JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1987, p. 1985–1988
0095-1137/87/101985-04$02.00/0

Vol. 25, No. 10

Improved Selective Culture Media for *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*

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Received 16 March 1987/Accepted 30 June 1987

By modifying the previously described media tryptic soy-serum-bacitracin-vancomycin (TSBV) agar and tryptic soy-serum-bacitracin-vancomycin-fluoride (TSBVF) agar, two improved selective culture media were developed for isolation and enumeration of *Actinobacillus actinomycetemcomitans* (A medium) and *Haemophilus aphrophilus* (H medium) in oral specimens. Both media were supplemented with fusidic acid and spiramycin, and carbenicillin was also added to A medium. The growth yields of pure cultures of *A. actinomycetemcomitans* on A medium and of *H. aphrophilus* on H medium were comparable with those on the reference media. Compared with blood agar, the selective media inhibited these species about 10-fold or less. In addition, A and H media suppressed the growth of pure cultures of *Capnocytophaga* spp. and *Neisseria* spp., commonly found as contaminants on TSBV and TSBVF, 10^5 times or more compared with that on blood agar. In samples from diseased periodontal pockets, the recoveries of *A. actinomycetemcomitans* on A medium and *H. aphrophilus* on H medium equaled those on TSBV and TSBVF, respectively. In about 50% of the cultures on the reference media, contaminating bacteria were detected at levels higher than 10^4 CFU/ml of sample. The corresponding value for both A and H media was about 2%.

Actinobacillus actinomycetemcomitans is considered strongly associated with periodontal diseases, especially the localized juvenile form (4, 26). *Haemophilus aphrophilus*, a closely related species (18) and a normal inhabitant of the human oral cavity (9–11), is also recovered from diseased periodontal pockets (14, 24) and various types of infections (23). Selective media have been devised for improved recovery of these two species (7, 11, 21, 24). Although these media are selective, growth of other gram-negative microorganisms is observed when samples from periodontal pockets are cultured. In a screening of such samples with tryptic soy-serum-bacitracin-vancomycin (TSBV) agar, designed for isolation of *A. actinomycetemcomitans* (21), and tryptic soy-serum-bacitracin-vancomycin-fluoride (TSBVF) agar, selective for *H. aphrophilus* (24), we found that *Capnocytophaga*- and *Neisseria*-like organisms frequently grew as contaminants, sometimes in large numbers. Similar observations have been reported (13, 16, 19, 21, 24). The present study was therefore undertaken with the purpose of improving the selectivity of the culture media for detection of *A. actinomycetemcomitans* and *H. aphrophilus*.

MATERIALS AND METHODS

Culture media. Two selective media have been developed, one for recovery of *A. actinomycetemcomitans* (A medium) and the other for recovery of *H. aphrophilus* (H medium). The media are modifications of previously described selective substrates for these two species (21, 24). The base in both media contained 4% (wt/vol) tryptic soy agar (Difco Laboratories, Detroit, Mich.) and 0.1% (wt/vol) yeast extract (Difco). The ingredients were gently boiled in distilled water until dissolved and were then autoclaved in 500-ml volumes at 121°C for 15 min. After cooling to 45°C, the A medium was supplemented with bacitracin (Kemiintressen AB, Sundbyberg, Sweden) at 75 μg/ml, carbenicillin (Fugacillin; Astra, Södertälje, Sweden) at 0.16 μg/ml, fusidic acid (Sigma Chemical Co., St. Louis, Mo.) at 1 μg/ml, spiramycin (Leo, Helsingborg, Sweden) at 10 μg/ml, and vancomycin (Eli Lilly & Co., Indianapolis, Ind.) at 5 μg/ml. The H medium was supplemented with bacitracin (75 μg/ml), hemin (5 μg/ml), fusidic acid (1.5 μg/ml), sodium fluoride (75 μg/ml), spiramycin (10 μg/ml), and vancomycin (5 μg/ml). Determination of the concentrations of the antibacterial agents employed in the media was performed by the macrobroth dilution method (25). The broth used was tryptic soy broth supplemented with 0.1% (wt/vol) yeast extract and 10% inactivated horse serum. Stock solutions of the antibiotics were used, prepared in sterile water at 1,000-fold-higher concentrations than the final solutions in the media. Solubilization of spiramycin was obtained by acidifying with glacial acetic acid (5 μl of acid per ml). The concentration of sodium fluoride in the stock solution was 2% (wt/vol). Stock solution for hemin was prepared as previously described (6). Inactivated horse serum was added to the two media to give a final concentration of 10% (vol/vol). The plates were stored at 4°C for periods not exceeding 1 week.

