Rifampin-Blood-Agar as a Selective Medium for the Isolation of Certain Anaerobic Bacteria

VERA L. SUTTER, PAUL T. SUGIHARA, AND SYDNEY M. FINEGOLD

Anaerobic Bacteriology Laboratory and Department of Medicine, Wadsworth General Hospital, Veterans Administration, Los Angeles, California 90073, and Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

A selective medium which allows detection of relatively small numbers of Fusobacterium varium in fecal specimens is described. Blood-agar containing 50 µg of rifampin per ml inhibits the growth of many species of Bacteroides and of F. fusiforme/nucleatum but allows good growth of F. varium and most strains of F. mortiferum. Quantitative cultures of 11 fecal specimens were done on rifampin and other selective and nonselective media. F. varium was recovered in counts of 10^4 and 10^5 per gram from two specimens on rifampin only. A third specimen yielded 10^9 F. varium on several media, including rifampin. Some Eubacterium and Clostridium species also grew on rifampin, and these ordinarily were distinguished from the Fusobacterium by colony morphology. This medium is of value in fecal flora studies and should be useful with other kinds of specimens where mixtures of organisms are common.

Studies of the incidence and normal distribution of Fusobacterium varium and other Fusobacterium species and the role of these organisms in health and disease would be greatly aided by the use of selective media. To recover these bacteria when they are outnumbered by other members of a mixed flora, the major components of the flora must be inhibited.

Blood-agar plates containing neomycin or kanamycin, with or without vancomycin (2), are sometimes useful for selecting out Fusobacterium from mixed flora; however, Bacteroides persist to varying degrees, and some gram-positive anaerobes (and aerobes) may also survive, particularly if vancomycin is not used. Lahee's selective medium (4) had not proved useful to us in selecting small numbers of Fusobacterium from among much larger numbers of B. fragilis in specimens of intestinal contents and feces.

Data regarding the susceptibility of various anaerobic bacteria to rifampin indicated that the minimal inhibitory concentration (MIC) against F. varium and F. mortiferum species was equal to or greater than 100 µg/ml, whereas the MIC against strains of Bacteroides and other Fusobacterium species was 3.2 µg/ml or less. Anaerobic cocci, Actinomyces, Bifidobacterium, Corynebacterium, and some Clostridium, were also quite susceptible to rifampin (S. M. Finegold, V. L. Sutter, and P. T. Sugihara, Bacteriol. Proc., p. 73, 1969). Others (1, 3, 5) have reported that most strains of facultative bacteria likely to be encountered in the intestine were inhibited by 50 µg or less of rifampin per ml. We felt, therefore, that the resistance of F. varium and F. mortiferum to rifampin would make this drug very useful in a selective medium for these bacilli.

MATERIALS AND METHODS

Bacterial strains. A total of 92 strains of gram-negative, anaerobic, nonsporeforming bacilli were used in this study. Most were from human sources; a few were from animal or unknown sources. Methods of identification are those used in a previous publication from this laboratory (7); species designations are those given previously (8), except that F. fusiforme and F. nucleatum are grouped together as F. fusiforme/nucleatum and F. mortiferum and F. ridiculosum are also grouped as F. mortiferum/ridiculosum because of the difficulty of separating them on the basis of biochemical tests.

Rifampin. Rifampin (Ciba Pharmaceutical Co.) was prepared by dissolving 100 mg of rifampin in 20 ml of absolute ethyl alcohol; 80 ml of distilled water was then added to obtain a stock solution of 1,000 µg/ml. This was stored at 4°C for up to 8 weeks (6).

Media. All media were prepared using Brucella agar (Pfizer Diagnostics) as a base and adding 5% defibrinated sheep blood after autoclaving. Various concentrations of rifampin were added to the base both before and after autoclaving to determine the stability of rifampin under these conditions and optimum concentration to be used. Brilliant Green-blood-agar (BG) was prepared by adding 100 µg of Brilliant Green per ml to the base before autoclaving (4). Kanamycin-vancomycin-blood-agar (KV) was prepared by adding 100 µg of kanamycin base per ml to
the base medium prior to autoclaving and 7.5 µg of vancomycin per ml after autoclaving. Neomycin-
blood-agar (NEO) was prepared by adding 100 µg of neomycin base per ml to the base medium prior to
autoclaving (2).

**Stability studies.** Twofold dilutions of rifampin
(12.5 to 0.4 µg/ml) were prepared in the blood-agar
base. The rifampin was added to one set prior to
autoclaving at 121 C for 15 min and to another after
autoclaving. Five strains of *B. fragilis* were inoculated
to each set of plates which were then incubated anaerobically (80% N₂, 10% H₂, and 10% CO₂) for
48 hr.

