

Selective Medium for Isolation of *Actinobacillus actinomycetemcomitans*

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A selective medium, TSBV (tryptic soy-serum-bacitracin-vancomycin) agar, was developed for the isolation of *Actinobacillus actinomycetemcomitans*. TSBV agar contained (per liter) 40 g of tryptic soy agar, 1 g of yeast extract, 100 ml of horse serum, 75 mg of bacitracin, and 5 mg of vancomycin. The TSBV medium suppressed most oral species and permitted significantly higher recovery of *A. actinomycetemcomitans* than nonselective blood agar medium. The distinct colonial morphology and positive catalase reaction of *A. actinomycetemcomitans* easily distinguished this bacterium from *Haemophilus aphrophilus*, *Campylobacter* species, and a few other contaminating organisms. With the TSBV medium, even modestly equipped laboratories will be able to isolate and identify *A. actinomycetemcomitans* from clinical specimens.

Isolation and identification of *Actinobacillus actinomycetemcomitans* has become increasingly important because of this organism's suspected role in certain types of human periodontal disease. Patients with localized juvenile periodontitis frequently harbor high numbers of *A. actinomycetemcomitans* in the subgingival plaque (6) and demonstrate high levels of serum antibodies against *A. actinomycetemcomitans* (2; P. Hammond, J. Slots, and R. J. Genco, *J. Dent. Res.* **60A**:498, 1981). Toxic substances from this organism including a potent endotoxin (4) and leukocidin (1, 7, 8) may play a role in pathogenesis.

Development of a selective medium for the specific recovery of *A. actinomycetemcomitans* would be of particular value because the organism forms small, translucent colonies which will be readily overlooked if other organisms outnumber it significantly. Nevertheless, even if *A. actinomycetemcomitans* is present only in small proportions, it may be of etiological significance due to its virulence. Furthermore, the growth of *A. actinomycetemcomitans* can be inhibited *in vitro* by common oral streptococcal species (J. D. Hillman and S. S. Socransky, *J. Dent. Res.* **60A**:603, 1981; Y. Yamamoto, P. A. Mashimo, H. Reynolds, and R. J. Genco, *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1981, D41, p. 50). A bacterial medium which suppresses species that are inhibitory to *A. actinomycetemcomitans* should facilitate the recovery of the organism.

This paper describes and evaluates an improved medium for selective culturing of *A. actinomycetemcomitans*. This medium, which is designated TSBV, contains tryptic soy agar

(Difco Laboratories, Detroit, Mich.), 10% serum, and 0.1% yeast extract, and it includes the selective agents bacitracin (75 µg/ml) and vancomycin (5 µg/ml). *A. actinomycetemcomitans*, unlike most oral bacterial species, is highly resistant to bacitracin and vancomycin (J. Slots, *Arch. Microbiol.*, in press).

MATERIALS AND METHODS

Bacterial strains. The *A. actinomycetemcomitans* strains used were: ATCC 29522, ATCC 29523, and ATCC 29524, obtained from the American Type Culture Collection, Rockville, Md.; NCTC 9709 and NCTC 9710, obtained from the National Collection of Type Cultures, London, England; Y4, obtained from S. S. Socransky, Boston, Mass.; and fresh clinical isolates 1, 15, 67, 90, 107, and 125, from our laboratory. The strains were maintained by weekly subculture on tryptic soy agar supplemented with 5% sheep blood and 0.1% yeast extract (BBL Microbiology Systems, Cockeysville, Md.) (TB medium) in an atmosphere of 90% air-10% CO₂.

Medium. TSBV was prepared with tryptic soy agar to which was added 1.0 g of yeast extract per liter. The pH was adjusted to 7.2, and the medium was autoclaved for 15 min at 121°C. The medium was cooled to 50°C, and horse serum and filter-sterilized bacitracin (Sigma Chemical Co., St. Louis, Mo.) and vancomycin (Sigma) were added to give final concentrations of 10%, 75 µg/ml, and 5 µg/ml, respectively. Preliminary studies with pure cultures of *A. actinomycetemcomitans* and dental plaque samples showed that these concentrations of serum, bacitracin, and vancomycin were optimal with respect to supporting growth of *A. actinomycetemcomitans* and suppressing growth of other oral species. The plates were stored aerobically at 5°C and used within 7 days of preparation.

