Journal of Microbiological Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Mutans streptococci enumeration and genotype selection using different bacitracin-containing media

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ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 5 May 2014
Accepted 6 May 2014
Available online xxxx

Keywords:
Genotypes
rep-PCR
Selective media
Streptococcus mutans
Streptococcus sobrinus
Total streptococci

ABSTRACT

The primary etiological agents associated with dental caries include the mutans streptococci (MS) comprised of *Streptococcus mutans* and *Streptococcus sobrinus*. The effective cultivation and isolation of MS are necessary for the study of MS, including their proper clinical assessment in the epidemiological study of dental caries. Several selective media have been developed for the isolation, enumeration, and characterization of MS. However, inhibition of MS may occur, reducing counts and perhaps limiting selection of some strains. The purpose of this study was to compare five culture media containing bacitracin recommended for the isolation of MS.
Five commonly used bacitracin-containing media (MSB, MSKB, GTSB, TYS20B, and TYCSB) used for MS isolation were quantitatively evaluated. Standard plate counts were performed in duplicate for 2 prototype MS strains (*S. mutans* UA159 and *S. sobrinus* 6715) and for MS isolates from clinical saliva samples obtained from 16 children (approximate age 5 years) to determine total plate counts, and total *S. mutans* count. Selected isolates ($n = 249$) from all of the five media from 5 saliva samples were further confirmed as *S. mutans* with real-time PCR then subsequently evaluated qualitatively with rep-PCR for genotype determination.
All media resulted in variable enumeration with no significant difference in MS counts. MS prototype strains grew well on all five media; clinical isolates demonstrated more variability in counts but no overall significant differences were found. MSB demonstrated comparable ability to grow *S. mutans* but allowed for more non-*S. mutans* growth. All 5 media identified a consistent predominant genotype by rep-PCR. Recovery of minor genotypes was not inhibited by media type.

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1. Introduction

The mutans streptococci (MS) are primary etiological agents associated with the initiation of dental caries (Loesche, 1986). *Streptococcus mutans* and *Streptococcus sobrinus* are the two clinically relevant species of MS found in the oral cavity. These two species are typically identified and quantified in "caries epidemiological and early intervention studies (Saravia et al., 2013).

Numerous selective media have been introduced for the isolation, quantitation, and characterization of MS. Typically these media are supplemented with bacitracin and sucrose as selective agents for clinically relevant streptococci (Gold et al., 1973; Hildebrandt and Bretz, 2006). However, unintentional inhibition of MS may occur, reducing counts and perhaps limiting the quantity of detection and isolation of some strains (Hildebrandt and Bretz, 2006; Tanzer et al., 1984). Initially, mitis salivarius with bacitracin (MSB) medium was considered a reliable selective media for isolation of MS (Gold et al., 1973). As such, MSB medium was selected as the primary selective medium for a large-scale epidemiological study of *S. mutans* by our laboratory. However, several studies have suggested that MSB medium results in lower MS counts than other available selective media and can possibly result in false-negatives (Saravia et al., 2013; Schaeken, 1986; Tanzer et al., 1984; Van Palenstein Helderman et al., 1983; Wan et al., 2002).

Abbreviations: DL, DiversiLab™; GSTB, glucose, sucrose, tellurite, bacitracin media; MS, mutans streptococci; MSB, mitis salivarius-bacitracin media; MSKB, mitis salivarius, kanamycin, bacitracin media; OD, optical density; rep-PCR, repetitive extragenic palindromic polymerase chain reaction; SmTC, *S. mutans* total plate count; THB, Todd Hewitt Broth; TPC, total plate count; TYCSB, tryptone, yeast, cysteine, sucrose, bacitracin media; TYS20B, trypticase soy, sucrose, bacitracin media.

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http://dx.doi.org/10.1016/j.mimet.2014.05.010
0167-7012/© 2014 Published by Elsevier B.V.

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In this study, five selective media are evaluated: mitis salivarius-bacitracin (MSB), mitis salivarius-kanamycin-bacitracin (MSKB), glucose-sucrose-tellurite-bacitracin (GSTB), trypticase soy-sucrose-bacitracin (TYS20B), and tryptone-yeast-cysteine-sucrose-bacitracin (TYCSB) (Gold et al., 1973; Kimmel and Tinanoff, 1991; Schaeken, 1986; Tanzer et al., 1984; Van Palenstein Helderman et al., 1983). Detailed descriptions of these media can be found in the literature, but briefly, these media typically are supplemented with bacitracin and high concentrations of sucrose that act as selective agents for clinically relevant MS. Sucrose allows MS to be distinguished while inhibiting other organisms and bacitracin is known to inhibit other oral streptococci (Gold et al., 1973; Hildebrandt and Bretz, 2006; Schaeken, 1986). Mitis salivarius media (in MSB, MSKB) is commonly used as a base medium since it is selective for streptococci and because MS colony morphology is easily distinguished on this media (Gold et al., 1973). Sucrose is replaced by sorbitol in MSKB and the addition of kanamycin was incorporated to enhance the activity of bacitracin (Kimmel and Tinanoff, 1991).

The purpose of this study is to evaluate these five media quantitatively and qualitatively using MS prototype strains and clinical isolates. In addition, evaluation of MSB in comparison to the other four media will be assessed.

