Synergistic haemolysis test for presumptive identification and differentiation of *Clostridium perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens*

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SUMMARY A new test for the presumptive identification of *Clostridium perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens* is described. The test is based on the synergistic haemolysis shown by the clostridia and group B streptococci on sheep and human and CaCl₂-supplemented human blood agar. *C. perfringens* gave crescent-shaped synergistic lytic zones (7 to over 10 mm in length), and *C. paraperfringens* usually small-sized (3 mm), bullet-shaped reactions on all three types of media. *C. bifermentans* showed a horseshoe-shaped synergistic reaction only on human blood containing media, and *C. sordellii* only on CaCl₂-supplemented human blood agar. *C. perfringens* type A antiserum inhibited synergistic lytic activities of the four species. The test provided a reliable method for presumptive identification and differentiation of the four clostridial species and may obviate the need for the Nagler test.

Most clinical microbiology laboratories use the Nagler test^{1,2,3} for presumptive identification of *Clostridium perfringens* and other phospholipase C (lecithinase C) producing clostridia. Phospholipase C hydrolyses phosphatidylcholine (lecithin) to phosphorylcholine and a water-insoluble diglyceride⁴ that manifests as a precipitation of a 'lecithovitelin complex' in the egg-yolk or human serum containing media.⁵ Phospholipase C activity of *C. perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens* can be inhibited by *C. perfringens* alpha-antitoxin.^{1,2,6} A further differentiation within this group of clostridia must be made by the use of several other criteria, such as lactose fermentation and production of gelatinase, tryptophanase, and urease.^{7,8} *C. absonum* phospholipase C activity is only slightly inhibited by the alpha-antitoxin,⁶ and phospholipase C produced by some other clostridia, for example, *C. novyi*, is not antigenically related to that of *C. perfringens*.^{4,9}

The purpose of this study is to offer a simple test as an aid in the presumptive identification of *C. perfringens* and other clostridia that produce anti-

genically related phospholipase C. The test is based on the synergistic haemolysis due to the interaction of the CAMP-factor of group B streptococci and clostridial phospholipase C on sheep blood agar (SBA), human blood agar (HBA), and CaCl₂-supplemented human blood agar (CaHBA). The lytic phenomenon and its application in the presumptive grouping of beta-haemolytic streptococci were described earlier.¹⁰

Material and methods

BACTERIA

In this study, 81 isolates of *C. perfringens*, 10 of *C. bifermentans*, 11 of *C. sordellii*, and four of *C. paraperfringens* were used. All isolates, except for five *C. bifermentans* and six *C. sordellii*, were obtained from routine clinical specimens from different patients in the Kingston General Hospital Microbiology Laboratory over a period of four years. D Muncey, of the Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, donated one *C. bifermentans* (E41) and four *C. sordellii* (E16, E17, E18, and E19) strains; DE Mahony, of the Microbiology Department, Dal-

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housie University, Halifax, Nova Scotia, donated three *C. bifermentans* (1124, 2221A, and 2549) and one *C. sordellii* (9714) strain, and M Magus, of the Special Reference Bacteriology Laboratory Services Branch, Toronto, Ontario, donated one *C. bifermentans* (SC286) and one *C. sordellii* (SL279) strain. The group B streptococcus was isolated from a routine clinical specimen.

Identification of the clostridia to the species level was based on Gram stain, spore formation, colony morphology, and type of haemolysis on human and sheep blood agar, half-antitoxin Nagler plate, lactose fermentation, gelatinase, tryptophanase, and urease activity.^{7,8} Motility tests were not performed on the majority of isolates. Routine clinical isolates were kept in chopped meat broth as stock cultures, and all donated strains came in lyophilised form. During the study all isolates were kept on blood agar plates in air at 4°C and subcultured on a fresh blood agar before testing.

MEDIA

Egg-yolk emulsion agar was a modified McClung and Toabe medium,³ using a fresh hen's egg and a tryptose blood agar base (Oxoid, London, UK).

For the synergistic haemolysis test, 15-20 ml of the tryptose blood agar base containing 5% (v/v) of citrated sheep blood (Woodlands Laboratory Ltd, Guelph, Ontario, Canada) or outdated whole human blood bank blood was poured into disposable Petri dishes (15 × 85 mm). Washed erythrocytes were not used. Quality control of the plates was done as described earlier.¹⁰

To overcome chelating activity of sodium citrate in the whole human blood bank blood, and to supply necessary divalent cations for the phospholipase C activity and production^{4,9,11-13} additional sets of plates were supplemented by CaCl₂·2H₂O, MgSO₄·7H₂O, and ZnSO₄·7H₂O. Preliminary experiments indicated that useful concentrations varied in a relatively broad range for each tested salt, but final w/v concentrations in the base medium of 0.066% for CaCl₂, 0.0066% for MgSO₄, and 0.00066% for ZnSO₄ were the most beneficial and were subsequently used in the study. Only one salt was added to one batch of the medium. When blood was mixed with the base medium containing additional CaCl₂, defibrination was done by sterile glass beads to avoid formation of fibrin deposits in poured plates. After initial trials, Ca²⁺ supplemented HBA was chosen as a part of the set of media, and Mg²⁺ supplemented HBA was used only when quality control of the new human blood batch showed some inferiority in the haemolytic patterns. In routine work, HBA could be easily supplemented if the well-dried plates were evenly flooded with 1 ml of sterile aqueous solution

of either 1% CaCl₂ or 0.1% MgSO₄, allowed to soak for about 15 minutes at room temperature, and redried at 37°C.