**Optimum concentration studies.** Twenty-six strains
of the anaerobic, gram-negative bacilli were streaked
on blood-agar (BA) and BA containing 50 and 75 µg
of rifampin per ml and incubated anaerobically for
48 to 72 hr. The inoculum consisted of a 4-mm loopful
of growth from a 24- to 48-hr culture in fluid thioglycollate medium enriched with 25% ascitic fluid.
The amount of growth on the two selective media was
compared to that on BA. Growth was graded as 4+ (heavy confluent growth), 3+ (semiconfluent growth),
2+ (>100 colonies), 1+ (10 to 100 colonies), ±
(<10 colonies), and no growth. Growth was consid-
ered inhibited when it was two grades or more below
that of the control.

**Comparison of selective media.** The 92 strains of
gram-negative, anaerobic, nonsporeforming bacilli
were tested as above on BA containing rifampin, BG,
and NEO (50 µg/ml) with BA as the control medium.

**Selectivity and specificity of rifampin in studies of
fecal flora.** Dilutions of fecal specimens from 11
individuals were plated on rifampin, KV, NEO, BA,
and Eugonagar with 1% maltose. Cultures were incu-
bated anaerobically and examined after 48 to 72 hr.
Colonies of stock strains of *F. varium* and *F. mort-
tiferum* on rifampin were usually 2 to 3 mm in diam-
eter and had a domed, opaque center with a flat, trans-
lucent, irregular edge. This type of colony, as well as
any others that were 1 mm or more in diameter, was
isolated, tested for aerotolerance, and identified pre-
sumptively by colonial and microscopic morphology
and by susceptibility to antibiotics (7). Representative
strains were further characterized by other biochem-
ical methods and end products of glucose fermenta-
tion, as were the stock culture strains. Small colonies
(less than 1 mm in diameter) were examined by mi-
roscope and subcultured for determination of viability
and aerotolerance. All isolates from the other
media were identified presumptively, and representa-
tive strains were further characterized as above.

**RESULTS**

When 100-mg amounts of rifampin were dis-
solved in ethanol and water, a slight precipitate of
insoluble material was observed. This had not
been apparent with 10-mg quantities of rifampin
dissolved in comparable ratios of ethanol and
water. The rifampin used was completely soluble
when methanol (suggested by the manufacturer)
was substituted for ethanol. Others (9) have also
observed solubility problems with different lots of
rifampin but have used alternative solvents and
have not determined whether microbiological
activity is involved in the insoluble material. A
comparison of microbiological activity of rif-
ampin by plate-dilution method dissolving (i) 10
mg in methanol, (ii) 10 mg in ethanol, and (iii) 100
mg in ethanol showed that all solutions were
equally active against 12 strains of *B. fragilis*.
The ethanol-insoluble material was apparently
biologically inactive.

**Stability studies.** Autoclaving rifampin-contain-
ing media resulted in loss of rifampin activity. Of five
strains of *B. fragilis* which were inhibited by
0.4 µg or less of fresh rifampin per ml, two re-
quired 1.6 µg/ml, two required 0.8 µg/ml, and
one required 0.4 µg/ml or less for inhibition
when autoclaved rifampin was used. Rifampin
added to the autoclaved base was used in the
remainder of the studies.

**Optimum concentration studies.** Results indi-
cated that both 50 and 75 µg of rifampin per
ml inhibited all 13 strains of *B. fragilis* and five
strains of *F. fusiforme/nucleatum* tested. Three
strains of *F. varium* were not inhibited by 50 µg
of rifampin per ml, whereas 75 µg of rifampin per
ml markedly inhibited growth of one and resulted
in moderately inhibited growth of another. Of
two strains of *F. mortiferum* tested, one was
inhibited by both concentrations, and the other
was inhibited only by 75 µg of rifampin per ml.
One strain of *F. ridiculosum* was inhibited by
both concentrations of rifampin. Although inhib-
ited as defined above, light growth did occur
with each strain of *F. mortiferum* and *F. ridicu-
losum*. The concentration of 50 µg of rifampin
per ml nevertheless was selected for further studies
because it was thought that a lesser amount
might not have been sufficiently inhibitory to
facultatives present in feces.

**Comparison of selective media.** The ability of
the stock strains of *Bacteroides* species to grow on
the various selective media is shown in Table 1. All
strains of *Bacteroides* species except *B. trichoides*
were unable to grow or had only a few inhibited
colonies on rifampin. Growth on other media
varied (Table 1). Although 22 of the *B. fragilis*
strains were inhibited on BG, three of these had
2+ growth with 4+ growth on the control BA.

