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New Medium Selective for *Fusobacterium* Species and Differential for *Fusobacterium necrophorum*

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Fusobacterium egg yolk agar is a new medium selective for *Fusobacterium* species and differential for *Fusobacterium necrophorum*. The medium is a Brucella Agar base (Difco Laboratories, Detroit, Mich.) containing vancomycin, neomycin, josamycin, and egg yolk. All species of fusobacteria grew with only minimal inhibition. The mean $\log_{10}$ difference in counts between *Fusobacterium* egg yolk agar and control media for 30 strains of seven species of fusobacteria was 0.1922. *F. necrophorum* typically showed a strong lipase reaction. Most other organisms were significantly inhibited by the medium.

Fusobacteria are frequently isolated from clinically significant anaerobic infections (1, 2, 4). Fusobacteria have been involved in oral and dental infections, peritonsillar abscesses, brain abscesses, aspiration pneumonia, lung abscesses, empyema, hepatic abscesses, intraabdominal abscesses, septicemia, and endocarditis, as well as other less common infections. Several species of fusobacteria have been isolated as causes of human disease; the most commonly encountered is *Fusobacterium nucleatum*.

Our laboratory has become aware of nine cases of sepsis or other serious infection caused by *Fusobacterium necrophorum* within a 2-year period. We have undertaken studies to determine human carriage of *F. necrophorum* and its possible role in upper respiratory tract disease. To facilitate these studies, we have developed a new selective and differential medium. This medium, *Fusobacterium* egg yolk agar (FEA), contains josamycin, vancomycin, and neomycin and is highly selective for *Fusobacterium* species and differential for *F. necrophorum*.

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MATERIALS AND METHODS

Bacterial strains. Clinical isolates from the Wadsworth Anaerobic Bacteriology Laboratory Collection and American Type Culture Collection strains were kept frozen in 20% skim milk at −70°C until use. The organisms had been identified by previously detailed methods (11).

Strains were thawed and inoculated into thioglycolate broth 135C supplemented with hemin (5 μg/ml), vitamin K_1 (0.1 μg/ml), and a calcium carbonate chip (THIO) (Clinical Standards Laboratory, Torrance, Calif.). These were subcultured at least once before being used in experiments and were maintained by weekly subculture on Brucella Agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood, hemin (5 μg/ml), and vitamin K_1 (10 μg/ml) (BAK).

Patients. Subjects were students treated at the UCLA Student Health Service for either pharyngitis or an unrelated problem. Informed consent was obtained, and specimens were collected as described below.

Clinical specimens. Throat swabs from the tonsillar fossae were obtained on prereduced cotton swabs and transported in Port-A-Cul tubes (BBL Microbiology Systems, Cockeysville, Md.). These were subsequently inoculated onto BAK and FEA media (see below) and incubated in GasPak jars (BBL Microbiology Systems) for 48 h. Anaerobic isolates were identified by techniques described elsewhere (11). *Enterobacteriaceae* and *Pseudomonas* species were obtained from clinical specimens (urine, sputum, wound cultures) submitted to the Wadsworth Clinical Microbiology Laboratory. *Enterobacteriaceae* and *Pseudomonas* were identified as described elsewhere (5).

Medium. FEA was prepared with Brucella Agar base, to which was added Na_2HPO_4 (5.0 g/liter), KH_2PO_4 (1.0 g/liter), $MgSO_4$ (0.1 g/liter), hemin (5 μg/ml), and polysorbate 80 (BBL) (1 ml/liter). The pH was adjusted to 7.6 with 20% NaOH, and the preparation was autoclaved at 15 lb. (ca. 6.8 kg) for 15 min at 121°C. The medium was cooled to 50°C, and vancomycin (Eli Lilly & Co., Indianapolis, Ind.), neomycin (The Upjohn Co., Kalamazoo, Mich.), and josamycin (Yamanouchi Pharmaceutical Co., Tokyo) were added to obtain final concentrations of 5 μg/ml, 100 μg/ml, and 3 μg/ml, respectively. Sterile egg yolk suspension was added to a final concentration of 2.5% (vol/vol).

