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Antagonistic activity of a multi-functional gold standard chlorhexidine against *Lactobacillus acidophilus* isolated from childhood caries

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Abstract
The background of this study chlorhexidine gluconate is an effective bactericidal agent and broad-spectrum antimicrobial drug. It has been extensively researched and is the "gold standard" antimicrobial in oral hygiene. Among different kind of microorganisms the cariogenic *Lactobacillus acidophilus* is recognized as a predominant pathogen in childhood caries. So plaque samples were collected from different dental clinics. The isolates were identified by biochemical characterization and 16SrDNA gene sequencing. Further, the antimicrobial production was assessed. Lactic acid production by *L. acidophilus* is partially growth associated followed by quantification of organic acid and determination of *p*H value indicates that organic acid production was increased with the highest acidity (1.8%) and determination of H_2O_2 production by the specific 5 isolates generated H_20_2. The highest proportion was found 5 out of 5 strain (100%) being positive. Finally antimicrobial efficacy of CHX was used at 2% concentration (50µl, 100µl and 150µl) against *L. acidophilus*.

Keywords: dental caries, CHX, *Lactobacillus* acidophilus, anti-microbial activity

1. Introduction

Childhood caries is a chronic and transmissible disease characterized by demineralization of tooth owing to production of acids by bacteria in biofilms formed on its surface. The elimination of cariogenic microorganisms is one of the vital factors for the primary caries prevention (Bjorndal and Larsen, 2000) [1].

Modern studies demonstrate that the microbes include *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus*, and *L. rhamnosus* are the most common cariogenic microorganisms involved in caries progression (Oda *et al*, 2015) [2]. The progression of caries associated with the microbe and is also isolated both from enamel carious lesions and hidden dentin caries (Simon-Soro and Mira, 2015) [3].

Mainly causative agent of *Lactobacillus* is a set of bacteria that occur at elevated levels in deep childhood caries lesions (Byun *et al*, 2004) [4]. The analysis shows that among the genus *Lactobacillus*, *L. acidophilus* is most prevalent, and *L. paracasei*, *L. rhamnosus*, and *L. fermentum* are also present in deep caries lesions and promote caries progression (Callaway, 2013) [5]. If these caries lesions progress, they may eventually result in pain and pulp exposure in young children. For this reason to diminish the possible of chief caries and enhance the postoperative sensitivity, antibacterial agents may be chosen according to their ability to reduce the possibility of existing bacteria. The use of antimicrobial solutions as an oral rinse disinfectant for reducing cariogenic microorganisms according to the target of the application has been recommended (Esra Uzer Celik *et al*, 2016) [6].

Chlorhexidine gluconate (chlorhexidine) is a gold standard broad-spectrum antimicrobial drug. Acting as an antiseptic, it is an effective bactericidal agent against all categories of microbes, including bacteria, yeast, and viruses and also commonly used antimicrobial agent because of its ability to significantly reduce the levels of cariogenic microorganisms (Obata *et al*, 2014) [7]. Chlorhexidine molecules are positively charged and most bacteria and surface structures in the oral cavity, including the surfaces of teeth and mucous membranes, are negatively charged. In accordance with the principle that opposite charges attract, chlorhexidine binds strongly to all these surface structures of microbes easily.

When chlorhexidine binds to microbial cell walls it induces changes, damaging the surface structure, leading to an osmotic imbalance with consequent precipitation of cytoplasm causing cell death. The substantively of chlorhexidine enhances this bactericidal effect, which allows for the retention of chlorhexidine in the oral cavity and a prolonged residual antimicrobial effect for up to 12 hours or longer depending on the dosage and form. As a result chlorhexidine can be used repeatedly and over long periods of time to eliminate the oral bacteria. Furthermore, it destroys all categories of microbes, not just bacteria, and there is little risk for the development of opportunistic infections. So hence the present study has planned to evaluate the antimicrobial activity of multifunctional gold standard chlorhexidine against the caries infections.

