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Evaluation of three selective media for isolation of *Aggregatibacter actinomycetemcomitans*

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Background and Objective: Aggregatibacter actinomycetemcomitans is a pathogen in oral and nonoral infections. Detection and quantification of this pathogen can be performed using selective culture techniques. The aim of this study was to establish the efficacy of two known selective media in their ability to select and support the growth of *A. actinomycetemcomitans*.

Material and Methods: Trypticase soy bacitracin vancomycin (TSBV) medium and brain–heart infusion agar with vancomycin (Dentaid-1), as well as a modified Dentaid-1 medium (in which the brain–heart infusion agar was substituted with brain–heart infusion broth), were compared. Two-hundred and eighteen clinical samples were used to establish the recovery rate, the number of colony-forming units (CFUs) of *A. actinomycetemcomitans* as well as the total number of CFUs on the three different types of medium. In addition, the numbers of gram-negative aerobic rods and yeasts were determined.

Results: Both types of Dentaid-1 medium showed a higher recovery of *A. actinomycetemcomitans* compared with TSBV. However, these differences did not reach statistical significance. The total number of CFUs of *A. actinomycetemcomitans* recovered was significantly higher on Dentaid-1 compared with TSBV ($p = 0.029$). The mean number of gram-negative aerobic rods recovered was statistically higher on both types of Dentaid-1 medium in comparison with TSBV. Low numbers of yeasts were recovered occasionally on all test plates.

Conclusion: Dentaid-1 is a low-cost effective alternative to TSBV for the isolation and growth of *A. actinomycetemcomitans* from clinical samples, such as dental plaque, which contain a complex microflora.

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Aggregatibacter actinomycetemcomitans is a capnophilic, gram-negative rod that is primarily associated with human periodontal disease. The organism can also be involved in non-oral infections such as endocarditis, prosthetic heart valve infection (1) and brain abscesses (2). *A. actinomycetemcomitans* has a low prevalence in periodontal health (3,4). However, high levels of the organism are found in aggressive periodontitis (5). Detection of *A. actinomycetemcomitans* has been facilitated by the use of selective isolation media. Mandel & Socransky (6) described on a trypticase soy agar based-medium supplemented with sheep blood, malachite green and bacitracin for the isolation if *A. actinomycetemcomitans*. Slots (7) reported on a tryptic soy agar elective medium supplemented with horse serum, yeast, bacitracin and vancomycin (TSBV), and this medium has become the stan-

dard medium for the isolation and growth of *A. actinomycetemcomitans*. The TSBV medium was modified by Holm *et al*. (8), who added carbenicillin, fusidic acid and spiramycin to give better suppression of bacterial species other than *A. actinomycetemcomitans*. This medium is, however, complex and expensive to prepare. In order to produce a low-cost, but effective, isolation medium for *A. actinomycetemcomitans*, Alsina *et al*. (9) developed a new medium, Dentaid-1, which has brain–heart infusion agar as a base and is supplemented with yeast, sodium fumarate, sodium formate and vancomycin. This new medium was shown to support the growth of *A. actinomycetemcomitans* and the number of contaminants was comparable with the TSBV medium. Although detection of *A. actinomycetemcomitans* is currently often performed using molecular techniques, such as the PCR, isolation of *A. actinomycetemcomitans* by culture is still necessary for antibiotic-susceptibility testing (10) and strain typing (11). The purpose of the present study was to evaluate the recovery of *A. actinomycetemcomitans* from a large sample of clinical specimens ($n = 218$) after culture on Dentaid-1 medium prepared using two different brain–heart infusion bases and to compare the recovery of *A. actinomycetemcomitans* on these media with recovery on the TSBV medium. Attention was also paid to the concomitant growth of yeasts and gram-negative aerobic rods on the three selective media.

Material and methods

Selective media

Dentaid-1 and modified Dentaid-1 (mDentaid-1) were prepared with brain–heart infusion agar and brain–heart infusion broth, respectively, and were supplemented with the compounds listed in Table 1. TSBV medium was prepared according to the original description by Slots (7) and checked for the ability to support the growth of *A. actinomycetemcomitans* using a reference strain (DSM 11123). Plates were used within 2 d of preparation.