Growth of pure cultures. Growth of 21 strains of *A. actinomycetemcomitans*, 21 strains of *H. aphrophilus*, 6 strains of *Capnocytophaga* spp., and 6 strains of *Neisseria* spp. was examined on A and H media and compared with that obtained on blood agar (6), TSBV agar (21), and TSBVF agar (24).

Besides the type cultures of *A. actinomycetemcomitans* (ATCC 29522, ATCC 29523, and NCTC 9710) and strain HK 309 of *H. aphrophilus* (kindly provided by M. Kilian, Aarhus, Denmark), all other strains were human isolates from diseased periodontal sites. Those isolates designated *A. actinomycetemcomitans* were gram-negative, facultatively anaerobic short rods requiring neither NAD nor hemin as a growth factor, producing catalase but not oxidase, urease, or tryptophanase, and fermenting glucose but not lactose or sucrose. They belonged to biotypes I, II, IV, V, and VI, determined as described previously (22). The *H. aphrophilus* strains possessed the same characteristics as *A. actinomycetemcomitans* with the exceptions of inability to produce

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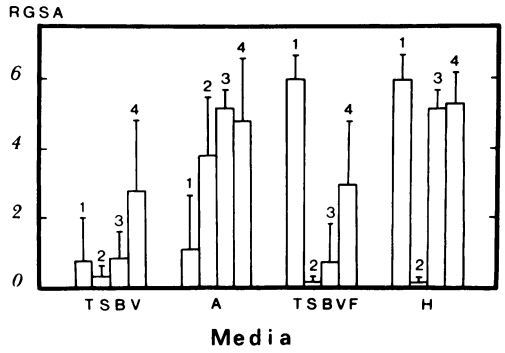

FIG. 1. Growth-inhibitory ability of the four selective media, evaluated with pure cultures and expressed as RGSA values (8). Mean values and standard deviations for: 1, *A. actinomycetemcomitans*; 2, *H. aphrophilus*; 3, *Capnocytophaga* spp.; and 4, *Neisseria* spp.

catalase and to ferment mannitol, and ability to ferment lactose and sucrose. The *Capnocytophaga* strains were gram-negative rods, often with tapered ends, growing anaerobically and in 95% N_2–5% CO_2, being catalase and oxidase negative (13) and showing enzymatic activities characteristic for *Capnocytophaga* spp. with the API ZYM system (12). The *Neisseria* strains were gram-negative diplococci, facultatively anaerobic, and catalase and oxidase positive (5).

Acid production from carbohydrates by *Actinobacillus* and *Haemophilus* strains was examined by means of a modified micromethod. Briefly, filter-sterilized phenol red broth (Difco), 2.2% (wt/vol) in distilled water, was inoculated with the test organism from a 24-h blood agar culture to a final density of approximately 10^8 viable cells per ml. Volumes of 0.05 ml of filter-sterilized 5% (wt/vol) carbohydrate solutions in distilled water were mixed with 0.2 ml of the suspension in wells in Minitek disposable plates (BBL Microbiology Systems, Cockeysville, Md.). The plates were aerobically incubated in a humid atmosphere at 37°C for 24 to 48 h. Change in color of the broth in the wells from red to lime yellow was considered to indicate a positive fermentation reaction.

For preparation of the inocula, the test organisms were cultured on blood agar incubated for 24 h in 95% N_2–5% CO_2. From these cultures suspensions were made by diluting the cells in 0.1 M phosphate buffer (pH 6.8) to a density corresponding to 10^8 cells per ml and then inoculated on the TSBV, TSBVF, A, H, and blood agars from appropriate dilutions. The plates were incubated in jars (95% N_2–5% CO_2) at 37°C for 4 days, after which the numbers of CFU were counted. For each strain the yield of growth on the four selective media was compared with that on blood agar and expressed as relative growth-supporting ability (RGSA) value, which was determined as the logarithm of the ratio of the number of colonies on blood agar to the number of colonies on the selective agar from equivalent dilutions (8).

