg yolk-free tryptose-sulfite-cycloserine agar pour plates (13) which were incubated anaerobically in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) at either 37 or 45°C. Resulting black colonies were gram stained and identified as *C. perfringens* if they produced a typical double zone of beta-hemolysis on Trypticase soy sheep blood agar (BBL) plates and gave appropriate reactions in supplemented nitrate-motility medium (13) and lactose-gelatin medium (14). If equivocal reactions were obtained in these media, fermentation products were determined by gas-liquid chromatography (15) or additional characteristics were tested with the BBL-Minitek system (19).

One strain of *S. agalactiae*, obtained from the microbiology laboratory at Greenview Hospital, Bowling Green, Ky. and shown to produce the CAMP factor was used as the indicator organism. Strains of clostridia tested are listed in Table 1. Overnight cultures of the clostridial isolates grown anaerobically on egg yolk-free tryptose-sulfite-cycloserine or sheep blood agar plates or in Fluid Thioglycolate Medium (Difco Laboratories, Detroit, Mich.) were used for the test inoculum. Part of a colony or a loopful of liquid culture was transferred to a sheep blood agar plate (BBL or GIBCO Diagnostics, Madison, Wis.) and streaked in a straight line near the center of the plate. Four cultures could be accommodated per plate (Fig. 1). Part of a colony of an overnight (or older) culture of *S. agalactiae* was then streaked at right angles to each of the clostridial streaks. Care was exercised so that the streaks did not touch, but were separated by a distance of about 1 to 2 mm. The inoculated plates were incubated anaerobically in GasPak jars at 37°C for 18 to 24 h and then examined and interpreted. A positive (+) reverse CAMP test (RCT) was indicated by the formation of an "arrowhead" between the streaks of the clostridial isolate and *S. agalactiae*, a nega-

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TABLE 1. *Source of Clostridium species tested using the reverse CAMP test*

Source	Species	No. of isolates
Sewage-water samples, Bowling Green, Ky.	*C. perfringens*	123
Sewage-water samples, Bowling Green, Ky.	Non-*C. perfringens*	45[a]
Clinical samples, Baptist Hospital, Nashville, Tenn.	*Clostridium sporogenes*	1
	Clostridium difficile	1
	Clostridium tetani	1
	C. tertium	1
	C. bifermentans	1
	C. novyi	1
	C. sordellii	1
	Clostridium septicum	1
Clinical samples, VA Hospital, Durham, N.C.	*Clostridium cadaveris*	1
	C. sordellii	1
	C. tetani	1
	C. ramosum	1
	C. perfringens	1
Clinical samples, Dalhousie University, Department of Microbiology, Halifax, Nova Scotia, Canada	*C. perfringens*	11[b]
Food-animal samples, Food Research Institute, University of Wisconsin, Madison, Wis.	*C. perfringens*	4[c]
Stock strains, American Type Culture Collection, Rockville, Md.	*Clostridium paraperfringens*	2[d]

[a] Includes seven randomly picked organisms identified as *C. bifermentans*.
[b] Includes one isolate each of strains 2, 4, 28, 43, 48, 55, 63, 73, 75, 78, and 96.
[c] Includes one isolate each of strains CW143, CW569, CS459, and NCTC8798.
[d] Includes one isolate each of strains ATCC 27639 and ATCC 27640.

tive (−) RCT was indicated by the lack of any zone of beta-hemolysis, and a bullet-shaped zone of synergistic beta-hemolysis was denoted as a plus-minus (+/−) RCT. Figure 1 illustrates the three types of reactions obtained in an actual test plate.

