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Improved, Low-Cost Selective Culture Medium for *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans **is considered to be one of the major oral putative pathogens, especially in cases of juvenile periodontitis. This microorganism requires nutritionally complex media for growth, and therefore the media for its primary isolation usually include blood agar or serum in their base. In this study we present a new medium, Dentaid-1, which improves the detection of *A. actinomycetemcomitans* in periodontal samples. In its composition, blood and serum have been omitted, hence reducing its cost and making it a more restrictive medium against the growth of other microorganisms with high nutritional requirements. The growth yields of pure cultures of the bacteria on Dentaid-1 were comparable to those on nonselective blood agar. Moreover, clinical efficacy was evaluated in subgingival samples from 77 subjects with adult periodontitis. Dentaid-1 detected *A. actinomycetemcomitans* in 24 subjects, while a previously described tryptic soy-serum-bacitracin-vancomycin agar detected the microorganism in only 19 subjects (79.1%). Dentaid-1 is a low-cost, noninhibitory formula for the improved diagnosis and monitoring of patients subgingivally infected by this important oral putative pathogen.**

In the last few years, substantial evidence has emerged that *Actinobacillus actinomycetemcomitans* may be, along with *Porphyromonas gingivalis*, a major oral putative pathogen, as judged by this organism's rare occurrence in periodontally healthy individuals (25). *A. actinomycetemcomitans* has been isolated from adult periodontitis lesions, but less frequently and in lower numbers than from lesions in juvenile periodontitis subjects (21, 26). Treatment failures have been associated with the failure to reduce the amount of the microorganism in treated sites (14, 17). Furthermore, data on the transmission of *A. actinomycetemcomitans* from host to host (5, 6, 32) and new evidence of its role as an infectious agent involved in disease development at extraoral sites (35) are providing one of the strongest associations between this oral pathogen and periodontal and systemic diseases.

Existing data on the presence of *A. actinomycetemcomitans* in clinical infections have been obtained by using both selective and nonselective media for their isolation. Since *A. actinomycetemcomitans* is found in small proportions and because its growth can be inhibited in vitro by common oral streptococcal species (23), selective culture media are useful tools in the detection and enumeration of this bacterium. Although new molecular techniques are extremely sensitive in the detection of target bacteria (32, 33, 34), culture techniques are still the methods of reference for studying viable cells and a prerequisite for determining the antimicrobial susceptibility of a given pathogen.

Among the selective culture media described for the isolation and enumeration of *A. actinomycetemcomitans* (11, 16, 23), tryptic soy-serum-bacitracin-vancomycin (TSBV) medium (23) has been the most widely used in the analysis of any kind of oral samples (4, 19) in studies throughout the world (2, 10, 13, 26). TSBV medium has been defined as a low-inhibitory medium compared with a nonselective blood agar medium (23). Moreover, it allows for the direct detection of catalase activity on the primary isolation plate, facilitating the presumptive identification of *A. actinomycetemcomitans* (23). Catalase activity is a key assay in distinguishing between *A. actinomycetemcomitans* and the morphologically similar *Haemophilus aprophilus* (23). The genus *Actinobacillus* is included in the *Pasteurellaceae* family. These microorganisms require nutritionally complex media for primary isolation (15). Usually blood agar or serum has been the base for the design of selective media (11, 16, 23).

The aim of the present study was to evaluate a new selective culture medium for *A. actinomycetemcomitans* that lacks both blood and serum. It was designed to confirm the following expectations: optimal growth of *A. actinomycetemcomitans* and suppression of oral flora that should be equal to or better than what is observed when using TSBV medium as the reference medium.

MATERIALS AND METHODS

Bacterial strains. The *A. actinomycetemcomitans* strains used were ATCC 33383 and NCTC 10981, obtained from the American Culture Collection, Rockville, Md., and from the National Collection of Type Cultures, London, England, respectively. Additionally, 20 clinical isolates from our laboratory were included. The 22 strains were maintained at −85°C and subcultured three times at 48-h intervals on brain heart infusion agar (BHIA) (Difco Laboratories, Detroit, Mich.) before testing. Plates were incubated in a CO_2 incubator (5% CO_2) (Sanyo Electric Co., Ltd.).

Selective media. Dentaid-1 was prepared using BHIA to which the following compounds were added: 5 g of yeast extract, 1.5 g of sodium fumarate (Sigma Chemical Co., St. Louis, Mo.), and 1 g of sodium formate (Sigma) per liter. The medium was autoclaved for 15 min at 121°C. The final pH was 7.2 ± 0.2. Once the medium was cooled to 50°C, vancomycin (Sigma) was added to a final concentration of 9 μg/ml. TSBV medium was prepared according to the original description of the medium (23).

