Effect of Bile and Desoxycholate on Gram-Negative Anaerobic Bacteria

KAORU SHIMADA,† VERA L. SUTTER, AND SYDNEY M. FINEGOLD
Department of Medicine and Anaerobic Bacteriology Laboratory, Wadsworth Hospital, Veterans Administration, and the Department of Medicine, UCLA School of Medicine, Los Angeles, California 90073

Received for publication 20 May 1970

The bile tests for characterizing gram-negative anaerobic bacilli were reevaluated in prereduced anaerobically sterilized peptone-yeast-glucose broth, in thiglycollate broth, and on blood agar plates. Blood agar plates were unsatisfactory. The combination of 20% bile with 0.1% desoxycholate inhibited *Fusobacterium*, *Bacteroides melaninogenicus*, and *B. oralis* and sometimes *Sphaerophorus necrophorus*, but not *B. fragilis* or other *Sphaerophorus* species studied. Ten per cent bile with 0.05% desoxycholate was less satisfactory. There was no significant difference between fresh and commercial powdered bile. Desoxycholate (0.1% in thiglycollate broth) inhibited *B. fragilis*, *Fusobacterium*, *B. melaninogenicus*, *B. oralis*, and *S. necrophorus*, but not *S. varius* or *S. morifera/S. ridiculous*. The bile and desoxycholate tests are simple to perform and helpful for characterization and classification of gram-negative anaerobic bacilli.

It has long been recognized that bile and bile acids have antibacterial properties against certain species of microorganisms, but that they are without effect on others (15). These actions have been utilized in tests for differentiation of aerobes as well as gram-negative anaerobic bacilli and in selective media. It is recognized that, among gram-negative anaerobic bacilli, *Bacteroides fragilis* strains are not inhibited (and may be stimulated) by bile, whereas *B. oralis* and *Fusobacterium fusiforme* strains are inhibited (9, 14). Disagreement and lack of information still exist concerning the effect of bile on *Sphaerophorus* species and on *B. melaninogenicus*. There is also disagreement as to the concentrations of bile or bile and desoxycholate which should be used, and there is uncertainty as to whether one must use fresh bile rather than the commercially available product. Some of the confusion in the literature undoubtedly relates to failure to speculate properly organisms of the genus *Sphaerophorus*.

In the present study, the action of bile, sodium desoxycholate, and bile with sodium desoxycholate on gram-negative anaerobic bacteria has been investigated by using a number of well-characterized strains.

MATERIALS AND METHODS

Most strains used in this study were isolated from humans, but one strain of *Sphaerophorus varius* was isolated from a chinchilla. The three *S. necrophorus* strains were sent to us and the source is uncertain. Strains were characterized on the basis of morphology, production of lecithinase and lipase and proteolysis on egg yolk agar, production of indole, nitrate reduction, fermentation of carbohydrates, esculin hydrolysis, and end products of glucose fermentation (1). We also used antibiotic susceptibility patterns (8; V. L. Sutter and S. M. Finegold, Bacteriol. Proc., 1969, p. 73). The species or group designations used in this study are those used in the current *Berger's Manual* (7th ed.) except as follows. (i) *B. fragilis* includes *B. fragilis*, *B. convexus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, and *B. vulgatus*. (ii) *B. oralis* is as described by Losche and others (8). (iii) *F. fusiforme* and *F. nucleatum* are grouped together and *S. moriferus* and *S. ridiculous* are also grouped together because of the difficulty in separating them on the basis of the tests used. They may be separated on the basis of differences in nucleic acid base ratios (L. V. Holdeman and W. E. C. Moore, personal communication).

Oxgall and sodium desoxycholate (Difco) were added singly or in combination to various media as follows. Oxgall representing final concentrations of 10 and 20% bile was added to blood agar plates, prereduced anaerobically sterilized peptone-yeast-glucose broth (10), and Fluid Thioglycollate Medium (BBL 11260). Fresh beef bile from several animals, obtained from a slaughterhouse in winter, was added to Fluid Thioglycollate Medium at a concentration of 20%. Final concentrations of 0.05 and 0.1% sodium desoxycholate were also added to some sets of the above media, with or without oxgall. The bile acids in the Oxgall (Difco) and fresh beef bile used in this study were analyzed on thin-layer chromatograms by using
a solvent system of iso-octane, ethyl acetate, and acetic acid (5:5:1, reference 6).