Recovery from clinical specimens. Using the four selective media, the presence of *A. actinomycetemcomitans* and *H. aphrophilus* was analyzed in 58 and 48 samples, respectively, from diseased periodontal pockets (depth, >4 mm). The samples were taken with either three paper points or a curette as previously described (4, 15). The samples were transported to the laboratory in 1.8 ml of prereduced 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (3). The agar plates were inoculated with 0.05-ml volumes from appropriate 10-fold serial dilutions in MOPS buffer and incubated in 95% N_2–5% CO_2 for 4 days. With the aid of a stereomicroscope, colonies with different morphologies were counted. Representative colonies were Gram stained, and those formed by short gram-negative rods were isolated and identified using the tests described above.

RESULTS

Figure 1 illustrates the growth yields of the test organisms on the four media in comparison with those on blood agar. The mean RGSA values for *A. actinomycetemcomitans* on TSBV and A agars were 0.76 and 1.12, respectively. The difference was statistically significant ($P < 0.01$, Wilcoxon rank sum test). On TSBV the mean RGSA values for *H. aphrophilus* and *Capnocytophaga* spp. were 0.30 and 0.82, respectively, which corresponds to a growth inhibition of less than 10 times in comparison with blood agar. The growth of the *Neisseria* strains was suppressed about 10^3 times (mean RGSA, 2.78) on TSBV. On A agar these bacterial groups were inhibited 10^4 times or more.

The mean RGSA values for *H. aphrophilus* were 0.08 and 0.12, respectively ($P < 0.05$), on TSBVF and H agars. The two media inhibited *A. actinomycetemcomitans* more than 10^6 times. On TSBVF and H agars the growth inhibition of the other bacterial groups was comparable to those on TSBV and A agar, respectively.

The colony morphologies of *A. actinomycetemcomitans* on A agar and *H. aphrophilus* on H agar were similar to those observed on TSBV and TSBVF, respectively, as described earlier (21, 24).

In specimens collected from periodontal pockets, *A. actinomycetemcomitans* was found in 40% of the samples when assayed with A agar and in 35% when TSBV agar was used. *H. aphrophilus* was recovered in 33% of the samples cultured on both TSBVF and H agars. A good relationship was found between the recoveries of *A. actinomycetemcomitans* on TSBV and A agars (Fig. 2) as well as of *H. aphrophilus* on TSBVF and H agars (Fig. 3). No significant difference in the recoveries of *A. actinomycetemcomitans* was found between TSBV and A media. Significantly ($P < 0.05$) higher numbers of *H. aphrophilus* were detected with H agar than with TSBVF medium. In all samples, differences of less than 10-fold were found between the new and the previously described selective media. In three samples *A. actinomycetemcomitans* was detected at low levels only on A agar. Furthermore, two of these samples showed growth

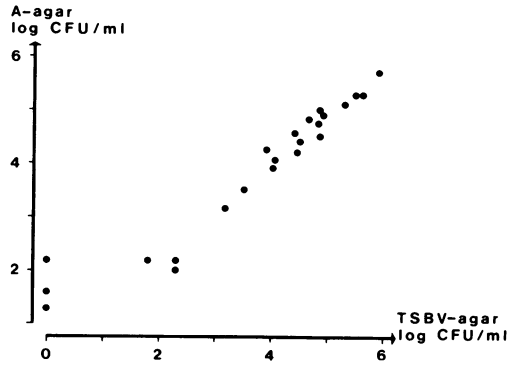

FIG. 2. Comparison between A and TSBV media in recovering *A. actinomycetemcomitans* from periodontal samples ($n = 23$).

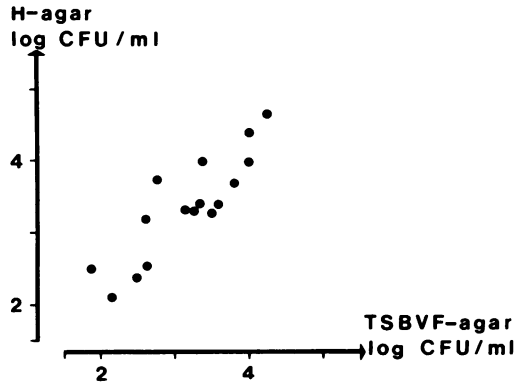

FIG. 3. Comparison between H and TSBVF media in recovering *H. aphrophilus* from periodontal samples (n = 16).

of contaminants of more than 10^3 CFU/ml of sample on TSBV plates.