Recovery efficiency of pure *A. actinomycetemcomitans* cultures. BHIA subcultures of the 22 previously described strains were inoculated into freshly prepared

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tubes containing brain heart infusion broth (Difco), and the cell density was adjusted to 10^8 to 10^9 cells per ml. Suspensions were dispersed by mixing with a vortex mixer for 30 s and then serial 10-fold diluted in phosphate-buffered saline (PBS). Adequate dilutions were spread in duplicate by using a spiral plater (Countermat; IUL Instruments) and incubated for 72 h in a 5% CO_2 incubator. The media used were; TSBV, Dentaid-1, and an anaerobic blood agar (BHIA, 5% horse blood, 5 mg of hemin per liter, 10 mg of menadione per liter). Bacterial counts were numbered as CFU/milliliter. For each strain, the yield of growth on the two selective media was compared with that found on the blood agar and is expressed as a relative-growth-supporting-ability (RGSA) value, which was determined as the logarithm of the ratio of the number of colonies on the blood agar to the number of colonies on the selective agar (11, 12).

Clinical specimens. Subgingival plaque samples from 77 patients with untreated (before mechanical treatment of scaling and root planning) or treated (1 to 3 months after treatment) adult periodontitis were received for microbiological diagnosis at our laboratory from several private dental clinics. One pooled sample per patient was obtained by sampling the four deepest periodontal pockets (one in each quadrant) and using paper points (two paper points per site) as previously described (9). The eight paper points per patient were transferred to 2 ml of reduced transport fluid (28), which was transported and processed within 24 h. In the laboratory, samples were dispersed with a vortex mixer for 30 s and serially diluted in 10-fold steps in prereduced PBS. Appropriate dilutions were plated in parallel on TSBV and Dentaid-1. After incubation for 72 h at 37°C in a 5% CO_2 incubator, the plates were examined for the presence and enumeration of *A. actinomycetemcomitans*. The contaminant flora (rest of the flora) was quantified in positive samples. Counts on clinical samples were also numbered as CFU/milliliter.

An *A. actinomycetemcomitans* presumptive identification was first made on the basis of colonial morphology. Presumptive identification continued with determining the catalase activity at 72 h of incubation on discrete colonies on the primary isolation plate. Catalase-positive and catalase-negative colonies resembling *A. actinomycetemcomitans* were subcultured on BHIA (Difco), and after 24 to 48 h of incubation the catalase activity was confirmed upon subculture. Strains were also tested for lactose fermentation (ONPG [*o*-nitrophenyl-β-D-galactopyranoside]) (27), and ONPG-positive strains were identified by Gram stain, aerotolerance, and rapid enzymatic methods (Innovative Diagnostic Systems, Inc., Norcross, Ga.). Subsequently, lactose-negative colonies resembling *A. actinomycetemcomitans* were confirmed by using a species-specific PCR (8, 20).

PCR. For PCR analysis of presumptive *A. actinomycetemcomitans*, a colony was resuspended in 100 μl of sterile distilled water. Then, 3.2 μl of this suspension was used in each PCR reaction. The primers used for PCR were designed to identify *A. actinomycetemcomitans* targeted 16S rRNA (3) were as follows: the forward primer sequence was 5′-AAACCCATCTCTGAGTTCTTCTTC-3′, and the reverse primer sequence was 5′-ATGCCAACTTGACGTTAAAT-3′. These primers gave an expected amplification product of 557 bp. PCR amplification was carried out in a reaction volume of 25 μl consisting of 3.2 μl of the initial sample in water for a final volume of 20.1 μl and 4.9 μl of the reaction mixture containing 1× PCR buffer [67 mM Tris-HCl, pH 8.8; 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20; 1.5 mM $MgCl_2$], 0.6 U of EcoTaq DNA polymerase (ECOGEN), 0.25 mM concentrations of the deoxynucleoside triphosphates, and 80 pmol of each primer. PCR cycling was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Barcelona, Spain). After an initial denaturation step of 94°C for 5 min, 35 amplification cycles of denaturation at 94°C for 30 s, annealing of primers at 55°C for 30 s, and primer extension at 72°C for 30 s were carried out, followed by a final primer extension step at 72°C for 7 min. Reaction products were either stored at −20°C or analyzed immediately.

Negative control samples consisting of the standard mixture with 3.2 μl of sterile distilled water were included in each batch of samples analyzed by PCR.