Table 1. Composition of Dentaid-1, modified Dentaid-1 (mDentaid-1) and trypticase soy bacitracin vancomycin (TSBV) isolation media

TSBV	Dentaid-1	mDentaid-1
Tryptic soy agar: 40 g/L Yeast extract: 1.0 g/L Autoclave: 121°C for 15 min pH 7.2 Horse serum: 10% Bacitracin: 75 μg/mL Vancomycin: 5 μg/mL	BHI agar: 52 g/L Yeast extract: 5 g/L Sodium fumarate: 1.5 g/L Sodium formate: 1 g/L pH 7.2 Autoclave: 121°C for 15 min Vancomycin: 9 μg/mL	BHI broth: 37 g/L Agar: 15 g/L Yeast extract: 5 g/L Sodium fumarate: 1.5 g/L Sodium formate: 1 g/L pH 7.2 Autoclave: 121°C for 15 min Vancomycin: 9 μg/mL

BHI, brain–heart infusion.

Design

The study was performed in two separate experiments: in the first experiment, the recovery of *A. actinomycetemcomitans* on TSBV and Dentaid-1 media was compared; and in the second experiment, mDentaid-1 medium was compared with TSBV medium for the recovery of *A. actinomycetemcomitans*. The outcome variables of these experiments included: total numbers of colony-forming units (CFUs); total counts of *A. actinomycetemcomitans*; total counts of gram-negative aerobic rods; and total counts of yeasts.

Clinical samples

Subgingival samples ($n = 218$) were obtained from patients with untreated, chronic or aggressive destructive periodontal disease with deep and bleeding pockets (> 5 mm). Samples were obtained after mechanical removal of supragingival plaque deposits and air drying of the sampling sites. Sampling sites were than isolated with cotton rolls. Two sterile paper points were subsequently inserted into the deepest and bleeding pocket in each quadrant of the dentition and left *in situ* for 15 s. Paper points were pooled in 2 mL of reduced transport fluid (12).

Sample processing

Upon arrival in the laboratory, the samples were vortexed for 45 s to dislodge bacteria from the paper points. Tenfold serial dilutions were prepared in sterile saline (1/10, 1/100 and 1/1000 dilutions) and 100-μL aliquots of the dilutions were spread on the three selective isolation media. The plates were incubated in air + 5% CO_2 at 35°C for up to 5 d.

Identification and counting

A. actinomycetemcomitans was identified on the basis of its typical colony morphology (a star-like inner structure) and the ability to degrade 3% hydrogen peroxide by catalase production (7). For further identification, the API-ZYM (Biomérieux, Marcy l'Etoile, France) was used (13).

The total number of CFUs was determined on plates containing at least 100 CFUs. Then, the numbers of CFUs of yeast species and of gram-negative rods were established using gram staining. The total number of CFUs of gram-negative rods was recorded using the following semi-quantitative scale: 0, no gram-negative rods; 1, < 100 CFUs; 2, 10–300 CFUs; and 3, > 300 CFUs.

Data analyses

The numbers of total CFUs, total counts of *A. actinomycetemcomitans* and total counts of gram-negative rods were log-transformed (GraphPad Instat 3; GraphPad Software, Inc., La Jolla, CA, USA). Differences in mean counts were tested for statistical significance using the nonparametric Mann–Whitney *U*-test. Detection frequencies on the different plates were compared using Fisher's

exact test. The level of significance was set at 5% ($p < 0.05$).

Results

A. actinomycetemcomitans was isolated more frequently from Dentaid-1 (24%) than from TSBV medium (19%; p = not significant). Also, in the second experiment the number of *A. actinomycetemcomitans*-positive samples was higher on mDentaid-1 (18%) than on TSBV medium (16%; p = not significant) (Table 2). Comparison of *A. actinomycetemcomitans* counts revealed a significantly higher mean count on Dentaid-1 in comparison with TSBV medium ($p = 0.029$, Table 3). The mean total *A. actinomycetemcomitans* count on the mDentaid-1 medium was also higher than on TSBV medium but the difference did not reach significance. Significantly higher counts of gram-negative aerobic rods were observed on both Dentaid-1 ($p = 0.0142$) and mDentaid-1 ($p = 0.0001$) media in comparison with TSBV medium. No significant differences were noted in the total numbers of CFUs between the two types of Dentaid-1 medium and TSBV medium. The number of samples with detectable yeast was low on Dentaid-1 and TSBV medium (12/111 vs. 7/111; p = not significant) and on mDentaid-1 and TSBV medium (15/107 vs. 14/107; p = not significant).