Recovery efficiency of pure cultures of *A. actinomycetemcomitans*. Test strains of *A. actinomycetemcomi-*

TABLE 1. Comparative growth on nonselective TB medium and on TSBV medium of 12 pure cultures of *A. actinomycetemcomitans*

Medium	Incubation	Geometric mean growth \pm SD in % of the TB medium growth	Median growth in % of the TB medium growth
TB	85% N ₂ -10% H ₂ -5% CO ₂	100	100
TSBV	85% N ₂ -10% H ₂ -5% CO ₂	99 \pm 14	103
TSBV	90% air-10% CO ₂	96 \pm 43	89
TSBV	Candle jar	85 \pm 23	84

tans were inoculated into prereduced, anaerobically sterilized brain heart infusion broth (BBL). Overnight cultures were dispersed by mixing with a Vortex mixer at the maximal setting for 60 s and were serially 10-fold diluted in anaerobic Ringer solution. Using a bent-glass rod, 0.1-ml portions of 10⁻⁵ and 10⁻⁶ dilutions were spread on TB and TSBV plates. TB plates were incubated for 72 h at 37°C in a Coy anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.) containing 85% N₂-10% H₂-5% CO₂. TSBV plates were incubated for 72 h at 37°C in a Coy anaerobic chamber, in a Torbal jar (The Torsion Balance Co., Clifton, N.J.) with no catalyst and containing 90% air-10% CO₂, and in a candle jar.

Clinical specimens. Subjects were adolescents who were admitted to our dental clinic for treatment of rapidly advancing periodontal disease on molars and incisors (localized juvenile periodontitis). Samples from deep periodontal pockets were obtained by using paper points as previously described (6). These were transferred to 9 ml of anaerobic Ringer solution, the bacteria were dispersed by mixing with a Vortex mixer at the maximal setting for 60 s, and the bacterial suspension was serially diluted in 10-fold steps in anaerobic Ringer solution. Using a bent-glass rod, 0.1-ml portions of appropriate dilutions were plated on TB and TSBV. After incubation for 72 h at 37°C in a Coy anaerobic chamber, the plates were examined for *A. actinomycetemcomitans*. Randomly selected isolates suspected of being *A. actinomycetemcomitans* were

subcultured and confirmed as *A. actinomycetemcomitans* if they were gram-negative, capnophilic (exhibited scant growth in air but grew well in 10% CO₂), fermentative, catalase-positive coccobacilli which did not require X (hemin) and V (NAD⁺) factors for growth.

RESULTS

Table 1 shows the recovery efficacy of 12 strains of *A. actinomycetemcomitans* on TB after anaerobic chamber incubation and on TSBV after anaerobic chamber, Torbal jar with 90% air-10% CO₂, and candle jar incubation. For each strain tested, the viable count on TB in the anaerobic chamber (nonselective culturing) was designated 100%, and the viable counts on the TSBV plates were compared with this. Under anaerobic incubation, growth on TSBV was similar to that on TB. Some inhibition tended to occur when incubation took place in 90% air-10% CO₂ or in a candle jar; however, the observed differences in recovery rates were not statistically significant at the 5% significance level as analyzed by the Wilcoxon signed-ranks test for matched pairs.

Fourteen specimens of advanced juvenile periodontitis lesions were obtained. Table 2 shows the number of *A. actinomycetemcomitans* re-

TABLE 2. Recovery of *A. actinomycetemcomitans* from dental plaque on TSBV medium and on TB medium after 72 h of anaerobic incubation

Specimen	<i>A. actinomycetemcomitans</i> recovery (colony-forming units)		% Increase in recovery on TSBV medium ^a
	TSBV medium	TB medium	
1	144 \times 10 ²	88 \times 10 ²	64
2	120 \times 10 ²	27 \times 10 ²	344
3	450 \times 10 ³	42 \times 10 ³	971
4	3 \times 10 ²	<10 ²	
5	11 \times 10 ²	2 \times 10 ²	450
6	528 \times 10 ²	106 \times 10 ²	398
7	251 \times 10 ³	206 \times 10 ³	22
8	180 \times 10 ³	19 \times 10 ³	847
9	24 \times 10 ²	32 \times 10 ²	-25
10	294 \times 10 ³	140 \times 10 ³	110
11	488 \times 10 ²	100 \times 10 ²	388
12	772 \times 10 ²	152 \times 10 ²	408
13	16 \times 10 ²	<10 ²	
14	14 \times 10 ²	<10 ²	

^a Data were obtained by subtracting the value for TB medium from the value for TSBV medium, dividing the resulting number by the value for TB medium, and then multiplying by 100.