Desoxycholate agar (BBL) plates which contain 0.1% sodium desoxycholate were also tested.

Agar plates were streaked with a loopful of growth from a 48-hr fluid thioglycollate culture supplemented with 25% ascitic fluid. Subsequently, it was found necessary to inoculate the desoxycholate agar plates with a loopful of colonial growth or 0.5 ml of fluid culture to obtain growth. Plates were incubated anaerobically in Brewer jars in an atmosphere of 90% N₂ and 10% CO₂ after seven flushings to displace oxygen. Liquid media were inoculated with two or three drops of the above thioglycollate cultures by means of a Pasteur pipette. Prerred media were flushed with N₂ during inoculation since CO₂ decreased the pH of the desoxycholate peptone-yeast-glucose broth and produced precipitation. Ascitic fluid (25%) and menadione (0.5 μg/ml) were added to thioglycollate broth in studies utilizing B. melaninogenicus.

After incubation for 1 to 5 days at 37 °C, the bacterial growth in the various test media was compared with that obtained in comparable control media which contained neither bile nor desoxycholate. Growth on agar plates was graded as 4+ (heavy, confluent growth), 3+ (semiconfluent growth), 2+ (>100 colonies), 1+ (10 to 100 colonies), ± (<10 colonies), or no growth. It was considered inhibited if less growth appeared on test plates as compared with the control. Growth in liquid media was assessed visually as none to 4+, with 4+ turbidity corresponding to that of a No. 10 McFarland nephelometer standard. Growth was considered inhibited if test media were less turbid than was the control.

**Table 1. Effect of bile and desoxycholate on gram-negative anaerobic bacilli**

| Bacteria        | No. of strains tested | 0.1% Desoxycholate | 10% Oxgall̄ | 20% Oxgall̄ | 10% Oxgall + 0.05% desoxycholate | 20% Oxgall + 0.1% desoxycholate |
|-----------------|-----------------------|--------------------|-------------|-------------|---------------------------------|---------------------------------|
| Bacteroides fragilis | 31                    | 28                 | 1           | 1           | 1                               | 1                               |
| B. melaninogenicus   | 10                    | 10                 | 10          | 10          | 10                             | 10                             |
| B. oralis         | 5                     | 5                  | 5           | 5           | 5                               | 5                               |
| Fusobacterium fusiforme/ nucleatum | 14             | 14                 | 14          | 14          | 14                             | 14                             |
| Sphaerophorus varius | 7                     | 1                  | 1           | 1           | 1                               | 3                               |
| S. mortiferus/ ridiculosis | 5              | 2                  | 0           | 0           | 0                               | 0                               |
| S. necrophorus   | 2                     | 2                  | 1           | 1           | 2                               | 2                               |

* Test medium: blood agar plate.

**Table 2. Effect of bile and desoxycholate on gram-negative anaerobic bacilli**

| Bacteria            | No. of strains tested | 0.1% Desoxycholate | 10% Oxgall | 20% Oxgall | 10% Oxgall + 0.05% desoxycholate | 20% Oxgall + 0.1% desoxycholate |
|---------------------|-----------------------|--------------------|------------|------------|---------------------------------|---------------------------------|
| Bacteroides fragilis | 31                    | 25                 | 31         | 0          | 1                               |                                 |
| B. melaninogenicus   | 11                    | 11                 | 10         | 10         | 10                             | 10                             |
| B. oralis           | 6                     | 6                  | 6          | 6          | 6                               | 6                               |
| Fusobacterium fusiforme/ nucleatum | 14          | 14                 | 14         | 14         | 14                             | 14                             |
| Sphaerophorus varius | 8                     | 0                  | 0          | 0          | 0                               | 0                               |
| S. mortiferus/ ridiculosis | 6              | 0                  | 0          | 0          | 0                               | 0                               |
| S. necrophorus      | 3                     | 3                  | 3          | 0          | 0                               | 1                               |

* Test medium: Fluid Thioglycollate Medium (BBL 11260).

