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MODIFIED TSBB CULTURE MEDIA ENHANCE FASTER GROWTH OF *STREPTOCOCCI MUTANS* AS COMPARED TO EXISTING CULTURE MEDIA

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ABSTRACT: There are various studies on growth of *Streptococcus mutans* which was done by different scientists on different media but it has been found that there is still some deficiency in existing medium. In our study we have tried to prepare a modified, less laborious and more selective medium for *Streptococcus mutans* which is specific for their growth. We studied 10 plaque samples from caries active school children and 2 reference strains of *Streptococcus mutans* ATCC 25175 (serotype c) and OMZ 61 (serotype a) to evaluate the selectivity and sensitivity of our modified medium i.e TSBB (Thioglycolate Sucrose Blood Bacitracin) agar. To check efficiency of our modified TSBB medium, both reference strains and plaque samples were plated on TSBB and other four old existing media i.e TYCSB, TSY20B, MM10, and GSTB for their comparison and evaluation. In our study it was found that highest recovery of *Streptococcus mutans* was observed on TSBB medium followed by TYCSB medium. TSBB medium inhibits the all *Streptococcus* species other than *Streptococcus mutans* present in the oral cavity. Another advantage of TSBB medium is that it does not require anaerobic conditions for isolation of *Streptococcus mutans* as thioglycolate medium itself is an anaerobic medium. By this we can conclude that this modified TSBB media is more efficient, selective and sensitive for isolating *Streptococcus mutans* in comparison to other four existing media.

INTRODUCTION: *Streptococcus mutans* has emerged as one of the most significant caries causing organism^{10, 3, 1, 18}. Mostly researches are related with *Streptococcus mutans* etiology, epidemiology, transmission of caries and many molecular biological studies which require its easy and quick isolation.

For this purpose several selective media have been developed. Among all the media MSB(Mitis salivarius bacitracin) was the first widely accepted medium⁵ but it has several disadvantages like sometimes it is tough to differentiate visually between *S. mutans* and non- *S. mutans* colonies and serotype a of *Streptococcus mutans* is failed to grow on MSB^{4, 15, 8}.

Kimmel & Tinaoff 1991⁷ and Llena et al 2007⁹ modified MSB and gave the names MSKB and MSBTPF respectively. MSKB was supplemented Mitis salivarius agar with kanamycin sulfate, sorbitol, potesium tellurite & bacitracin and MSBTPF by adding 5% potassium tellurite and 64

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$\mu\text{g/ml}$ fluconazol, but result of this MSKSB is not satisfactory as selectivity of this media for *Streptococcus mutans* very less but MSBTPF's results were relatively better. There are many media which were prepared to overcome the shortcomings of MSB medium. Such as tryptone-yeast extract- cysteine with sucrose and bacitracin (TYCSB)^{19, 20, 21, 22} trypticase soy with sucrose and bacitracin (TSY 20B) [14], MM 10⁴, Mannitol containing broth², glucose-sucrose-tellurite-bacitracin (GSTB)¹⁷.

Wade et al 1986²¹ proved best recovery of *S. mutans* with a final sucrose concentration of 20% and bacitracin 2 units /ml. Sucrose is added before autoclaving the media and bacitracin is added after sterilization.

The aim of the present study was to compare the growth ability of *S. mutans* on five media including new modified medium named as Thioglycolate sucrose blood bacitracin agar (TSBB) which was isolated from clinical samples along with two reference strains.

MATERIAL AND METHODOLOGY:

Bacterial strain:

The standard strain of *S. mutans* which was used in this study was ATCC 25175. It was stored in -70°C, thawed and sub-cultured onto Thioglycolate agar. As this is anaerobic medium there was no need to give anaerobic condition. Gram staining and biochemical tests were done to confirm the purity of the culture. Further bacterial strain was grown on same above mentioned medium Thioglycolate agar in candle jar.

Clinical samples:

The dental plaque sample were collected from all surfaces of the teeth and tongue by sterilized cotton swabs. 10 samples were collected from 10 caries active school going children. Each sample was placed in test tubes containing fluid thioglycolate medium and stores at 4°C till plated.

Media preparation:

TYCSB, TSY 20B, MM 10, GSTB were prepared according to manufacturer's and developer's instructions.