Discussion

The aim of this study was to compare the efficacy of three selective media for the isolation and growth of *A. actinomycetemcomitans*. The high costs of the TSBV medium were a major reason for this comparative study. The TSBV medium contains 10% horse serum and two antibiotics, whereas Dentaid-1 needs no serum for growth of *A. actinomycetemcomitans*. To further reduce the costs of Dentaid-1 we replaced the brain–heart infusion agar with brain–heart infusion broth. *A. actinomycetemcomitans* was recovered more frequently on both types of Dentaid-1 medium than on TSBV medium. The reason why *A. actinomycetemcomitans* may not be isolated on TSBV medium in some cases is unclear. *A. actinomycetemcomitans* isolates from samples recovered from Dentaid-1 plates, but not from TSBV plates, were transferred to freshly prepared TSBV medium and in all instances these isolates produced growth on TSBV. Therefore, it is unlikely that suppression of the growth of feeder bacteria on TSBV medium is the explanation for the lack of initial growth of *A. actinomycetemcomitans* in some samples. We also checked *A. actinomycetemcomitans* strains isolated from Dentaid-1 media, but not from TSBV medium, for susceptibility to bacitracin and vancomycin; however, none of these isolates were susceptible to these antibiotics at the concentrations used in the isolation media. Reduced growth (i.e. 2–5 logs of inhibition) of *A. actinomycetemcomitans* on TSBV medium, but not on blood agar, has been described (9).

Compared with TSBV medium, a significantly higher number of *A. actinomycetemcomitans* colonies were isolated from Dentaid-1 but not from mDentaid-1. One potentially negative characteristic was the recovery of a significantly higher number of gram-negative aerobic rods from both Dentaid-1 and mDentaid-1. Although this phenomenon is a disadvantage, it did not result in a lower recovery rate of *A. actinomycetemcomitans* and did not reduce the total numbers of *A. actinomycetemcomitans*.

We did not include the malachite-green bacitracin selective medium in this study because it has been shown that the recovery rate of *A. actinomycetemcomitans* on this medium is much lower than on TSBV medium (14). We incubated all plates in air + 5% CO_2 because it has been shown that anaerobic incubation significantly reduces the recovery of *A. actinomycetemcomitans* on all selective media tested (14). Our results are in full agreement with the observations of Alsina *et al.* (9), who first described the low-cost Dentaid-1 culture medium and who also found a

Table 2. Frequency of isolation of *Aggregatibacter actinomycetemcomitans* on Dentaid-1, modified Dentaid-1 (mDentaid-1) and trypticase soy bacitracin vancomycin (TSBV) isolation media

Experiment 1	No. of samples	Experiment 2	No. of samples
Dentaid-1 (+), TSBV (+)	22	mDentaid-1 (+), TSBV (+)	14
Dentaid-1 (+), TSBV (–)	5	mDentaid-1 (+), TSBV (-)	5
Dentaid-1 (-), TSBV (+)	0	mDentaid-1 (-), TSBV (+)	3
Dentaid-1 (-), TSBV (–)	84	mDentaid-1 (-), TSBV (-)	85
Total samples	111		107

(+), *A. actinomycetemcomitans* recovered. (–), *A. actinomycetemcomitans* not recovered. Two different sets of TSBV plates were used in the experiments.

Table 3. Total counts of *Aggregatibacter actinomycetemcomitans*, gram-negative aerobic rods and total colony-forming units on Dentaid-1, modified Dentaid-1 (mDentaid-1) and trypticase soy bacitracin vancomycin (TSBV) isolation media

	Experiment 1		Experiment 2	
Bacterial species	Dentaid-1	TSBV	mDentaid-1	TSBV
Total *A. actinomycetemcomitans* counts	4.73(0.62)[a]	4.54(1.12)	4.92(1.14)	4.84(0.65)
Gram-negative aerobic rods	1.3(1.1)[b]	0.96(1.1)	1.8(1.0) [c]	1.4(1.2)
Total counts	4.25(1.19)	4.48(0.92)	5.07(0.85)	5.08(0.89)

Values are mean (standard deviation) of log-transformed total counts.
[a]Significant difference between Dentaid-1 and TSBV in experiment 1 ($p = 0.029$).
[b]Significant difference between Dentaid-1 and TSBV in experiment 1 ($p = 0.0142$).
[c]Significant difference between mDentaid-1 and TSBV in experiment 2 ($p = 0.0001$).

significantly lower recovery (79%) of *A. actinomycetemcomitans* on TSBV medium.

We confirm that Dentaid-1 medium is highly selective and cost effective (approximately 50% of the cost of TSBV medium) and may therefore be a useful tool for the isolation and growth of *A. actinomycetemcomitans* from both oral and nonoral clinical samples.

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