The recovery of *H. aphrophilus* on TSBVF equaled that on TSBV, whereas on A agar this microorganism was found in cultures from only two samples and at concentrations of less than 10^2 CFU/ml.

With the exception of two samples taken from the same patient, *A. actinomycetemcomitans* was not observed on TSBVF or H agar. The *A. actinomycetemcomitans* strain isolated from these samples could tolerate NaF concentrations of more than 300 μg/ml, as revealed by further studies in liquid media.

In approximately 50% of the samples cultured on TSBV and 52% of those on TSBVF, contaminating bacteria were detected at levels of more than 10^4 CFU/ml of sample (Fig. 4). The corresponding value for A and H agars was 2% of the samples.

DISCUSSION

The present results indicate that A and H media considerably suppress the growth of contaminants without reducing growth yields of *A. actinomycetemcomitans* or *H. aphrophilus*, compared with the findings on TSBV and TSBVF.

On the basis of previous publications dealing with antibiotic susceptibility of the periodontal flora (1, 2, 17), spiramycin and fusidic acid were added to TSBV and TSBVF to inhibit growth of *Capnocytophaga* spp. and *Neisseria* spp., respectively. The A medium was further supplemented with carbenicillin to suppress growth of *H. aphrophilus*. The concentration used is rather low compared with the inhibiting concentration reported by Baker et al. (1). These authors stated that *A. actinomycetemcomitans* should be considered resistant to carbenicillin. According to our experience, a 10-fold-higher concentration of carbenicillin

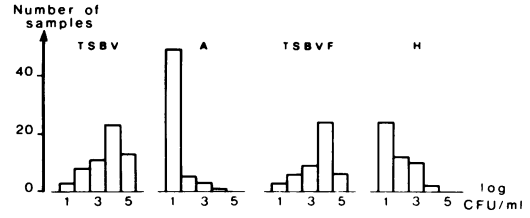

FIG. 4. Distribution of the clinical samples, based on recoveries of contaminants on the four selective culture media.

than that used in A agar resulted in growth inhibition of the strains tested. At the same time, a synergetic effect between carbenicillin and fusidic acid was observed. Similar results have been reported in the past (17).

Since carbenicillin is not added to H agar, the medium was supplemented with a higher amount of fusidic acid than A agar to achieve similar selectivity. H agar was further modified by increasing the concentration of NaF compared with that in TSBVF. The higher concentration was preferred since it does not affect the growth of *H. aphrophilus* (24), while it results in a better inhibition of *A. actinomycetemcomitans* (20). However, strains of *A. actinomycetemcomitans* resistant to NaF can be recovered on H agar, although at low frequencies.

Regarding the results with the pure cultures, both TSBV and A agars appeared to suppress growth of *A. actinomycetemcomitans* compared with blood agar. In contrast to our findings, Slots (21) reported equally good growth of *A. actinomycetemcomitans* strains on TSBV and blood agar. It is possible that methodological differences are the factor responsible. A agar showed a slightly higher inhibition of the growth of *A. actinomycetemcomitans* compared with TSBV. It was found that decrease of the carbenicillin concentration in the medium to 0.08 to 0.1 μg/ml improves the growth yields of *A. actinomycetemcomitans* strains to the level of those observed on TSBV. However, this modification will result in an increased contamination of *H. aphrophilus* on A plates.

Without affecting the recovery of *A. actinomycetemcomitans* and *H. aphrophilus* from periodontal samples, A and H media considerably suppressed the growth of contaminants and enabled bacteriological examination of undiluted samples. This fact is illustrated by the observations with three samples for which low numbers of *A. actinomycetemcomitans* were found only on the A medium. The finding that pure cultures of *A. actinomycetemcomitans* and *H. aphrophilus* were inhibited by A and H media to a greater extent than by TSBV and TSBVF, respectively, seems to be of minor clinical importance since this observation was not confirmed by the results with the periodontal specimens. Occasionally, yeasts, *Neisseria* spp., and some gram-negative rods could be recovered on A and H media. However, because of their occurrence at low numbers, their growth did not interfere with the bacteriological analysis of the samples.

Because of their selectivity, the improved media promote the recovery of *A. actinomycetemcomitans* and *H. aphrophilus*, and they should be valuable in studying the two bacterial groups in relation to various oral diseases.

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