Mutans sanguis agar (Hi Media) was used in this study as reference medium, which was autoclaved and dispersed in sterile petri plate. In an effort to improve the selectivity and accuracy of the isolation of *Streptococcus mutans* we have formulated an enriched and selective medium TSBB which contains - Thioglycolate agar which consists of Trypticase soy agar 4g, Yeast extracts 1g, Thioglycolate 3g, Sucrose 20g, D/W 100ml.

After the medium was autoclave at 121°C, 15 lbs pressure for 15 mins, cool it at 55°C. Add two disk of bacitracin (each contains 10 U) for at least 15 mins, so that antibiotic get suspends in the medium. For maintaining temperature of medium at 55°C, it was kept in water bath and then 2 ml of blood was added to it.

Dilution and inoculation of reference strains ATCC 25175 and OMZ 61:

Colonies of reference strains were pelleted by centrifugation (10,000 rpm for 20 minutes) and then re-suspended in sterile PBS. These inocula were disaggregated by sonication bath for 3 min for 40 kHz. Bacteria were finally suspended in PBS to make a suspension equal to a 0.5 McFarland standard (1.2×10^8 CFU/ml). It was then further diluted from 1:10 to 1:10⁷. 0.1 ml portion of each dilution were plated on different media by spread plate method. Plates were incubated for 48 hours at 37° C in candle jar.

Dilutions and inoculation of *Streptococcus mutans* isolated from clinical samples:

The samples were homogenized by vortexing for 5 minutes, 1 ml samples were diluted from 1:10 to 1:10⁶. After dilution, 0.1 ml portion of appropriate dilution was plated by using a bent glass rod on different agar media (triplets of every medium). After incubation of 48 hr suspected colonies were isolated and identified by using following tests – Gram staining, Catalase test, Esculine hydrolysis test, sugars (mannitol, sorbitol, melibiose and inulin) fermentation test.

Colony counting:

Each medium was inoculated by 0.1 ml of each dilution of reference strain of *Streptococcus mutans* and 10⁻⁵ and 10⁻⁶ dilution of each clinical sample.

The colonies on plates were enumerated by colony counter.

Statistical analysis:

Analysis of variance for multiple comparison (ANOVA) using stata 6.0, statsoft (Tulsa USA) was performing in order to confirm statistical significance of differences among samples ($P < 0.05$). Mean values were compared using the Tukey test at $P < 0.05$.

RESULT:

A total of 10 caries material were taken from low socioeconomic caries active school active children. All the children were of 5-6 years. Every sample and reference strain of *Streptococcus mutans* were plated on all the media. Growths on agar plates were monitored by counting the colony forming unit (CFU) after incubation period of 48 hours.

Recovery and enumeration of *Streptococcus mutans* from clinical samples:

Recovery of *Streptococcus mutans* on different media from diluted dental plaque samples obtained presented on **Fig. 1**. The *S. mutans* was recovered from all the five media. The best recovery was in TSBB (87%) medium which was almost similar with TYCSB (81.5%), followed by TSY20 (68%) GSTB (60%). MM10 (29%) showed the minimum recovery.

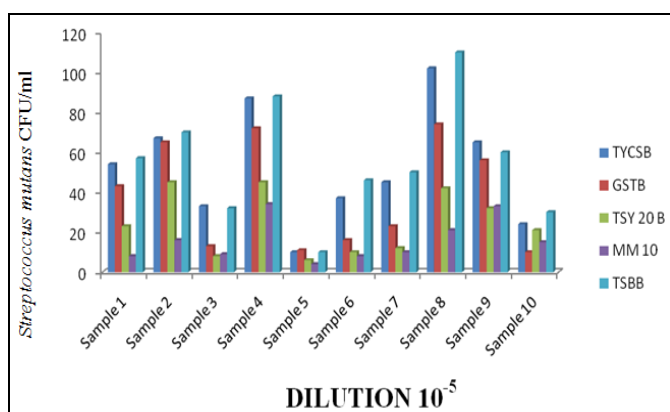


FIG.1: ENUMERATION OF STREPTOCOCCUS MUTANS FROM 10 CARIES ACTIVE CHILDREN IN 10⁻⁵ DILUTION. (MEAN NUMBER OF COLONIES ON THREE PLATED OF EACH MEDIUM).

Relative growth index (RGI) which was calculated for evaluating the selectivity of media.