Growth of stock strains of *Fusobacterium*
species on the various selective media is shown in
Table 2. Strains of *F. fusiforme/nucleatum* were
unable to grow on rifampin. Half of them were
inhibited or unable to grow on BG and KV,
whereas all grew well on NEO. All *F. varium*
strains and all but one *F. mortiferum* strain grew
well on rifampin. The one known strain of *F.
ridiculosum* was inhibited. Results with these
species on BG and KV were similar to those
observed with *F. fusiforme/nucleatum*, and most
strains grew well on NEO. The incompletely identified strains of *F. mortiferum*/*ridiculosum* gave varying results.

One strain of *Vibrio sputorum* was also tested. It grew well on BG and KV but was unable to grow on rifampin or NEO.

**Selectivity and specificity of rifampin in studies of fecal flora.** Three of the 11 fecal specimens yielded *F. varium*. Species of *Fusobacterium* were not recovered from the other eight specimens. The selectivity and specificity of rifampin medium with six of the specimens is illustrated in Table 3.

### Table 1. Growth of Bacteroides species on selective media

| Medium                                      | B. corrodens | B. fragilis | B. hypermegae | B. melaninogenicus | B. oralis | B. trichoides |
|---------------------------------------------|--------------|-------------|----------------|--------------------|-----------|--------------|
| Rifampin (50 μg/ml)                        | 0/4          | 0/24        | 0/1            | 0/5                | 0/4       | 1/1          |
| Brilliant Green (100 μg/ml)                | 0/4          | 2/24        | 1/1            | 0/5                | 0/4       | 1/1          |
| Kanamycin (100 μg/ml) + vancomycin (7.5 μg/ml) | 0/4          | 24/24       | 0/1            | 5/5                | 4/4       | 0/1          |
| Neomycin (100 μg/ml)                       | 0/4          | 24/24       | 0/1            | 2/5                | 4/4       | 1/1          |

* Number of strains showing uninhibited growth/number of strains tested.

### Table 2. Growth of *Fusobacterium* species on selective media

| Medium                                      | *F. necrophorum* | *F. necrophorum* | *F. necrophorum* | *F. necrophorum* | *F. necrophorum* | *F. necrophorum* |
|---------------------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| Rifampin (50 μg/ml)                        | 0/14             | 0/1              | 0/8              | 15/15            | 5/6              | 0/1              |
| Brilliant Green (100 μg/ml)                | 7/14             | 0/1              | 1/8              | 15/15            | 4/6              | 0/1              |
| Kanamycin (100 μg/ml) + vancomycin (7.5 μg/ml) | 7/14             | 0/1              | 1/8              | 14/15            | 6/6              | 0/1              |
| Neomycin (100 μg/ml)                       | 14/14            | 0/1              | 7/8              | 15/15            | 5/6              | 0/1              |

* Number of strains showing uninhibited growth/number of strains tested.

### Table 3. Selectivity and specificity of rifampin blood agar

| Specimen no. | Bacteria recovered from rifampin-blood-agar | Count* | Other anaerobes present in large numbers* | Count* |
|--------------|---------------------------------------------|--------|------------------------------------------|--------|
| 211          | *Fusobacterium varium*                      | 10⁷    | *Bacteroides fragilis*                   | 10⁷    |
| 220          | *F. varium*                                 | 10⁴    | *B. fragilis*                            | 10⁴    |
| 249          | *Clostridium ramosum*                       | 10⁴    | *Bifidobacterium*                        | 10⁴    |
| 259          | *F. varium*                                 | 10⁹    | *B. fragilis*                            | 10⁹    |
| 265          | *Eubacterium limosum*                       | 10⁴    | *Clostridium*                            | 10⁴    |
| 401          | *Clostridium innocuum*                      | 10⁴    | *B. fragilis*                            | 10⁴    |
|              | *E. filamentosum*                           | 10⁴    | *Peptostreptococcus intermedius*         | 10⁴    |
|              | *E. biforme*                                | 10⁴    | *B. fragilis*                            | 10⁴    |

* Number of bacteria per gram of dry feces.

* Not recovered from rifampin-blood-agar.
Clostridium species. No viable B. fragilis or facultative bacteria were recovered.

DISCUSSION

Studies of normal flora and distribution of specific bacteria are greatly aided by the use of selective media. These media enable the microbiologist to isolate small numbers of some bacteria from masses of others. They may also be sufficiently specific or incorporate adequate differential features to allow presumptive identification of the isolates.