2. Materials and Methods

2.1 Sample Collection

Dental samples were obtained from different dental clinics around Tirupur district. It was kept in glass vial with saline solution and was brought to the laboratory of PG and Research Dept. of Zoology, Division of Microbial Technology, Chikkanna Govt. Arts College, Tirupur. Then, the samples were incubated at 37°C for 24 hours and the samples were used for isolation of cariogenic bacteria.

2.2 Identification of *Lactobacillus acidophilus*

The organism isolated by Man Rogosa Sharpe agar was the selective media for *Lactobacillus acidophilus* (Fig. 2). After, the identification was done by Gram staining, IMViC test, Nitrate reduction test, Sugar fermentation test, Catalase test, antimicrobial production (H_2O_2, Lactic acid and Organic acid), and genotypic characterization.

2.3 Production of antimicrobial compound by oral *Lactobacillus acidophilus*

2.3.1 Determination of lactic acid production by *Lactobacillus acidophilus*

The strains were grown in MRS broth for 48 h and supernatant was collected by centrifuging at 10,000 rpm for 15 m at 4°C. Phenolphthalein was added in to the 20 ml of supernatant as an indicator for titrimetric estimation. One ml of 0.1M NaOH is equivalent to 90.08 mg of lactic acid.

2.3.2 Determination of hydrogen peroxide by *Lactobacillus acidophilus*

Take 25 ml of dilute sulphuric acid were added to 25ml of MRS broth culture of test organisms. Titration was carried out with 0.1 N of Potassium permanganate. Each ml of Potassium permanganate is equivalent to 1.070 mg of H_2O_2. A decolourization of the sample was regarded as end point.

2.3.3 Quantification of organic acid and determination of *p*H value by *Lactobacillus acidophilus*

In this study 1% (V ⁄ V) of 24 h active culture of *Lactobacillus* was used to inoculate 10% sterilized skim milk and initial *p*H (6.6) was determined by digital electrode *p*H meter. The inoculated skim milk was incubated at 37°C for 72 h and samples were collected in every 24h; 48 h, 72 h and liquid of coagulated milk were separated by filtration. The *p*H of the separated liquid was recorded using a digital electrode *p*H meter. The quantification of organic acid was performed through titration with 0.1 N NaOH using phenolphthalein as *p*H indicator.

2.4 Preparation of gold standard CHX stock solution

In this study the 2% CHX powder was weighed and it was thoroughly dissolved in 100ml of sterile distilled water. Further, it was used against dental pathogen *Lactobacillus acidophilus*.

2.5 Antimicrobial efficacy of gold standard CHX against *Lactobacillus acidophilus*

The antimicrobial activity of CHX was done by well diffusion method. The solid medium was prepared with using Muller Hinton agar. After solidification of medium the 0.1ml overnight culture was spread over the surface of agar. After puncture was made by using different range of Cork borer (50µl, 100 µl and 150µl). Finally the hole was filled with 2% con. of CHX at different µl (50, 100 and 150). After it was kept in incubator at 37◦C, 24h for its antimicrobial activity. Measure the zone of inhibition around the well.

3. Results

Isolation and identification of *Lactobacillus acidophilus*

In this study decay samples were collected using sterile transport medium (Fig. 1). Totally ten isolates were used. The *Lactobacillus acidophilus* was identified by Man Rogosa Sharpe agar (Fig. 2), grams reaction (Fig.3) and biochemical characterization, genotypic characterization.

Production of antimicrobial compound by oral *Lactobacillus acidophilus*

Lactic acid estimation

Lactic acid production of EPS producing cariogenic plasmid strains were studied by titrimetric estimation which include MTVG08, MTVG13, MTVG14, MTVG15, MTVG22, MTVG47 and MTVG48 produce equal amount of lactic acid 180.16 mg/ml in the oral cavity of rural caries patients (Fig. 4).