$RGI = \frac{\text{Test medium colony count (CFU)}}{\text{Control medium colony count (CFU)}} \times 100$.

It was observed that moderate growth of *Streptococcus mitis*, and *Streptococcus salivarius* in the, TSY 20B, MM 10, GSTB media (RGI < 10%) and in TSBB (RGI < 5%) in relation to control medium. Recovery of *Streptococcus sobrinus* in TYCSB was high (RGI > 35%). While *Staphylococcus aureus*, *Lactobacillus*, *Actinomyces* were inhibited (RGI < .05) in TSY20B, MM10 and GSTB media. RGI was less than 0.001% in TYCSB and TSBB.

Mean relative growth index of *Streptococcus mutans* was 95.6 and 92.85 for TSBB and TYCSB respectively and 66.3 % for TSY 20 B, 46 % for MM10 and 38.4 % for GSTB.

Recovery and enumeration of Reference strain of *Streptococcus mutans*:

Efficacy of different media for Reference strain ATCC 25175 and OMZ 61 were observed by calculating CFU /ml. Strains were enumerated on different dilutions range 1:10 to 1:10⁷, shown in **Fig. 2**.

Both the reference strains revealed higher in number in TSBB agar and TYCSB as compared to other media. The average recovery rate of standard strains on above mention dilutions are describe in **Fig. 3**

RGI of ATCC 25175 and OMZ 61 were 97.2% and 96.4 % in TSBB and 92.1 and 89.3% in TCSB medium respectively (**Fig. 4**).

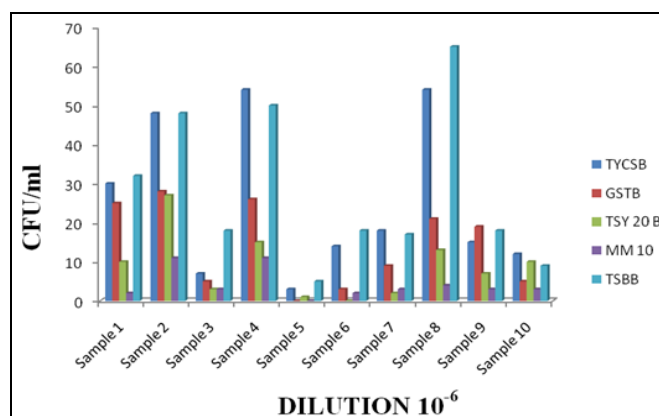


FIG. 2: ENUMERATION OF STREPTOCOCCUS MUTANS FROM 10 CARIES ACTIVE CHILDREN IN 10⁻⁶ DILUTION. (MEAN NUMBER OF COLONIES ON THREE PLATED OF EACH MEDIUM).