Powdered oxgall representing final concentrations of 10 and 20% bile in the medium.

**RESULTS**

Growth of gram-negative anaerobic rods on blood agar plates with incorporated sodium desoxycholate, oxgall, or both, is noted in Table 1. All 14 strains of Fusobacterium, 10 strains of B. melaninogenicus, and 5 strains of B. oralis were inhibited by either 10 or 20% oxgall, with or without desoxycholate. Most B. fragilis strains were sensitive to desoxycholate, but not to oxgall, with or without desoxycholate. Twenty-eight of 31 B. fragilis strains were inhibited by 0.1% desoxycholate, whereas only one strain was inhibited by 10 and 20% bile. Most S. varius or S. mortiferus/ ridiculosis strains were not affected by bile or desoxycholate, but strains of S. necrophorus were inhibited in all media containing desoxycholate.

The data indicate that the effect of bile and desoxycholate may be helpful in differentiation of gram-negative anaerobic bacilli. However, the blood agar plate is not always satisfactory as the base medium for this test because of inconsistent results with a few B. fragilis and Sphaerophorus strains.

On commercial desoxycholate agar (0.1% desoxycholate content), only S. varius and S. mortiferus/ridiculosis, among the gram-negative anaerobic rods tested, could grow. Even some of these Sphaerophorus strains failed to grow when inoculated lightly, so it is necessary to streak out at least a loopful of colonial growth from solid media or 0.5 ml of undiluted broth culture and to incubate the tests for 3 to 4 days to obtain growth.
TABLE 3. Effect of bile and desoxycholate on gram-negative anaerobic bacilli

| Bacteria                      | No. of strains tested | No. of strains inhibited | 0.05% Desoxycholate | 0.1% Desoxycholate | 10% Oxgall B. | 0.05% Desoxycholate | 20% Oxgall B. | 0.1% Desoxycholate |
|-------------------------------|-----------------------|--------------------------|---------------------|-------------------|---------------|---------------------|---------------|-------------------|
| Bacteroides fragilis          | 30                    | 23                       | 29                  | 0                 | 0             | 0                   | 0             | 0                 |
| B. melaninogenicus            | 8                     | 8                        | 8                   | 8                 | 8             | 8                   | 8             | 8                 |
| B. oralis                     | 3                     | 3                        | 3                   | 3                 | 3             | 3                   | 3             | 3                 |
| Fusobacterium fusiforme/ nucleatum | 14               | 11                       | 14                  | 12                | 13            | 13                  | 13            | 13                |
| Sphaerophorus varius          | 8                     | 0                        | 0                   | 0                 | 0             | 0                   | 0             | 0                 |
| S. moriferus/ridiculosus      | 6                     | 0                        | 0                   | 0                 | 0             | 0                   | 0             | 0                 |
| S. necrophorus                | 3                     | 3                        | 3                   | 1                 | 2             | 2                   | 2             | 2                 |

a Test medium: peptone-yeast-glucose.
b Powdered oxgall representing final concentrations of 10 and 20% bile in the medium.

TABLE 4. Comparison of commercial Oxgall (Difco) and fresh ox bile: effect on gram-negative anaerobic bacilli

| Bacteria                      | No. of strains | 20% Difco Oxgall, with or without 0.1% desoxycholate | 20% Fresh ox bile, with or without 0.1% desoxycholate |
|-------------------------------|----------------|------------------------------------------------------|------------------------------------------------------|
| Bacteroides fragilis          | 7              | 1                                                    | 1                                                   |
| B. melaninogenicus            | 3              | 3                                                    | 3                                                    |
| B. oralis                     | 3              | 3                                                    | 3                                                    |
| Fusobacterium fusiforme/ nucleatum | 9              | 9                                                    | 9                                                    |
| Sphaerophorus varius          | 3              | 0                                                    | 0                                                    |
| S. moriferus/ridiculosus      | 3              | 0                                                    | 0                                                    |
| S. necrophorus                | 2              | 2                                                    | 0                                                    |

a Test medium: Fluid Thioglycollate Medium (BBL 11260).
b Powdered oxgall representing a final concentration of 20% bile in the medium.
c Slight inhibition.