Quantification of organic acid and determination of *p*H value

The present experiment indicates that organic acid production was increased with the incubation time. On the other hand, *p*H of the media decreased with the increasing acid production. In this study highest acidity (1.8%) was observed after 72 h of incubation at 37°C for *Lactobacillus acidophilus* isolated from childhood caries (Fig.5).

Determination of H_20_2 production by oral *Lactobacillus acidophilus*

The finding from the present studies of H_2O_2 production by oral *Lactobacillus acidophilus*. A substantial proportion of the isolates MTVG02, MTVG20, MTVG25, MTVG38 and MTVG46 are generated H_20_2 (Table: 08), as revealed by decolourization of pink color that appeared during titration. The highest proportion was found among all cariogenic isolates, 5 out of 5 strain (100%) being positive. Among five strains including only one strain namely MTVGO2 (340.20 mg / ml) strongly to produce maximum amount of H_20_2 compare than other cariogenic isolates namely (MTVG20 - 255.15 mg / ml), (MTVG25 - 212.625 mg / ml), (MTVG46 - 212.625 mg / ml), and (MTVG38 – 127.575 mg / ml) (Fig. 6).

Antimicrobial activity of Gold standard Chlorhexidine

Antibacterial activity of 2% concentration of CHX was performed by Agar Well Diffusion assay against cariogenic *Lactobacillus acidophilus*. Three different concentrations (50µl, 100µl and 150µl) were used in this assay against oral *Lactobacillus acidophilus*. The maximum zone of inhibition 22.5mm, 35mm and 31mm was observed in strain no. MTVG75 followed by the minimum zone of inhibition 25mm, 27mm and 30mm was observed in strain no. MTVG06 at different µl concentration of (50,100 and 150) CHX (Table. 1, Fig. 7).

4. Discussion

Isolation of cariogenic *Lactobacillus acidophilus*

The author stating that the analysis of *Lactobacilli* by culture under micro aerophilic conditions in 65 deep caries samples indicated that *Lactobacillus acidophilus* was numerically dominant compare than other cariogenic isolates were also present in many samples. This is due to the presence of *Lactobacilli* in the oral cavity depends on numerous factors such as the presence of ecological niches e.g. natural anfractuosities of the teeth (Loesche *et al*, 1984) [8], partly erupted third molars or orthodontic device. In this study the decay causing *Lactobacillus acidophilus* were collected from tooth decay of children's and processed for antibacterial activity of CHX. The microbial colonies were counted in plaque samples by standard plate count.

Identification of cariogenic *Lactobacillus acidophilus*

The isolated bacteria were primarily identified on the basis of the Gram staining, IMViC test, oxidase test, nitrate reduction test, motility test, and different carbohydrate fermentation test in the Lactobacilli de Mann Rogosa and Sharpe (MRS) broth as described as Bergy's Manual of Systematic Bacteriology. Nigatu *et al*, (2000) [9] stated that since such biochemical methods depend on environmental and culture conditions, they sometimes lead to ambiguous results or even misidentifications. Quere *et al*, (1997) [10] reported that the increasing number of *Lactobacillus* strains with only slight variations makes the task more difficult.

Antimicrobial production - Determination of lactic acid production by *Lactobacillus acidophilus*

In this study dissimilar result was obtained, the maximum rate of lactic acid production 180.16 mg/ml was obtained with varied culture at 37°C in the oral cavity of rural caries patients because, the instigator Miloud Hadadji and Ahmed Bensoltane, (2006) [11] found their study have observed that the fitness of particular strains of *Lactobacilli, Bifidobacteria* and *Streptococci* for commercial utilization depends on its rapid growth and acidification of milk as well as its acid and oxygen tolerance. The production of lactic acid was compared with pure and mixed cultures and the values were 90 and 64 mM at 37°C; 82 and 140 mM at 45°C. The maximum rate of lactic acid production was obtained with mixed culture at 45°C.