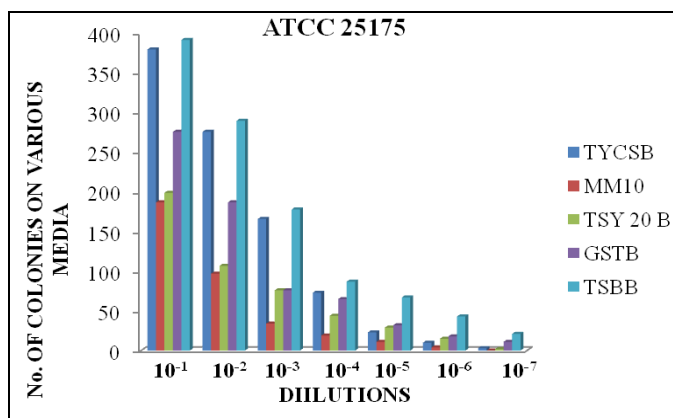


FIG. 3: ENUMERATION OF REFERENCE STRAIN ATCC 25175 ON DIFFERENT DILUTION ON DIFFERENT MEDIA

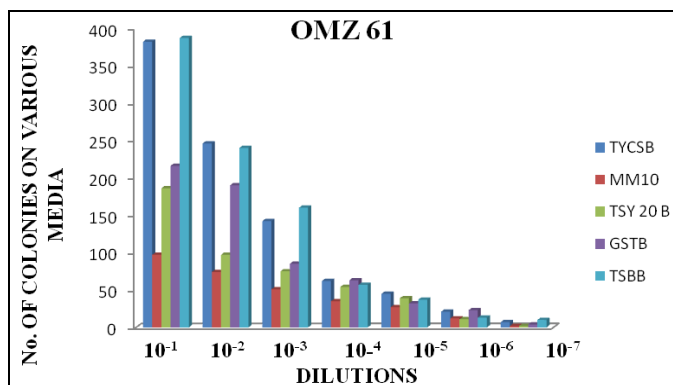


FIG. 4: ENUMERATION OF REFERENCE STRAIN OMZ 61 ON DIFFERENT DILUTION ON DIFFERENT MEDIA

One more advantage of TSBB medium is easy identifiable colonies of *Streptococcus mutans*. After the incubation period of 48 – 72 hours, colonies were examined, because medium was containing both, blood and sucrose and because of its presence most strains of *S. mutans* produced colonies of 1 mm in diameter with beads, droplets or puddles (dew drop like colonies) containing soluble extracellular polysaccharide (Hardie 1986) with α and γ (Fig. 5)



FIG. 5: SHOWING COLONIES OF 1MM IN DIAMETER, WITH BEADS, DROPLETS OR PUDDLES WITH γ HEMOLYTIC ON MODIFIED THIOGLYCOLATE AGAR.

DISCUSSION: This study intended to compare the sensitivity and selectivity of different culture media to isolate and identify *Streptococcus mutans* in caries active children and from reference strains.

Streptococcus mutans was grown on all the above mention media, but percentage of recovery was different. In the present study TSBB proved to be the best medium among the all with its recovery rate of 87%, followed by TYCSB (81.5%) from clinical samples.

And 68.7% and 70.2 % recovery rate on TSBB media for strain ATCC 25175 and OMZ 61 respectively which were highest as compared to other media and on TYCSB it were 53.2 % & 62.3% for both the strains.

These results may vary if compared to different studies. For examples, Wade et al 1986²¹ showed highest recovery on GSTB (64%), followed by TYCSB (60%). Wan et al 2000²² proved TYCSB is best medium with its recovery rate of 50± 7% for NCTC 10449 and 82 % from clinical samples. Hidebrandt & Bretz 2006⁶ also showed TYCSB as a better medium than MSB. Momeni et al 2014¹¹ observed no significant difference in *S. mutans* counting on all 5 bacitracin containing (MSB, MSKB, GTSB, TYS20B, and TYCSB) but allowed for more non-*S. mutans*.

In this study on the basis of relative growth index, the inhibitory effect of TSBB medium was also observed for other *Streptococcus* species present in oral cavity. This is an advantage of TSBB medium as RGI is quite good for *Streptococcus mutans* rather than other bacteria. TSY20B and GSTB are good media as it is economic but this media do not inhibit the growth of *Streptococcus* other than *Streptococcus mutans*^{7,22}.

Recently two other media have been developed for *Streptococcus mutans* MS-MUTV and SB-20M^{16, 12}. Disadvantages to MS-MUTV are its high cost and difficulty in preparation. SB-20M is quite a good medium as recovery rate of *Streptococcus mutans* is high and morphologically it can differentiate between *S. mutans* and *S. sobrinus*. Two more advantages of TSBB medium are 1. It helps to easily identify the colonies of

Streptococcus mutans as on this medium colonies appears like beads droplet and puddle shape which contain soluble extracellular polysaccharide with α & γ haemolysis. 2. It was found that there was no need to incubate the cultures in anaerobic condition as thioglycolate present in media contains sodium thioglycolate which consumes oxygen and provide a favorable condition for growth of *Streptococcus mutans*.

After observing almost all the aspects of TSBB (modified media) we can conclude that this modified media was found to be more appropriate and favorable for the growth *Streptococcus mutans* in comparison to other existing media.

CONCLUSION: TSBB media give good recovery and count of *Streptococcus mutans*. It helps to easily identify the colonies of *Streptococcus mutans* as on this medium colonies appears like beads droplet and puddle shape which contain soluble extracellular polysaccharide with α & γ haemolysis. 2. It was found that there was no need to incubate the cultures in anaerobic condition as thioglycolate present in media contains sodium thioglycolate which consumes oxygen and provide a favorable condition for growth of *Streptococcus mutans*. After observing almost all the aspects of TSBB (modified media) we can conclude that this modified media was found to be more appropriate and favorable for the growth *Streptococcus mutans* in comparison to other existing media.

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