In liquid media, the difference in antibacterial action of bile and desoxycholate against groups of gram-negative anaerobic rods was more striking (Table 2, 3). Desoxycholate (0.1%) inhibited all gram-negative anaerobic rods other than the S. varius and S. moriferus/ridiculosus strains, except for one strain of B. fragilis in peptone-yeast-glucose broth. Sphaerophorus strains grow rapidly in 0.1% desoxycholate-thioglycollate broth (as in other media), so it is usually possible to evaluate the result of this test after overnight incubation. Twenty percent bile plus 0.1% desoxycholate showed inhibitory action against all strains of Fusobacterium, B. melaninogenicus, and B. oralis in both liquid media except for one fusiform strain in peptone-yeast-glucose. With B. fragilis, this combination failed to inhibit any strains in peptone-yeast-glucose medium and 30 of 31 strains in thioglycollate broth. None of the S. varius and S. moriferus/ridiculosus strains was inhibited, but S. necrophorus gave variable results. Ten per cent bile was less satisfactory than 20% since it occasionally allowed growth of Fusobacterium and B. melaninogenicus.

The action of fresh beef bile was almost the same as that of commercial powdered oxgall, but it was somewhat less inhibitory (Table 4). The bile acids in the fresh beef bile and Difco Oxgall used in this study were analysed by thin-layer chromatography. Both contained glycine and taurine-conjugated cholic and desoxycholic, chenodeoxycholic acids or both. With regard to free bile acids, cholic and a trace of desoxycholic, chenodeoxycholic acids, or both, were present in Difco Oxgall, whereas only a trace of cholic acid but no desoxycholic, chenodeoxycholic acid, or both was detected in fresh bile.

DISCUSSION

Some workers consider it desirable to evaluate the action of bile on bacterial growth in terms of stimulation, no effect, or inhibition. However, the facts that B. fragilis and Sphaerophorus sometimes produce precipitation in liquid bile-containing media and that strains in these two groups generally grow well in thioglycollate broth or the peptone-yeast-glucose medium make it difficult to judge whether they are stimulated or unaffected by bile. The solid medium tested in this study did not resolve the problem. We did not try to duplicate the results of Beerens et al. (4) in which growth stimulation by bile was noted on the surface of a plate culture (VL agar with 0.2% glucose). Accordingly, we evaluated the effect of bile and/or desoxycholate on bacterial growth as simply "inhibition" or "no inhibition".

Information on the effect of bile on gram-negative anaerobic bacilli is relatively scanty and conflicting. Confusion concerning the classification of these organisms and failure to distinguish between certain strains of human and animal origin have contributed to the uncertainty in this area.

The usefulness of the bile test for characterization of gram-negative anaerobic rods was emphasized by Beerens et al. (4). Smith and Holdeman...
found *B. fragilis* unaffected and sometimes stimulated by 10% bile. Our data certainly indicate that *B. fragilis* is only rarely inhibited by bile in liquid media.

Prévot noted that bile prevented growth of some "*Sphaerophorus funduliformis*" strains (11) but did not note effect on other *Sphaerophorus* species. Beeren and Tahon-Castel (5) indicate that the *Sphaerophorus* group (which they classify as Bacteroides) is either stimulated, unaffected, or inhibited by 20% bile plus 0.1% desoxycholate; this group includes four species and there is no breakdown as to the effect of bile on *B. necrophorus* or *B. funduliformis*, as compared to the other two species. However, in an earlier publication, Beeren and Castel (3) reported that bile had no effect on 50 strains of *Sphaerophorus*. Suzuki reported that 3% Oxgall Powder (Difco; equivalent to 30% oxgall) in media inhibited 14 of 15 *Sphaerophorus* strains (not speciated) isolated from normal human feces (16). Fievez, working with 225 *Sphaerophorus* strains (215 of animal origin and 10 of human origin), found that growth in 10% bile varied from strain to strain, ranging from complete inhibition to growth equal to that obtained in controls (7). The Virginia Polytechnic Institute group (1) reports that *F. necrophorum* (S. necrophorus) is variably affected by 20% bile plus 0.1% desoxycholate, whereas *F. varium* (S. varium), *F. mortiferum* (S. mortiferus), and *F. ridiculosum* (S. ridiculosus) are unaffected. Our data indicate that, in liquid media, *S. necrophorus* was variably affected by 20% bile plus 0.1% desoxycholate but that the other three species of *Sphaerophorus* tested were never inhibited. Thus, we are in agreement with the VPI group.