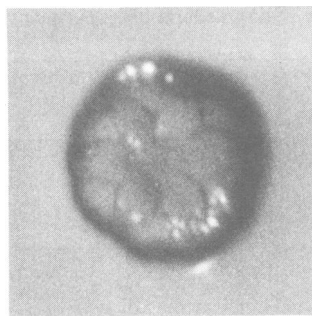


FIG. 1. Characteristic colony of *A. actinomycetemcomitans* after growth on TSBV for 72 h. Note starlike inner structure. Photography performed with light transmitted through the medium.

covered on TB and TSBV media. Significantly, eight samples yielded 2- to 10-times higher counts of *A. actinomycetemcomitans* on TSBV than on TB. Also, in three samples in which *A. actinomycetemcomitans* occurred in low numbers, the organism could be recovered only on the TSBV medium.

Colonies of *A. actinomycetemcomitans* on TSBV appeared as circular, convex, translucent, glistening, and 0.5 to 1.0 mm in diameter with slightly irregular edges after incubation for 3 days. On primary isolation, the colonies strongly adhered to the agar surface and commonly exhibited a starlike inner structure (Fig. 1). Continued subculture resulted in loss of the adherence and the starlike structure.

Organisms other than *A. actinomycetemcomitans* which grew on TSBV in 90% air–10% CO₂ or candle jar included mainly *Haemophilus aphrophilus* and *Campylobacter* species and occasionally *Neisseria* species, *Staphylococcus-Micrococcus* species, and yeasts. When incubated anaerobically, TSBV also supported growth of strains of fusobacteria and gram-negative anaerobic motile rods. The ability of *A. actinomycetemcomitans* to vigorously decompose 3% H₂O₂ applied on the primary isolation plate could distinguish this organism from the morphologically similar catalase-negative or weakly catalase-positive *H. aphrophilus*. The remaining contaminants could readily be distinguished from *A. actinomycetemcomitans* on the basis of major differences in colonial morphology.

DISCUSSION

Most existing data on *A. actinomycetemcomitans* in clinical infections have been obtained by using nonselective culture techniques. Since *A. actinomycetemcomitans* produces a small colony which readily can be overlooked and since

various streptococcal species can inhibit the organism's growth in vitro, it seems appropriate to use a special selective procedure for the isolation of *A. actinomycetemcomitans*.

Kilian and Schjøtt (5) recovered *A. actinomycetemcomitans* from dental plaque by using chocolate agar supplemented with 300 µg of bacitracin per ml, a medium developed by Hovig and Aandahl (3) for the selective recovery of haemophili. The isolation and identification of *A. actinomycetemcomitans* on this medium can be hampered by the growth of streptococci, several *Haemophilus* species, *Eikenella corrodens*, and neisseriae (5).

Mandell and Socransky (R. L. Mandell and S. S. Socransky, *J. Dent. Res.* 59A:512, 1980) isolated *A. actinomycetemcomitans* on Trypticase soy agar (BBL Microbiology Systems) supplemented with 128 µg of bacitracin per ml, 8 µg of malachite green per ml, and 5% sheep blood. This medium suppressed the growth of pure cultures of *A. actinomycetemcomitans* as much as 20%. The malachite green, which at relatively low concentrations can be inhibitory to *A. actinomycetemcomitans* (Slots, in press), may be responsible for this suppression. Mandell and Socransky's medium also may grow several contaminating *Haemophilus* species because of the blood supplement.

The TSBV medium overcomes several of these problems. The enrichment with 10% horse serum instead of with blood did not change the recovery rate of *A. actinomycetemcomitans*, but it suppressed hemin-requiring *Haemophilus* strains and allowed direct application of H₂O₂ to the primary isolation plate to verify the *A. actinomycetemcomitans* diagnosis. Bacitracin was added because many oral species are particularly susceptible to this antibiotic (Slots, unpublished data). The vancomycin content of 5 µg/ml suppressed the growth of potentially inhibitory streptococcal strains and other gram-positive species. Our finding of lower recovery rates of *A. actinomycetemcomitans* from clinical specimens on nonselective blood agar medium than on TSBV suggests that in vitro bacterial antagonism against *A. actinomycetemcomitans* can be of a significant magnitude. Additional suppression of contaminants could be achieved by incubating in 90% air–10% CO₂ or in a candle jar instead of anaerobically. The observation that the candle jar system provided good growth for *A. actinomycetemcomitans* may be of value in a clinical setting or in epidemiological studies where microbiological facilities are limited.

In conclusion, the TSBV medium described in this report can considerably aid the microbiological monitoring of *A. actinomycetemcomitans*; it is relevant in the diagnosis and treatment of *A. actinomycetemcomitans* infections.

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