Qualitative studies. For anaerobic organisms, one colony was picked and inoculated onto FEA with BAK

serving as a control. Plates were incubated in GasPak jars at 35°C for 48 h. Facultative anaerobic and aerobic gram-negative strains were inoculated onto FEA agar, and MacConkey agar (Clinical Standards Laboratories) was utilized for controls. Facultative anaerobic and aerobic organisms were incubated at 35°C for 24 h aerobically.

Quantitative studies. FEA and BAK plates were reduced in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.). Test organisms were inoculated into THIO, and overnight cultures were passed into the chamber. Serial 10-fold dilutions were made in sterile, reduced 0.05% yeast extract (BBL) with glass beads to facilitate mixing. Dilutions of 10^{-2} and 10^{-3} were plated on FEA and BAK using a Rotaplate (Fisher Scientific Co., Pittsburgh, Pa.). Plates were incubated in GasPak jars for 48 h at 35°C. Colonies were counted, and plates containing between 30 and 300 colonies were used for statistical analysis.

RESULTS

Qualitative studies. FEA supported the growth of a large number of strains of various *Fusobacterium* species (Tables 1 and 2). No strain failed to grow as well on FEA as on BAK control. Significantly, most *Bacteroides* sp. (including *Bacteroides fragilis*) were inhibited; only *Bacteroides thetaiotaomicron* grew on FEA. *Veillonella parvula* also was not inhibited by FEA. The only gram-positive anaerobe that grew on FEA was a multiple antibiotic-resistant *Clostridium* sp.

TABLE 1. *Growth of anaerobic organisms on FEA*

Organism	No. of strains	% of strains inhibited[a]	% of strains growing[b]
Actinomyces sp.	2	100	0
Bacteroides sp.	1	100	0
B. asaccharolyticus	2	100	0
B. bivius	2	100	0
B. disiens	2	100	0
B. distasonis	2	100	0
B. fragilis	5	100	0
B. melaninogenicus	6	100	0
B. oralis	1	100	0
B. ruminicola	3	100	0
B. thetaiotaomicron	3	0	100
B. ureolyticus	3	100	0
Clostridium sp.	6	83	17
Fusobacterium sp.	2	0	100
F. gonidiaformans	4	0	100
F. mortiferum	3	0	100
F. naviforme	6	0	100
F. necrophorum	31	0	100
F. necrogenes	1	0	100
F. nucleatum	48	0	100
F. russii	1	0	100
F. varium	4	0	100
Peptostreptococcus sp.	4	100	0
V. parvula	2	0	100

[a] No growth detectable.
[b] Growth equal to BAK.

TABLE 2. *Growth of aerobic and facultative enteric gram-negative rods on FEA under aerobic conditions*

Organism	No. of strains	% of strains inhibited[a]	% of strains growing[b]
Citrobacter sp.	3	100	0
Enterobacter sp.	8	75	25
E. coli	16	81	19
Klebsiella sp.	9	89	11
P. mirabilis	6	0	100[c]
Providencia sp.	1	0	100
Pseudomonas aeruginosa	7	0	100
Pseudomonas sp.	1	0	100
Serratia marcescens	5	0	100
Yersinia enterocolitica	1	0	100

[a] Growth markedly reduced compared with MacConkey agar control.
[b] Growth equal to MacConkey.
[c] Swarming inhibited.

TABLE 3. *Quantitative growth of Fusobacterium species on FEA and BAK media*

Organism	No. of strains	Log_{10} mean growth[a]	
		FEA	BAK
F. gonidiaformans	3	7.7853	7.8957
F. mortiferum	3	8.1883	8.3181
F. naviforme	3	7.7243	8.0257
F. necrogenes	1	8.2041	8.3502
F. necrophorum	9	8.2751	8.5123
F. nucleatum	7	7.9324	7.9664
F. varium	4	8.3790	8.5035

[a] Averages for all species on FEA and BAK were 8.1482 and 8.3404, respectively.

Facultative anaerobic and aerobic gram-negative rods obtained from hospitalized patients had less consistent growth patterns on FEA than did anaerobic bacteria. *Pseudomonas*, *Proteus*, *Providencia*, and *Serratia* consistently grew on FEA. Swarming of *Proteus mirabilis* was reduced. The majority of strains of *Escherichia coli*, *Klebsiella*, *Citrobacter*, and *Yersinia* were significantly inhibited as demonstrated by visual inspection of the plates.