TESTS

In the half-antitoxin Nagler test, and the synergistic haemolysis test, *C. welchii* (perfringens) type A antiserum (Wellcome Reagents Ltd, Beckenham, UK) was used as a source of the alpha-antitoxin.

Half-antitoxin blood plates (SBA, HBA, CaHBA) were prepared by smearing a swab saturated with the *C. perfringens* alpha-antitoxin, undiluted or diluted 1:6 or 1:10 in sterile broth, over one-half of the surface of the dried plate, which was then redried at 37°C. Two line inocula of group B streptococcus were made, one on the side without, and the other on the side with, the antiserum. Clostridial isolates were streaked at right-angles to the streptococcal lines, without touching, beginning on the antiserum-free side. Inoculated plates were incubated anaerobically overnight at 37°C in Gas Pak jars. Synergistic haemolysis reactions were read as 'large' (over 10 mm in length), 'intermediate' (5-10 mm), 'small' (less than 5 mm), or 'negative' (if no synergistic haemolysis could be detected).

Results

The results of the synergistic haemolysis test and the Nagler test are given in the Table.

In all 81 isolates of *C. perfringens*, a positive synergistic haemolysis reaction was crescent-shaped on SBA, HBA, and CaHBA (Figs 1, 2, and 3). The lytic zone was consistently large in size (15-18 mm) in the 57 isolates on SBA, and in 63 on HBA or CaHBA. On HBA, the lytic zone was least intensive. Twenty-four isolates on SBA and 18 on HBA or CaHBA showed intermediate or large haemolytic zones (7 mm or more) at a different time or on different batches of agar.

Small-sized, bullet-shaped lytic zones (3 mm) were given by three, and intermediate-sized, lytic zones (7 mm) by one isolate of *C. paraperfringens* on all three types of blood agar.

None of the 10 *C. bifermentans* or 11 *C. sordellii* isolates showed any lytic synergism with group B streptococci on SBA (Fig. 1). However, on HBA (Fig. 2), a narrow, horseshoe-shaped clearing appeared inside the marginal zone of the darkened erythrocytes surrounding the usual haemolytic pattern of *C. bifermentans* and was similar to, but somewhat separated from, the latter haemolysis. Two *C. bifermentans* isolates were strongly haemolytic on HBA, but the overlapping field of the CAMP-factor:*C. bifermentans* haemolysis was helmet-shaped, indicating the presence of the synergism.

Results of the Nagler test and synergistic haemolysis test with group B streptococci for presumptive identification and differentiation of *C. perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens*

Clostridial species	No. tested	Nagler test positive	Synergistic haemolysis test positive on:		
			SBA	HBA	CaHBA
<i>C. perfringens</i>	81	81	81	81	81
<i>C. bifermentans</i>	10	10	0	10	10
<i>C. sordellii</i>	11	11	0	0	11
<i>C. paraperfringens</i>	4	4	4	4	4

Fig. 1 SBA. The right side was smeared with *C. welchii* type A antiserum diluted 1:6 (AS). Group B streptococci inoculation lines (B)—left and right. *C. perfringens* (Cp), *C. bifermentans* (Cb), *C. sordellii* (Cs), *C. paraperfringens* (Cpp). Note the crescent-shaped synergistic lytic zone, and the target haemolysis of Cp at the left, and complete inhibition of both at the right, no synergism by Cb and Cs, and a small synergistic lytic zone of Cpp (left), only slightly inhibited by AS (right).

Fig. 2 HBA. Inoculations, antiserum, and legend as for Fig. 1. Note appearance of horseshoe-shaped zone of clearing of Cb (completely inhibited at the right), and a more pronounced usual haemolysis than on SBA (partially inhibited by AS).

Fig. 3 HBA supplemented with 0.066% of CaCl_2 . Inoculations, antiserum, and legend as for Fig. 1. Plates for Figs 1, 2, and 3 were inoculated with the same strain of either species (Cp-1, Cb-76, Cs-SL279, Cpp-8422). Note appearance of wide haemolysis and a weak synergistic clearing of Cs at the left. Inhibition by AS is similar to that of Cb.