2. Material and methods

2.1. MS prototype strains

S. mutans UA159 and *S. sobrinus* 6715 were selected as the MS prototype strains.

2.2. S. mutans clinical strains

For clinical isolates, saliva was collected from pre-school children ($n = 16$, approximate age 5 years) as part of an ongoing longitudinal epidemiological study of a high caries risk population located in Uniontown, AL, a small rural city. Sample collection was performed at a community health center during a scheduled follow-up visit. Inclusion criteria were that children were free of systemic disease. Human use approval was obtained from the University of Alabama at Birmingham (UAB) Institutional Review Board, with parents of participants providing informed consent, while children gave assent. Whole saliva samples were collected using sterile cotton swabs, which were then stored in 4.5 ml of reduced transport fluid (Syed and Loesche, 1972). Samples were shipped on ice to our laboratory at the University of Alabama at Birmingham School of Dentistry and stored at 4 °C until processed (within 24 h of collection).

2.3. Media & plating

All five media were prepared as originally describe in original publications (Gold et al., 1973; Kimmel and Tinanoff, 1991; Schaeken, 1986; Tanzer et al., 1984; Van Palenstein Helderman et al., 1983).

Prototype strains were inoculated from −80 °C frozen cultures into 5 ml Todd Hewitt Broth (THB) (Becton Dickinson, Sparks, MD, USA) and incubated anaerobically (10% CO_2, 10% H_2, and 80% N_2) at 37 °C for 24 h. A 500 μl aliquot of fresh culture was transferred to 4.5 ml of THB and grown to late log phase. Optical density was determined at 600 nm (OD_{600}) using a Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA), then adjusted to an OD_{600} of approximately 1.0 with THB, which corresponds to 1×10^9 cells/ml using electronic enumeration (Childers et al., 2011). Bacterial samples were serially diluted to 1×10^{-5} and plated in duplicate on each of the five media using a spiral plater (Spiral Systems Inc., Cincinnati, Ohio, USA) dispensing a 50 μl volume. This process was repeated in four independent tests.

Clinical saliva samples in reduced transport fluid were vortexed for 20 s on medium speed prior to plating. Undiluted samples were plated in duplicate on each of the five media using the spiral plater. All plates were incubated anaerobically at 37 °C for 48 h.

2.4. Enumeration

After incubation, all plates were counted using a stereomicroscope for total plate counts (TPC) and total *S. mutans* counts (SmTC) by manual counting according to the instructions for the spiral plater (Spiral Systems Inc., Cincinnati, Ohio, USA) to enumerate the colony-forming units per ml (CFUs/ml). Briefly, a quadrant sector-count method was used to enumerate bacteria using a minimum count of 20 colonies within a quadrant. Opposite quadrants in the same sector were counted and the bacterial density was found by adding the counts of the two sectors and dividing by the sample volume corresponding to the innermost sector counted.

Plates were randomly selected to perform counts without knowledge of counts on other media. The mean plate counts were calculated using duplicate plates. For the clinical isolates, SmTC were recorded based on colony morphology of the prototype strains (Fig. S1) (McGhee et al., 1982; Schaeken, 1986; Tanzer et al., 1984; Van Palenstein Helderman et al., 1983).

2.5. Genotype evaluation

For clinical samples that demonstrated sufficient *S. mutans* growth on all five selected media ($n = 5$), 10 presumptive *S. mutans* colonies were selected for genotype identification using repetitive extragenic palindromic polymerase chain reaction (rep-PCR) as described previously (Moser et al., 2010). Briefly, colonies were inoculated in 5 ml THB and grown anaerobically at 37 °C for 48 hours. DNA was extracted and checked for purity. All isolates were verified as *S. mutans* using SYBR Green™ PCR with sequence specific Yoshida primers before performing rep-PCR (Yoshida et al., 2003). Genotypes were determined using the DiversiLab™ (DL) system and cross-referenced with the library of known genotypes from the larger epidemiological study (Cheon et al., 2013).

2.6. Statistical analysis

Raw counts were transformed to approximate normality by taking logarithms of counts (count plus one, i.e. for counts of zero). Ratios of counts of *S. mutans* to total counts were based on raw counts, not on transformed counts. Statistical significance was ascertained using the GLM procedure in SAS version 9.2 using a significance cut-off value of $p < 0.05$.

3. Results

3.1. MS prototype strains

S. mutans UA159 and *S. sobrinus* 6715 grew equally well on all five media with no significant differences (Tables 1 & S1). For *S. mutans* UA159, the mean log recovery was marginally better on TYS20B (9.18 ± 0.10) than on the other media; MSB (9.16 ± 0.14), GSTB (9.08 ± 0.19), TYCSB (9.09 ± 0.12), and MSKB (9.05 ± 0.16) ($p = 0.69$). In terms of frequency, the highest counts were most often obtained on MSB than other media (2/4 replicates). When MSB was compared to the other media the difference was also non-significant ($p = 0.44$).

S. sobrinus 6715 resulted in comparable counts on all five media although at a slightly decreased level as compared to *S. mutans* UA159. The highest counts were most often observed on TYS20B (8.75 ± 0.41) than other media (2/4); MSB (8.79 ± 0.30), TYCSB (8.67 ± 0.29), MSKB (8.63 ± 0.25), and GSTB (8.64 ± 0.35) but not significantly different ($p = 0.94$) when all five media were compared. In

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Please cite this article as: Momeni, S.S., et al., Mutans streptococci enumeration and genotype selection using different bacitracin-containing media, J. Microbiol. Methods (2014), http://dx.doi.org/10.1016/j.mimet.2014.05.010

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