RESULTS

The 20 clinical isolates used for recovery efficiency purposes were all gram negative, capnophilic short rods, and non-lactose fermenting and rendered the band of a 557-bp amplification product by PCR assay (3). Catalase activity was positive for all but one.

The mean RGSA values for *A. actinomycetemcomitans* on TSBV and Dentaid-1 were 0.81 (Standard deviation [SD] = 1.61) and 0.06 (SD = 0.11), respectively. The difference was statistically significant ($P = 0.03$, Student paired *t* test). The RGSA value for Dentaid-1 suggests no inhibition compared to blood agar.

TABLE 1. Recovery and percentage of total cultivable *A. actinomycetemcomitans* microrganisms in positive subgingival samples from patients with adult periodontitis

Treatment group and medium	No. of positive samples ($n = 24$)	Mean CFU/ml ± SD (10^5)	Mean % ± SD
Before mechanical treatment			
TSBV	14	1.6 ± 2.8	43.6 ± 38.
Dentaid-1	14	3.3 ± 7.0	81.7 ± 31.
After mechanical treatment			
TSBV	5	0.9 ± 1.8	37.2 ± 38.0
Dentaid-1	10	1.0 ± 2.8	71.3 ± 40.6

For the 77 specimens collected from periodontal pockets, a good relationship was found in the detection of *A. actinomycetemcomitans* in TSBV and Dentaid-1. From 24 positive subgingival samples, 19 were detected in parallel by TSBV and Dentaid-1, and five additional positive samples were found on Dentaid-1. In summary, *A. actinomycetemcomitans* was found in 24.7% ($n = 19$) of the samples when they were assayed with TSBV and in 31.2% ($n = 24$) when Dentaid-1 was used.

Colonies on TSBV after 72 h of incubation are rough, circular, and convex with slightly irregular edges; they appear as small colonies with dark borders and a common star-shaped inner structure (23). In some strains, the morphology at 72 h on Dentaid-1 can differ from this appearance, showing smooth, circular, spherical colonies with an enhanced dark border and an incipient star inner structure. A total of 46 subcultures (21 from TSBV and 25 from Dentaid-1) from clinical specimens were performed for presumptive identification from selected colonies resembling *A. actinomycetemcomitans* on TSBV and Dentaid-1. From these, 43 subcultures (19 from TSBV and 24 from Dentaid-1) were ONPG negative and were confirmed by PCR as being *A. Actinomycetemcomitans* (3).

From the 43 subcultures, 41 subcultures on BHIA after 48 h of incubation in 5% CO_2 showed strong catalase activity, and 2 were catalase negative, corresponding to strains from TSBV and Dentaid-1 from the same sample. Three subcultures were ONPG positive: two strains isolated from TSBV were identified as *H. aphrophilus* and *Haemophilus segnis*, whereas one strain isolated from Dentaid-1 was identified as *H. segnis*.

When *A. actinomycetemcomitans*-positive samples were grouped according to patients before or after mechanical treatment, differences between the two media appeared only in the prevalence of the bacteria in the latter group, as shown in Table 1. Dentaid-1 detected twice as many *A. actinomycetemcomitans*-positive samples as did TSBV in these particular samples. As shown in Table 1, no differences in the mean recovery of viable CFU of *A. actinomycetemcomitans* per milliliter were observed ($P = 0.5$, Student paired *t* test). However, higher mean percentages of incidence of the bacteria were calculated from Dentaid-1 in both groups, which represents statistically significant differences ($P = 0.005$, Student paired *t* test). This suggests a lower recovery of contaminant flora by Dentaid-1 in comparison to TSBV. This is clearly shown in Fig. 1, where the mean log CFU of both *A. actinomycetemcomitans* and contaminant flora per milliliter are represented. The differences between contaminant flora on Dentaid-1 with respect to contam-