Quantitative studies. Thirty-two strains of *Fusobacterium* species were studied. Growth on FEA was similar to that on BAK, with only minimal inhibition being present on FEA (Table 3). Two strains of *F. nucleatum* did not grow on FEA in the quantitative study. However, they did grow without inhibition in the qualitative studies. These strains have not been included in the data analysis. For the thirty strains included,

the difference in the $\log_{10}$ of the mean colony counts was 0.1922.

Clinical study. Ninety throat swab specimens from the tonsillar fossae were obtained, of which 58 were from subjects with pharyngitis and 32 were from controls. Organisms recovered on FEA are shown in Table 4. FEA supported the growth of several species of *Fusobacterium*. *Leptotrichia buccalis* and *V. parvula* were also frequently recovered. Significantly, only two strains of *Bacteroides* grew on FEA. One was a *Bacteroides melaninogenicus* resistant to josamycin. The other isolate died before a species identification could be made. As anticipated, *Bacteroides* were usually present on BAK plates. Facultative and aerobic organisms were rarely recovered on FEA; only *Neisseria* sp. and one strain of *P. mirabilis* grew on the medium. No gram-positive organisms were recovered on FEA, whereas all 90 BAK plates contained streptococci.

Colonies of *Leptotrichia* and *Veillonella* could easily be recognized by colonial morphology. *Leptotrichia* formed 3- to 4-mm, white, raised, granular colonies, whereas *Veillonella* colonies were 1 mm and translucent. Fusobacteria typically grew as 2- to 3-mm translucent to white, convex, round, entire colonies. All isolates of *F. necrophorum* gave a strong lipase reaction, which allowed positive identification in 48 h.

DISCUSSION

Several other investigators have developed media selective for fusobacteria species. There are, however, major disadvantages to these media in terms of either their selectivity or utility for recovery of multiple *Fusobacterium* species.

Several attempts to develop a selective medium for fusobacteria have involved the use of crystal violet to inhibit gram-positive organisms (3, 7, 9, 13). Omata and Disraely reported on a medium containing crystal violet and streptomycin (9). They did not quantitate growth in comparison to control media, and both gram-negative rods and gram-negative cocci grew on the media. Subsequent studies have shown this to be a relatively ineffective selective medium.

TABLE 4. *Organisms recovered on FEA from human throat swabs*

Organism	No. of organisms recovered	
	Pharyngitis (n = 58)	Controls (n = 32)
Bacteroides sp.	1	0
B. melaninogenicus	1	0
Fusobacterium sp.	1	0
F. gonidiaformans	1	0
F. naviforme	3	0
F. necrophorum	6	4
F. nucleatum	15	21
L. buccalis	30	15
V. parvula	25	11
Neisseria sp.	5	1
P. mirabilis	0	1

Walker et al. recently developed a medium which includes crystal violet and erythromycin and is selective for oral *F. nucleatum* (13). Virtually no suppression of *F. nucleatum* was observed, as compared with control media. However, several species of gram-positive organisms (streptococci, peptostreptococci, *Actinomyces*, *Propionibacterium*) and gram-negative organisms (*Bacteroides*, *Capnocytophaga*, *Eikenella*, *Actinobacillus*, etc.) grew on their medium. This medium would not be appropriate for clinical specimens in which facultative anaerobes, gram-negative rods, or resistant gram-positive organisms are anticipated (e.g., hepatic abscesses, brain abscesses) as neither crystal violet nor erythromycin inhibit these rapidly growing organisms. Furthermore, no *Fusobacterium* species other than *F. nucleatum* was studied. Other species are more sensitive to erythromycin. For example, 20% of strains of *F. necrophorum* tested in our laboratory are susceptible to erythromycin at the level used. (Morgenstein et al., unpublished data).

Fales and Teresa (3) described a medium selective for *F. necrophorum* containing crystal violet and phenylethyl alcohol in an egg yolk base. No quantitative studies were performed, and growth of *Peptostreptococcus* and *Proteus* was noted. The clinical material studied was bovine hepatic abscesses which have a limited flora; therefore, no strains of *Bacteroidaceae* other than *F. necrophorum* were studied.