Determination of H_2O_2 production by *Lactobacillus acidophilus*

Production of H_2O_2 by *Lactobacillus* species is considered to represent a non specific antimicrobial defense mechanism of normal oral environment. Eschenbach *et al*, (1989) [12] detected 96% H_2O_2 – producing (LB^+) strains. In the present study, the highest proportion was found among all cariogenic isolates (MTVG02, MTVG20, MTVG25, MTVG38 and MTVG46), 5 out of 5 strain (LB^+ - 100%) being positive. Species with the largest number of LB^+ strains were *L. acidophilus*. These results agree with those published by Boris *et al*, (1998) [13]. Also, LB^+ strains and LB^- strains of *L. acidophilus* were isolated, as in McGroarty *et al*, (1992) [14].

Antibacterial activity of gold standard CHX used against *Lactobacillus acidophilus*

The existing study showed that dissimilar results was obtained in all different µl concentration (50, 100 and 150) of antimicrobial ability of gold standard CHX at 2% concentration used against oral pathogen *Lactobacillus acidophilus*. The maximum zone of inhibition was observed in 2% concentration of CHX, the strain no. MTVG75 emit maximum zone of inhibition 22.5mm, 35mm and 31mm followed by the minimum zone of inhibition 25mm, 27mm and 30mm was observed in strain no. MTVG06 at different µl concentration of (50,100 and 150) CHX. The CHX was used and it shows prominent antibacterial activity against decay causing organism. The author Mistry (2014) [15], reported that least concentrations of CHX were provide maximum zone of inhibition and the concentration often used in endodontic therapy is up to 2% and one more report was proved that found to be more effective in the least concentration and compared with other concentrations of CHX ranging from 0.002% to 2% (Lessa, 2010) [16].

5. Figures and Tables

Fig 1: Tooth Decay Sample

Fig 2: Isolation of *Lactobacillus acidophilus* from Man Rogosa Sharpe Agar

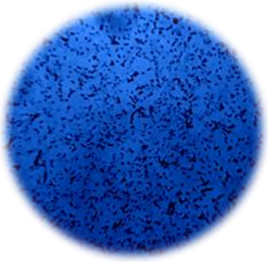


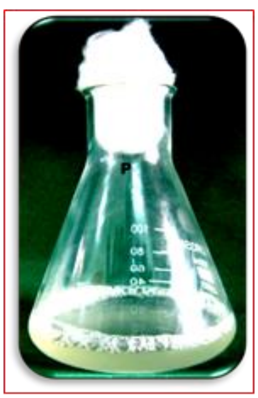
Fig 3: Gram staining of *L. acidophilus*

C: Control; P: Positive

Fig 4: Lactic acid production from *Lactobacillus acidophilus*

C: Control; P: Positive

Fig 5: Organic acid production by *Lactobacillus acidophilus*



P - Positive

Fig 6: Hydrogen peroxide production by *Lactobacillus acidophilus*

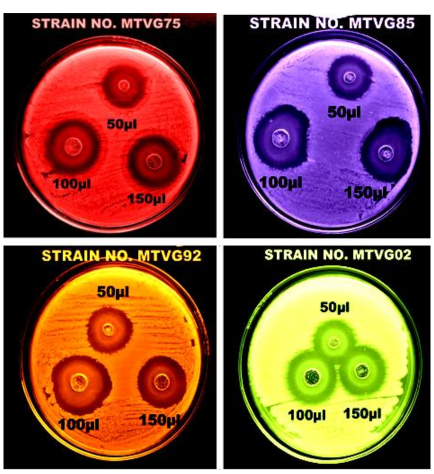


Fig 7: Antimicrobial activity of gold standard CHX against *Lactobacillus acidophilus*

6. Conclusion

The findings of the present study suggest that CHX is significantly effective against cariogenic microorganisms at 2% concentrations of different range of µl (50, 100 and 150). The gold standard multi-functional chlorhexidine can be considered as a broad spectrum activity to kill the cariogenic pathogen around the oral environment of school children's. The varied and effective applications of chlorhexidine make it a viable option for use in all dental settings and within a variety of dental procedures and pre-procedures with very few undesirable side effects.

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