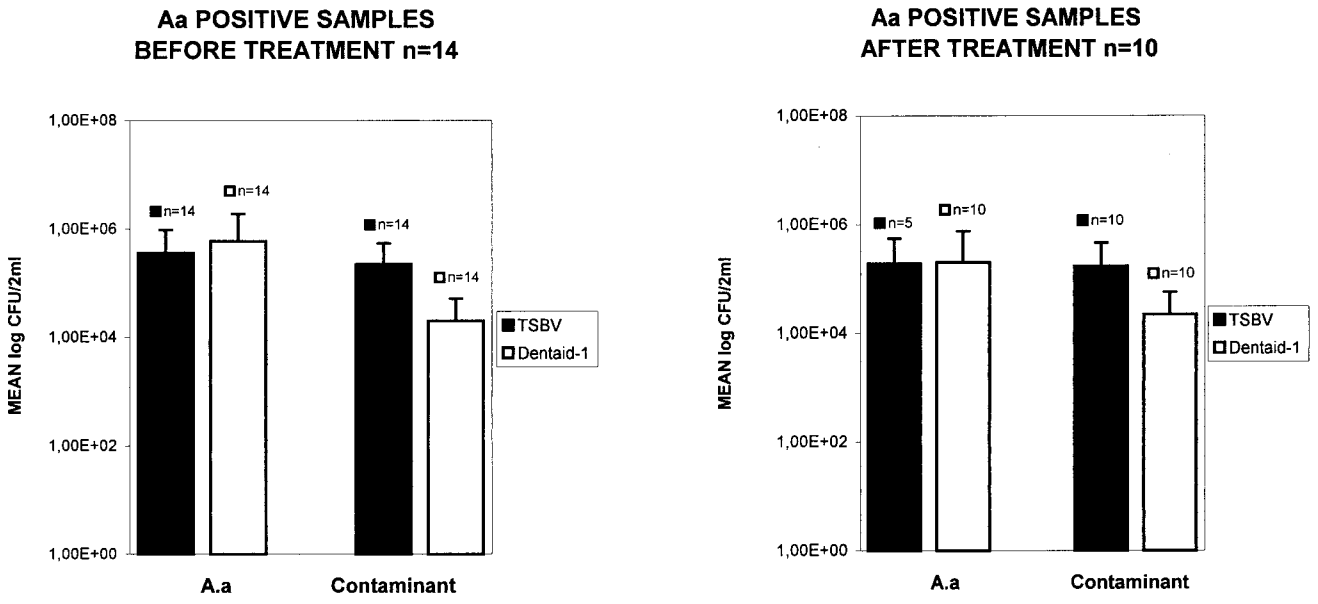

FIG. 1. Recovery of *A. actinomycetemcomitans* and contaminant flora (rest of flora) on Dentaid-1 in comparison to TSBV in samples before or after mechanical treatment.

inant flora on TSBV are statistically significant ($P = 0.007$, Student paired t test).

DISCUSSION

Development of a selective medium for the specific recovery of a given pathogen is primarily based on the selection of an adequate nutritive base and a proper inhibition system for reducing contaminant flora. Potential drawbacks with selective media are overselectivity or insufficient selectivity.

In the literature, Trypticase soy agar supplemented with either blood (16) or serum (11, 23) has been the nutritive base applied to selective media designed for the isolation of *A. actinomycetemcomitans*. Preliminary work carried out in our laboratory indicated that BHIA by itself is an excellent nutritive base that allows for good growth of pure cultures of the bacteria. When 5-g/liter doses of yeast extract are added, colonial development is comparable to that observed in TSBV (23). This fact allowed us to omit blood and serum from the nutritive base of the selective medium Dentaid-1. Moreover, if blood and serum are omitted from the formula, contaminant flora with high nutritional requirements are controlled and the cost per plate greatly decreased. The TSBV medium described by Slots (23) which omits the blood content of the previous MGB (malachite green-bacitracin) selective medium described by Mandell and Socransky (16), results in the inhibition of hemin-requiring *Haemophilus* species (23). Dentaid-1 suppresses the growth of *H. aphrophilus* and *Haemophilus paraphrophilus* by 3 log orders with respect to both anaerobic blood agar and TSBV (data not shown). Vancomycin plus formate and/or fumarate, as described below, and the use of a BHIA nutritive base lacking both blood and serum were found to be responsible for such suppression. Interestingly, *H. aphrophilus* is described as being morphologically similar to *A. actinomycetemcomitans* (23).

Bacitracin and vancomycin are antibiotics commonly used in the formulation of microbiological media (7) and particularly for the isolation of *A. actinomycetemcomitans* due to their expected role against a wide range of mainly gram-positive flora (19). Holm et al. (11) improved inhibition, including the inhibition of more gram-negative bacteria, by complementing bacitracin and vancomycin activity with carbenicillin, fusidic acid, and spiramycin in the same formula. This medium, called A-medium, is described as a modification of Slots' TSBV medium (23) and inhibits the growth of *Capnocytophaga* spp. and *Neisseria* spp. These species are found in large quantities as contaminant microorganisms in samples taken from periodontal pockets (22, 23, 31). In spite of the various antibiotics included, A-medium resulted only in slightly higher inhibition of the growth of *A. actinomycetemcomitans* compared with TSBV, as expressed by RGSA values of 1.12 and 0.76, respectively. These RGSA values for TSBV are similar to the value obtained in the present study. In consequence, our results also agree with the previous observations of Holm et al. (11), which indicated The suppression of *A. actinomycetemcomitans* grown on TSBV compared with blood agar. In our study, five pure cultures of *A. actinomycetemcomitans* (22.7%) experienced high suppression on TSBV. Between 2 and 5 logs of inhibition were observed, contributing to the high RGSA value obtained for TSBV. Possible explanations may be either methodological differences, as suggested by Holm et al. (11), or TSBV's overselectivity for some strains, as indicated by our results.