The initial RCTs were done on clostridia isolated from river water and sewage treatment plant effluent. Of 123 cultures identified as *C. perfringens*, 119 (96.8%) gave a positive RCT. Of the 45 environmental isolates identified as clostridia other than *C. perfringens*, none gave a positive RCT, whereas three such isolates were RCT +/−. Only one non-*C. perfringens* isolate gave a +/− RCT. This organism was identified

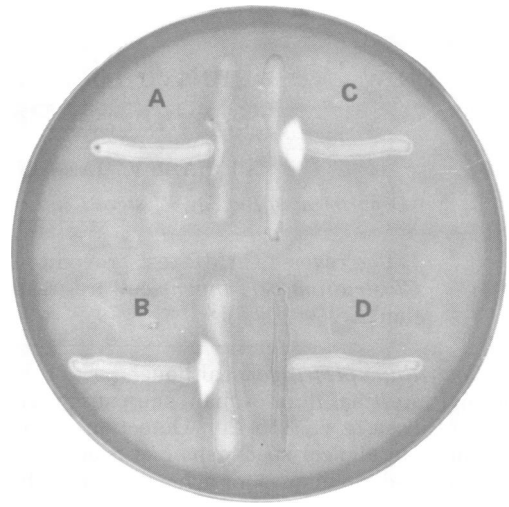

FIG. 1. *Zones of synergistic beta-hemolysis obtained with bacterial test isolates on sheep blood agar plates after 18 to 24 h of incubation in GasPak jars at 37°C. (A) C. perfringens no. 271 sewage isolate, +/− RCT; (B) C. perfringens strain 2 clinical isolate, + RCT; (C) C. perfringens NCTC8798 food isolate, + RCT; (D) C. bifermentans clinical isolate, − RCT.*

TABLE 2. *Results of RCT trials on sewage-water Clostridium species isolates*

Organism (no. of isolates)	No. of isolates with the following RCT result[a]		
	+	−	+/−
C. perfringens (123)	119 (96.8)	1 (0.8)	3 (2.4)
Non-*C. perfringens* (45)	0 (0.0)	44 (97.8)	1 (2.2)

[a] Numbers within parentheses indicate the percent of group giving a particular RCT result.

as *Clostridium beijerinckii* by the cultural, staining, and growth characteristics in *Bergey's Manual of Determinative Bacteriology* (18) (Table 2).

All *C. perfringens* cultures obtained from contributing laboratories gave positive RCTs. Likewise, all such clostridia other than *C. perfringens* gave negative RCTs.

This is, to our knowledge, the first time that the synergistic reaction between alpha-toxin-producing *C. perfringens* and CAMP factor-producing *S. agalactiae* has been tested with a large number of *C. perfringens* strains and other clostridia. Gubash (11), in his paper introducing the phenomenon of the synergistic hemolytic reaction, mentioned that the test described (an improved version of the CAMP test [6]) might be useful for identifying *C. perfringens* as well as Lancefield group B beta-hemolytic streptococci

on primary isolation, but apparently did not pursue this further.

The three strains of *C. perfringens* and the other clostridial culture that gave consistent +/− RCT results were interesting in that they gave zones of hemolysis similar to those described by Gubash (11) as occurring between *C. perfringens* and a Lancefield group A streptococcus, *Streptococcus pyogenes*. However, the *S. pyogenes* tested in this study (included initially as a negative synergistic beta-hemolysis control, but omitted during routine tests) did not give +/− RCT results.

Since Gubash presumed that the group A streptococci might have produced small amounts of the CAMP factor which produced the unique crescent-shaped zones of hemolysis, it is assumed that the peculiar +/− reactions observed in our experiments were due to some factor involving the clostridial strains. This follows since three different strains of group B streptococci used in all our tests had been shown to produce adequate amounts of the CAMP factor. The particular strains of *C. perfringens* that did not produce a good arrowhead may have produced smaller amounts of alpha-toxin. Alternatively, less than optimal ratios of enzyme to CAMP factor may have resulted in the +/− tests. On the other hand, the non-*C. perfringens* culture that gave a +/− test may have been producing low levels of the alpha-toxin. The single isolate of *C. perfringens* that failed to elicit any zone of synergistic hemolysis was presumed to be an alpha-toxin-negative strain, although the culture was lost before this was confirmed serologically.

It is not known how many clostridial species were represented in the 45 cultures obtained from the river, since the study was concerned primarily in identifying *C. perfringens*. However, three lecithinase-positive species (*Clostridium bifermentans*, *Clostridium sordellii*, *Clostridium novyi*), and two lecithinase-negative species (*Clostridium ramosum*, *Clostridium tertium*) were among them. Since *C. perfringens* is one of the clostridia most commonly encountered in clinical specimens, a simple and readily available test for distinguishing it from other clostridia would be valuable.