C. sordellii isolates did not show any synergistic lysis on HBA (Fig. 2), but on CaHBA all 11 isolates gave similar, but less intensive, haemolytic and synergistic haemolytic patterns to those of *C. bifermentans* (Fig. 3). ZnSO_4 or MgSO_4 did not induce synergistic haemolytic activity of *C. sordellii* but did improve the target haemolysis of *C. perfringens*, and the usual and synergistic haemolysis of *C. bifermentans* was most enhanced by CaCl_2 and MgSO_4 . None of the haemolytic reactions was affected by any of the tested salts on SBA.

All synergistic haemolysis reactions were always obtainable after an overnight incubation. Prolonged incubation increased the size of the haemolytic patterns but did not influence the appearance of *C. sordellii* synergistic haemolysis on SBA or Ca^{2+} -unsupplemented HBA.

When used undiluted, *C. perfringens* alpha-antitoxin inhibited all haemolytic activities of the four clostridial species (reactions given by some *C. paraperfringens* isolates were only partially inhibited on HBA). If diluted 1:6, the antitoxin completely

inhibited all lytic activities of *C. perfringens* and synergistic lysis of *C. bifermentans* and *C. sordellii* on appropriate media. The usual and, frequently, synergistic haemolysis of *C. paraperfringens*, and the usual haemolysis of *C. bifermentans* or *C. sordellii* were only partially inhibited (Figs 1, 2, and 3). Dilution 1:10 of the antitoxin completely inhibited all lytic activities of *C. perfringens* but did not affect any of the haemolytic patterns of the other three species.

Discussion

A synergistic haemolysis test, using group B streptococci, showed an advantageous ability to identify and differentiate simply and presumptively *C. perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens*. Moreover, the test offered a double control on the same plate in that the target, or other usual haemolysis pattern, and the synergistic haemolysis were both inhibited by the same antitoxin. The use of the diluted antitoxin helped clearly to separate *C. perfringens* from *C. bifermentans*, *C. sordellii*, and *C. paraperfringens*, and CaHBA differentiated between *C. bifermentans* and *C. sordellii*. It is conceivable that different batches of *C. perfringens* type A antiserum should first be titrated for optimal dilution due to a possible batch-to-batch variation. Strongly haemolytic clostridial isolates should be tested with the undiluted antitoxin, and also for tryptophanase and urease production. If only *C. perfringens* is to be identified, SBA could be used alone. If all four species are to be identified, HBA and CaHBA should be sufficient. Although the small-sized, bullet-shaped, synergistic haemolysis reaction indicated the presence of *C. paraperfringens*, the statement given for the egg-yolk emulsion plate reactions¹⁴ was also applicable here. Thus, reliance could not be placed solely on the differential quantitation of the lytic zone sizes in the identification of the species. Production of phospholipase C varied from isolate to isolate, and in the same isolate from time to time: this caused differences in size of the reactions, which were also affected by the condition of the medium. Further, a small-sized lytic zone occurred in mixed inocula when a clostridium that was not producing phospholipase C predominated over *C. perfringens*. The lack of inhibition by the diluted antitoxin was much more reliable than accurate measurement of zone sizes.

The choice of the base medium and the type of blood product seems to be important. Some trials with Columbia agar base (Oxoid, London, UK) and with packed human blood bank erythrocytes gave erratic haemolytic patterns. Therefore, tryptose

blood agar base and whole human blood are recommended.

Some variability in clarity of haemolysis occurred with different batches of the whole human blood bank blood. The addition of Mg^{2+} , Zn^{2+} , or Ca^{2+} was most beneficial to enhance uniformity of performance of HBA in that the haemolytic patterns of the tested clostridia were improved. Moreover, it indicated that *C. sordellii* phospholipase C was more Ca^{2+} dependent than that of *C. bifermentans*, and that the enzymes of both species could not attack sphingomyelin in the outer leaflet of intact sheep erythrocytes, even when aided by Mg^{2+} or the CAMP-factor. In short, sphingomyelin is the major phospholipid constituent of sheep erythrocyte membrane,¹⁵ whose hydrolysis is the prerequisite for this type of haemolysis,¹⁶ and sphingomyelinase C activity of type C phosphohydrolase possessing it, depends on the presence of Mg^{2+} .^{17,18} Furthermore, *C. perfringens*, whose phospholipase C has known sphingomyelinase activity,¹⁷ gave more intensive haemolysis on HBA and CaHBA than the former two species. Small-sized, synergistic, lytic zones of *C. paraperfringens* indicated to a small amount of phospholipase C produced, and its availability on both SBA and HBA suggested a similar activity to that of *C. perfringens*.

In conclusion, the synergistic haemolysis test proved simple and reliable for the presumptive identification and differentiation of *C. perfringens*, *C. bifermentans*, *C. sordellii*, and *C. paraperfringens* and may obviate the need for the Nagler test. There is an obvious economy in the use of diluted antitoxin and in the simplified differentiation of the four species. The eventual usefulness of the test in the presumptive identification of other phospholipase C producing clostridia has still to be determined.

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