The vancomycin content of Dentaid-1 was chosen for its efficacy in eliminating streptococcal species (16, 18, 23) and the high resistance of *A. actinomycetemcomitans* to this antibiotic, with a MIC_{90} value of ≥ 64 μg/ml (16).

Vancomycin as a sole inhibitory agent has been previously used in Hammond's selective medium for the oral putative pathogen *Campylobacter rectus* (B. F. Hammond and D. Mallonee, Abstract, J. Dent. Res. **67:**327, abstr. 1712). Our first observations of *A. actinomycetemcomitans* pure culture grown on Hammond's medium (Hammond and Mallonee, abstract)

formulated on a BHIA base indicated an unexpected slow colonial development, which seems to be due to an ingredient in the formula other than vancomycin. Hammond's medium incorporates vancomycin (9 μg/ml) as a selective agent and an SH_2 indicator system (ferrous sulfate, 0.2 g/liter, and sodium thiosulfate, 0.3 g/liter) as a differential marker with sodium formate (2 g/liter) and sodium fumarate (3 g/liter) as energy sources. Since our preliminary studies showed that formate and/or fumarate delays colonial development of *A. actinomycetemcomitans* from 24 to 48 h, the dosage of formate and fumarate was studied in a liquid medium (data not shown) to allow for good growth of *A. actinomycetemcomitans*. Furthermore, some strains belonging to other gram-negative species, mainly *H. aphrophilus* and *H. paraphrophilus*, were suppressed. Formate and fumarate sodium salts are usually included in cultivation media as an energy source for formate- and/or fumarate-requiring species (29; Hammond and Mallonee, abstract). Since vancomycin or formate-fumarate by themselves do not have such inhibitory qualities, we postulate that their combination can have a synergistic effect upon strains of certain gram-negative species in subgingival samples. No references have been found in the literature regarding this possible synergistic effect.

Adult periodontitis patients were chosen in order to challenge the efficacy of Dentaid-1 under the worst possible conditions. In the 24 positive clinical samples studied, Dentaid-1 suppressed contaminant flora by 10-fold compared to TSBV. Before mechanical treatment (scaling and root planning), *A. actinomycetemcomitans* was detected by Dentaid-1 and TSBV in the same patients and showed similar recovery rates. Surprisingly, after mechanical treatment, The prevalence of the bacteria was 100% higher in Dentaid-1 than in TSBV. Dentaid-1's greater suppression of contaminant flora, and TSBV's suppression of some strains of *A. actinomycetemcomitans* may be an explanation for these results. Furthermore, after mechanical treatment, subgingival pockets should be recolonized by species that are more inhibited by the new proposed medium. Streptococcal species are the most commonly encountered subgingival species, and they are known to be inhibitory for *A. actinomycetemcomitans* growth in vitro (23). Experimental work on comparing the species suppressed by Dentaid-1 and those suppressed by TSBV was beyond the scope of the present study and will be the subject of further specific study.

The presumptive identification performed here took into account colonial morphology, catalase activity, and lactose fermentation as previously described (1, 24) and is considered to help in the rapid and accurate screening of *A. actinomycetemcomitans* from either TSBV or Dentaid-1 selective media. We found two catalase-negative strains growing on both Dentaid-1 and on TSBV from clinical isolates. Although catalase-negative strains have been reported as relatively rare (30), they must be considered in order to achieve a correct microbiological diagnosis.

Finally, and in order to complement the presumptive identification of *A. actinomycetemcomitans* colonies isolated on Dentaid-1, we performed specific PCR which confirms our results. According to other authors (8, 20), the extreme specificity of PCR has been found to be particularly useful for the identification of suspected pathogens, supplying inconclusive or unexpected biochemical patterns as previously reported (20).

In conclusion, the new proposed medium, Dentaid-1, improves the detection of *A. actinomycetemcomitans* inexpensively, with a noninhibitory formula, and can be of considerable aid in microbiological diagnosis and in monitoring patients subgingivally infected with this bacterium.

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