Media containing either Brilliant Green or crystal violet have been recommended by Lahelle (4) and others for selection of Fusobacterium species, but have not proved useful in our laboratory in fecal flora studies. As can be seen from the comparison of growth of B. fragilis on BG and rifampin, rifampin was more inhibitory than BG for this organism, which is the most numerous member of the fecal flora. When rifampin was used in studies of the fecal flora of 11 individuals, B. fragilis was never recovered from rifampin plates yielding individual countable colonies. Although most strains of Fusobacterium were capable of growth on NEO and many were capable of growth on KV, these media are not generally useful for isolation of these bacteria in fecal flora studies because B. fragilis also grows well on them. Rifampin was the only medium on which Fusobacterium variurn was recovered from two of the three positive specimens. Because of larger numbers of B. fragilis or Bifidobacterium species or both, these strains of Fusobacterium variurn were not observed on the other media used. Rifampin medium should prove useful for isolation of F. variurn and possibly F. mortiferum from clinical specimens where mixtures of aerobes and anaerobes are often encountered.

Rifampin may also prove useful in selecting certain Eubacterium and Clostridium species from feces since these bacteria were isolated on rifampin from four of the specimens illustrated in Table 3 and from two additional specimens. These bacteria were not apparent on other media used. By using rifampin, Sugihara and co-workers from our laboratory (Bacteriol. Proc. p. 108, 1971) found Catenabacterium (Eubacterium) filamentosum in feces of 14 of 29 individuals. Counts ranged from $10^5$ to $10^6$ bacteria per gram of dry feces.

Further work is in progress to develop a more specific selective medium for certain Fusobacterium species and one that would not be as inhibitory to some strains of F. mortiferum and F. ridiculosum. Atlas and Turck (1), using a plate-dilution technique for susceptibility testing, suggested that a lower concentration of rifampin might be sufficient to inhibit facultative bacteria present in feces. Vancomycin can be used to inhibit gram-positive bacteria. Blood-agar containing 15 $\mu$g of rifampin per ml and 7.5 $\mu$g of vancomycin per ml has been found to inhibit the growth of stock strains of B. fragilis, B. melaninogenicus, B. oralis, F. fusiforme/nucleatum, F. necrophorum, Bifidobacterium, Clostridium, and anaerobic cocci, but to allow better growth of stock strains of F. mortiferum and F. ridiculosum than does rifampin. A trial of this medium with feces from human subjects yielded F. variurn or F. mortiferum/ridiculosum or both from 4 of 19 specimens in counts ranging from $10^5$ to $10^6$ bacteria per gram with total counts ranging from $10^5$ to $10^6$ bacteria per gram. Two of the specimens contained both species. Facultative bacteria were encountered on the rifampin-vancomycin media with nine of the specimens. This modified medium appears useful for isolating small numbers of these Fusobacterium species in bowel flora studies.

ACKNOWLEDGMENTS

Rifampin was supplied by Ciba Pharmaceutical Co., Summit, N.J. We thank the Anaerobe Laboratory of Virginia Polytechnic Institute for one strain each of B. trichoides and F. ridiculosum, two strains of F. mortiferum, and nine strains of F. variurn. We are also grateful for their confirmation of the identity of the C. ramosum strain and several F. variurn and mortiferum strains used in this study.

LITERATURE CITED

1. Atlas, E., and M. Turck. 1968. Laboratory and clinical evaluation of rifampin. Amer. J. Med. Sci. 256:246-254.
2. Finegold, S. M., A. B. Miller, and D. J. Posnick. 1965. Further studies on selective media for Bacteroides and other anaerobes. Ernaehrungsforschung 10:517-528.
3. Kunin, C. M., D. Brandt, and H. Wood. 1969. Bacteriologic studies of rifampin, a new semisynthetic antibiotic. J. Infect. Dis. 119:132-137.
4. Lahelle, O. 1947. Necrobacterium: a study of its bacteriology, serology and pathogenicity, and its relation to Fusobacterium. Acta Pathol. Microbiol. Scand. Suppl. 67:1-352.
5. McCabe, W. R., and V. Lorian. 1968. Comparison of the antibacterial activity of rifampicin and other antibiotics. Amer. J. Med. Sci. 256:255-265.
6. Stotmeier, K. D., G. P. Kubica, and C. L. Woodley. 1969. Antimycobacterial activity of rifampin under in vitro and simulated in vivo conditions. Appl. Microbiol. 17:861-865.
7. Sutter, V. L., and S. M. Finegold. 1971. Antibiotic disc susceptibility tests for rapid presumptive identification of gram-negative anaerobic bacilli. Appl. Microbiol. 21:13-20.
8. Virginia Polytechnic Institute and State University Anaerobe Laboratory. 1970. Outline of clinical methods in anaerobic bacteriology, 2nd ed. Virginia Polytech. Inst. and State Univ., Blacksburg.
9. Wiggins, G. L., J. V. McLaughlin, S. T. Bickham, W. L. Jones, and A. Balows. 1970. Susceptibility of Neisseria meningitidis strains from the civilian population to sulfadiazine, penicillin, and rifampin. Appl. Microbiol. 20:893-898.