In a brief note, McCarthy and Snyder described the use of vancomycin to eliminate the problem of incomplete inhibition of gram-positive bacteria by crystal violet (7). Streptomycin was also included in their medium. However, no data were presented to allow an appraisal of the selectivity or inhibitory properties of the medium.

To obviate these problems, we developed FEA. The vancomycin content of 5 μg/ml completely suppressed all gram-positive organisms in 90 clinical polymicrobial specimens. Josamycin is particularly useful in that all *Fusobacterium* species are relatively resistant, and many other *Bacteroidaceae* are sensitive (except *B. thetaiotaomicron* and *L. buccalis*) (6, 10, 12). The differential susceptibility of *Fusobacterium* sp. and *Bacteroides* sp. to josamycin is several

times greater than for erythromycin. We included neomycin at 100 μg/ml to allow for wider applicability of FEA to abdominal specimens. Neomycin did not completely inhibit enteric facultative organisms but did suppress the majority of strains. To make the medium differential for *F. necrophorum*, egg yolk was included, as the majority of strains are lipase positive (8).

FEA supported the growth of all strains of fusobacteria; it was indistinguishable on streak plates and quantitatively similar to standard media. The lipase reaction of *F. necrophorum* is readily visualized and allows presumptive identification within 48 h. Colonies of other *Fusobacterium* species can be rapidly differentiated from *Leptotrichia* and *Veillonella*.

FEA is a selective, minimally inhibitory agar for *Fusobacterium* species. It is differential for *F. necrophorum*. The medium has a wide range of applicability, including oral, pleuropulmonary, and intraabdominal specimens.

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LITERATURE CITED

1. **Bartlett, J. G., and S. M. Finegold.** 1972. Anaerobic pleuropulmonary infections. Medicine **51**:413–450.
2. **Chow, A. G., and L. B. Guze.** 1974. *Bacteroidaceae* bacteremia: clinical experience with 112 patients. Medicine **53**:93–126.
3. **Fales, W. H., and G. W. Teresa.** 1972. A selective medium for the isolation of *Sphaerophorus necrophorus*. Am. J. Vet. Res. **33**:2317–2321.
4. **Finegold, S. M.** 1977. Anaerobic bacteria in human disease. Academic Press, Inc., New York.
5. **Finegold, S. M., W. J. Martin, and E. G. Scott.** 1978. Bailey and Scott's diagnostic microbiology, 5th ed. C. V. Mosby Co., St. Louis.
6. **Long, S. S., S. Mueller, and R. M. Swenson.** 1976. In vitro susceptibilities of anaerobic bacteria to josamycin. Antimicrob. Agents Chemother. **9**:859–860.
7. **McCarthy, C., and M. L. Snyder.** 1963. Selective medium for *Fusobacterium* and *Leptotrichia*. J. Bacteriol. **86**:158–159.
8. **Moore, W. E. C., and L. V. Holdeman.** 1975. Fusobacterium, p. 404–416. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The William & Wilkins Co., Baltimore.
9. **Omata, R. R., and M. N. Disraely.** 1956. A selective medium for oral fusobacteria. J. Bacteriol. **72**:677–680.
10. **Reese, R. E., R. F. Betts, L. W. Goedde, and R. G. Douglas, Jr.** 1976. In vitro susceptibility of common clinical anaerobic and aerobic isolates against josamycin. Antimicrob. Agents Chemother. **10**:253–257.
11. **Sutter, V. L., D. M. Citron, and S. M. Finegold.** 1980. Wadsworth anaerobic bacteriology manual, 3rd ed. C. V. Mosby Co., St. Louis.
12. **Sutter, V. L., and S. M. Finegold.** 1976. Susceptibility of anaerobic bacteria to 23 antimicrobial agents. Antimicrob. Agents Chemother. **10**:736–752.
13. **Walker, C. B., D. Ratliff, D. Muller, R. Mandell, and S. S. Socransky.** 1979. Medium for selective isolation of *Fusobacterium nucleatum* from human periodontal pockets. J. Clin. Microbiol. **10**:844–849.