There is general agreement that *F. fusiforme* is inhibited by bile (5, 14). The VPI group (1) indicates that the results are variable with *F. fusiforme*, but these authors use inhibition in the sense of total inhibition of growth. Our results indicate consistent inhibition of *F. fusiforme* by bile (as per our definition of inhibition). In the case of several of our strains of *Fusobacterium*, there was some inhibition with the use of bile alone, but the inhibition was much more clear when desoxycholate was added.

Loesche et al. reported that 22 *B. oralis* strains were inhibited in 10% bile (Difco Oxgall) in Thioglycollate Medium without dextrose, supplemented with 0.2% yeast extract and 0.5% glucose (9). Smith and Holdeman (14) agree with this finding, and our results are similar with either 10 or 20% bile in blood agar or these concentrations with desoxycholate in fluid media.

Very little information is available concerning the effect of bile on *B. melaninogenicus*. Beeren and Tahon-Castel (5) place this organism in the genus *Ristella* with several other species; they state, for the genus *Ristella* as a whole, that organisms in it are unaffected or slightly inhibited. The VPI group (1) reports that *B. melaninogenicus* is inhibited by 20% bile with 0.1% desoxycholate. Our results also indicate that this organism is consistently inhibited by 20% bile, with or without desoxycholate.

An important result of the present investigation is the finding that 0.1% desoxycholate (in either thioglycollate broth or in commercial desoxycholate agar) inhibits all of the major groups of gram-negative anaerobic bacilli tested except three species of *Sphaerophorus* (*S. varius* and *S. mortiferus/ridiculosus*). This simple test should prove very useful in clinical laboratories. The concomitant use of bile overcomes the antibacterial activity of desoxycholate against *B. fragilis*; the component of bile responsible for this action is unknown.

It is interesting to note that *B. fragilis* and certain *Sphaerophorus* species which are not inhibited by bile are active in bile acid deconjugation, whereas *Fusobacterium*, *B. melaninogenicus* and *B. oralis*, all inhibited by bile, are inactive (13).

For the bile test, Beeren recommended fresh green oxgall which should be obtained in winter time; he added it to the media after autoclaving (2). Ueno added 10% sterile fresh ox bile, without autoclaving, to media and reported it was satisfactory for the bile test (17). We autoclaved fresh bile which was obtained in the winter time and added it to Fluid Thioglycollate Medium, with or without desoxycholate, and obtained almost the same activity as that of Difco Oxgall. Since it may be difficult to obtain uniformity with fresh materials, and since it is often inconvenient to obtain such material, commercial powdered oxgall is recommended for use.

It is still troublesome in clinical laboratories to identify gram-negative anaerobic bacilli. Certain tests used in identification, such as determination of end products of glucose fermentation and threonine deamination, can not readily be done in every clinical laboratory. Therefore, it is desirable to have other tests which are less difficult to perform. Our laboratory has reported that antibiotic susceptibility patterns are useful in characterization of gram-negative anaerobic rods (8; V. L. Sutter and S. M. Finegold, Bacteriol. Proc., 1969, p. 73). Data from the present study indicate that the bile test and susceptibility to desoxycholate also serve as additional characteristics for classification of these organisms. Use of 0.1% desoxycholate in thioglycollate broth provides a simple, distinct differentiation between three species of *Sphaerophorus* and the other
gram-negative anaerobic bacilli. _B. fragilis_ may be distinguished from the remaining organisms by uninhibited growth in the presence of 20% bile and 0.1% desoxycholate.