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LITERATURE CITED

1. **Akama, K., and S. Otani.** 1970. *Clostridium perfringens* as the flora in the intestine of healthy persons. Jpn. J. Med. Sci. Biol. **23**:161–175.
2. **Bisson, J. W., and V. J. Cabelli.** 1979. Membrane filter enumeration methods for *Clostridium perfringens*. Appl. Environ. Microbiol. **37**:55–66.
3. **Brummelkamp, W. H.** 1974. Treatment of anaerobic infections, p. 522–523. *In* A. Balows, R. M. DeHaan, V. R. Dowell, Jr., and L. B. Buze (ed.), Anaerobic bacteria: role in disease. Charles C Thomas Co., Springfield, Ill.
4. **Cabelli, V. J.** 1977. *Clostridium perfringens* as a water quality indicator, p. 65–79. *In* A. W. Hoadley and B. J. Dutka (ed.), Bacterial indicators/health hazards associated with water, STP 635. American Society for Testing and Materials, Washington, D.C.
5. **Center for Disease Control.** 1971. CDC foodborne outbreaks annual summary. Center for Disease Control, 1972. Atlanta, Ga.
6. **Darling, C. L.** 1975. Standardization and evaluation of the CAMP reaction for the prompt presumptive identification of *Streptococcus agalactiae* (Lancefield group B) in clinical material. J. Clin. Microbiol. **1**:171–174.
7. **Debevere, J. M.** 1979. A simple method for the isolation and determination of *Clostridium perfringens*. Eur. J. Appl. Microbiol. **6**:409–414.
8. **Erickson, J. E., and R. H. Diebel.** 1978. New medium for rapid screening and enumeration of *Clostridium perfringens* in foods. Appl. Environ. Microbiol. **36**:567–571.
9. **Finegold, S. M., W. J. Martin, and E. G. Scott.** 1978. Bailey and Scott's diagnostic microbiology, 5th ed., p. 231–232. C.V. Mosby Co., St. Louis, Mo.
10. **Fraser, A. G.** 1978. Neuraminidase production by clostridia. J. Med. Microbiol. **2**:269–280.
11. **Gubash, S. M.** 1978. Synergistic hemolysis phenomenon shown by an alpha-toxin-producing *Clostridium perfringens* and streptococcal CAMP factor in presumptive streptococcal grouping. J. Clin. Microbiol. **8**:480–488.
12. **Hauschild, A. H. W., R. J. Gilbert, S. M. Harmon, M. F. O'Keefe, and R. Vahlafeld.** 1977. ICMSF methods studies. VIII. Comparative study for the enumeration of *Clostridium perfringens* in foods. Can. J. Microbiol. **23**:884–892.
13. **Hauschild, A. H. W., and R. Hilsheimer.** 1974. Evaluations and modifications of media for enumeration of *Clostridium perfringens*. Appl. Microbiol. **27**:78–82.
14. **Hauschild, A. H. W., and R. Hilsheimer.** 1974. Enumeration of foodborne *Clostridium perfringens* in egg yolk-free tryptose sulfite cycloserine agar. Appl. Microbiol. **27**:521–526.
15. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
16. **McClung, L. S., and R. Toabe.** 1947. The egg yolk plate reaction for the presumptive diagnosis of *Clostridium sporogenes* and certain species of gangrene and botulinum groups. J. Bacteriol. **53**:139–147.
17. **Nagler, F. P. O.** 1939. Observations on a reaction between the lethal toxin of *C. welchii* (type A) and human serum. Br. J. Exp. Pathol. **20**:473–485.
18. **Smith, L. D. S., and G. Hobbs.** *Clostridium*, p. 553. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.
19. **Stargel, W. D., F. S. Thompson, S. E. Phillips, G. L. Lombard, and V. R. Dowell, Jr.** 1976. Modification of the minitek miniaturized differentiation system for characterization of anaerobic bacteria. J. Clin. Microbiol. **3**:29–30.
20. **Sutton, R. G. A., and B. C. Hobbs.** 1968. Food-poisoning caused by heat sensitive *Clostridium welchii*: a report of five recent outbreaks. J. Hyg. **66**:135–146.