Use of these tests together with other simple tests, such as type of growth in broth, and unique colonial and microscopic morphological features would provide reasonably reliable differentiation of gram-negative anaerobic bacilli for routine clinical purposes.

ACKNOWLEDGMENTS

We acknowledge the kindness of W. E. C. Moore and L. V. Holdeman in supplying us with a draft of their section on gram-negative anaerobic bacilli prepared for the eighth edition of _Bergey's Manual of Determinative Bacteriology_. This was very helpful to us in speculating certain of the gram-negative anaerobic bacilli.

We appreciate the kindness of H. Beerens, R. Gibbons, W. E. C. Moore, A. R. Prévot, and A. Sonnenwirth, all of whom sent one or more strains of the gram-negative anaerobic bacilli used in this study.

This investigation was supported by Public Health Service training grant AI309 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Anaerobe Laboratory. 1969. Outline of clinical methods in anaerobic bacteriology. Virginia Polytechnic Institute, Blacksburg, Va.
2. Beerens, H. *In N. Kosaki and S. Suzuki_. 1968. Anaerobes in clinical medicine, p. 110. Igaku Shoin, Tokyo.
3. Beerens, H., and M. M. Castel. 1960. _Action de la bile sur la croissance de certaines bacteries anaerobies a gram negatif_. Ann. Inst. Pasteur 99:454-456.
4. Beerens, H., Y. Schaffner, J. Guillaume, and M. M. Castel. 1963. Les bacilles anaerobies non sporules a gram negatif favorises par la bile. Ann. Inst. Pasteur Lille. 14:5-48.
5. Beerens, H., and M. M. Tahon-Castel. 1965. Infections humaines à bactéries anaérobies nontoxignes. Presses Académiques Européennes, Bruxelles.
6. Enroth, P. 1963. Thin layer chromatography of bile acids. J. Lipid Res. 4:11-16.
7. Fievez, L. 1963. Étude comparée des souches de _Sphaerophorus_, _necrophorus_ isolées chez l'homme et chez l'animal. Presses Académiques Européennes, Bruxelles.
8. Finegold, S. M., N. E. Harada, and L. G. Miller. 1967. Antibiotic susceptibility patterns as aids in classification and characterization of gram-negative anaerobic bacilli. J. Bacteriol. 94:1443-1450.
9. Loesche, W. J., S. S. Socransky, and R. J. Gibbons. 1964. _Bacteroides_ orvii, proposed new species isolated from oral cavity in man. J. Bacteriol. 88:1329-1337.
10. Moore, W. E. C. 1966. Techniques for routine culture of fastidious anaerobes. Int. J. Syst. Bacteriol. 16:173-190.
11. Prévot, A. 1966. Manual for the classification and determination of the anaerobic bacteria, 1st American ed. Lea and Febiger, Philadelphia.
12. Sébald, M. 1962. Étude sur les bactéries anaérobies gram-négatives sporulées. Imprimerie Barnéoud S. A. Laval.
13. Shimada, K., K. S. Bricknell, and S. M. Finegold. 1969. Deconjugation of bile acids by intestinal bacteria. Review of literature and additional studies. J. Infec. Dis. 119:273-281.
14. Smith, L. D. S., and L. V. Holdeman. 1969. The pathogenic anaerobic bacteria. Charles C Thomas, Publisher, Springfield, Ill.
15. Stancy, M. and M. Webb. 1947. Studies on the antibacterial properties of the bile acids and some compounds derived from cholic acid. Proc. Roy. Soc. Ser. B Biol. Sci. 134:523-537.
16. Suzuki, S., T. Ushijima, and H. Ichinose. 1966. Differentiation of _Bacteroides_ from _Sphaerophorus_ and _Fusobacterium_. Jap. J. Microbiol. 10:193-200.
17. Ueno, K. *In N. Kosaki and S. Suzuki_. 1968. Anaerobes in clinical medicine, p. 110. Igaku Shoin, Tokyo.