Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 21 (2020) 100198

Contents lists available at ScienceDirect

Journal of Clinical Tuberculosis and Other Mycobacterial Diseases

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

Validity of a CB-NAAT assay in diagnosing tuberculosis in comparison to culture: A study from an urban area of South India

Aishwarya Raj [a], Shrikala Baliga [b,c], M. Suchitra Shenoy [b,c,*], B. Dhanashree [b,c], P. Prasanna Mithra [d], Smitha K. Nambiar [b], Leesha Sharon [b]

[a] Department of Biophysics, National Institute of Mental Health and Neuro Sciences (Institute of National Importance), Bengaluru, India
[b] Department of Microbiology, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, India
[c] Manipal McGill Centre for Infectious Diseases, PSPH, Manipal, India
[d] Department of Community Medicine, Kasturba Medical College, Mangalore, India

1. Introduction

The world today is known for its advancements in technology in all social sectors, most importantly in the health sector and specifically in the field of Mycobacteriology. Clinically suspected TB cases are normally tested for the presence of *Mycobacterium tuberculosis* in appropriate samples by laboratory diagnostic methods. Conventionally, Acid fast bacilli (AFB) smear microscopy and the culture methods are employed for the diagnosis. Fluorescence microscopy of Auramine-O stained smears and liquid culture using tools like the BACTEC™ "Mycobacterial Growth Indicator Tube 960" (MGIT 960; Becton-Dickinson, Sparks, MD, USA) are the most commonly used conventional methods. Culture method is the most sensitive and specific method for the detection of *Mycobacterium tuberculosis*. Despite this, cultures are highly prone to contamination and the process can still take several days and does require expensive equipment, strict biosafety practices and well trained technical staff [1].

Among the currently available Nucleic Acid Amplification Tests (NAAT), the Xpert MTB/RIF Assay (CB-NAAT), the LINE Probe Assay (LPA) and the Loop-Mediated Isothermal Amplification (LAMP) are endorsed by WHO for *in vitro* diagnosis of TB. The GeneXpert® system powered by the Cepheid Innovations, for the CB-NAAT (Cartridge based), is an automated, semi-quantitative, hemi-nested, real-time PCR used for the simultaneous detection of the MTB complex and its rifampicin (RIF) resistance pattern associated with the mutation in the *rpoB* gene, in clinical samples with a 2 h turnaround time [2].

The present study was carried out to assess the performance of CB-NAAT (Cepheid GeneXpert®) system for the diagnosis of MTB in both pulmonary and extrapulmonary specimens, within the demographic area of Mangalore, in South Karnataka.

2. Materials and methods

The study was approved by the Institutional Ethics Committee of Kasturba Medical College, Mangalore. This cross-sectional study was conducted at the Department of Microbiology, Kasturba Medical College, Mangalore, Manipal Academy of Higher Education, Manipal, in Dakshina Kannada District of Karnataka State in India, over a period of 31 months from June 2016 to December 2018. The Department of Microbiology consists of Designated Microscopy Center as per the Revised National Tuberculosis Control Program (RNTCP) under the DOTS (Directly Observed Treatment, Short course), which caters to attached tertiary care hospitals of the study Institute. The health care provided in these hospitals includes people from neighboring Districts of Karnataka and Kerala States. Pulmonary & extrapulmonary specimens received at the Microbiology laboratory, in sterile containers, from the clinically suspected tuberculosis patients were used in the study. Saliva, blood, urine and stool samples were excluded from the study.

The samples received at the laboratory were divided into three portions, one part each was used for AFB direct smear preparation, CB-NAAT and MGIT liquid culture, respectively. Direct and concentrated smears were prepared, stained using the Fluorochrome acid-fast staining method, with the Auramine-O fluorescent stain and screened as per the guidelines [3]. A smear was reported positive if either the direct or the concentrated smear showed the presence of AFB. The NALC-NaOH method [4] was used for the sample digestion and decontamination. The concentrates were cultured on to BD BBL™ MGIT™ Mycobacteria Growth Indicator Tube using the BACTEC™ MGIT™ 320 system. Cultures were incubated for up to 8 weeks to confirm the negativity of *Mycobacteria* in the sample. If positive, the culture was subjected to a rapid immunological ID test using the BD MGIT™ TBc ID test device to differentiate MTB from *Mycobacterium* genus. CB-NAAT was done using

* Corresponding author at: Department of Microbiology, Kasturba Medical College, Mangalore, India.
E-mail addresses: shrikala.baliga@manipal.edu (S. Baliga), suchitra.u@manipal.edu (M.S. Shenoy), dhanashree@manipal.edu (B. Dhanashree), prasanna.mithra@manipal.edu (P.P. Mithra).

https://doi.org/10.1016/j.jctube.2020.100198

Available online 28 October 2020

the Cepheid GeneXpert® system, according to the manufacturer's instructions [2]. At the end of the test, the result was reported as MTB detected or MTB not detected along with Rifampin (RIF) resistance status.

The collected data were coded and entered onto Statistical Package for Social Sciences (IBM SPSS Statistics for Windows) version 25.0. Armonk, NY:IBM Corp. Results were expressed as proportions using tables. Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) were calculated, by considering the liquid culture results as the gold standard. For comparison across the CB-NAAT and culture groups, chi-square test was used and a p value of <0.05 was considered as statistically significant.

3. Results

The samples received from suspected TB patients were from different localities within and around the urban area of Mangalore, which included Bantwal, Puttur, Moodbidri, Belthangady, Sullia of Dakshina Kannada district, Udupi, Hassan, Chikmagalur districts of Karnataka and Kasaragod and Kannur districts of Kerala. Samples obtained from 831 patients were included in the study of which 507 (61.01%) were males, 324 (38.99%) were females. The mean age of the patients was 52.01. The pulmonary samples (n = 682) included in the study were Bronchial Alveolar Lavage (BAL) [N = 591], sputum [N = 84], endotracheal (ET) aspirate [n = 6], and bronchial biopsy [n = 1] (Fig. 1). The extrapulmonary samples (n = 149) were body fluids [n = 75], tissues [n = 34], pus [n = 30], lymph node aspirates [n = 8] and gastric lavage [n = 2] (Fig. 2).

Smears were positive in 72 patient samples, which consisted of 56 pulmonary and 16 extra-pulmonary cases. The true positive occurrence with the use of smear microscopy was 95.83%. A positive culture growth was observed in 148 pulmonary and 32 extrapulmonary samples. The rapid ID test had identified 122 cultures as MTB (95 pulmonary and 27 extrapulmonary) and the rest 58 (53 pulmonary and 5 extrapulmonary) as Genus *Mycobacterium* species. Table 1

The GeneXpert® system detected the presence of MTB in 106 pulmonary and 30 extrapulmonary samples. The true positive rate of detection of MTB by CB-NAAT was found to be 75.74%. Five cases of RIF resistance were also detected. 30 samples positive by CB-NAAT had grown Genus *Mycobacterium* species in culture. Additionally, CB-NAAT detected MTB in three samples which were smear positive and culture negative. CB-NAAT also detected MTB in 61 smear positive and culture positive samples, 42 smear negative and culture positive samples, and 33 smear negative and culture negative samples.

The sensitivity, specificity, PPV and NPV of smear microscopy were 38.33, 99.54, 95.83 and 85.38% respectively and that of the CB-NAAT assay is tabulated in Table 2.

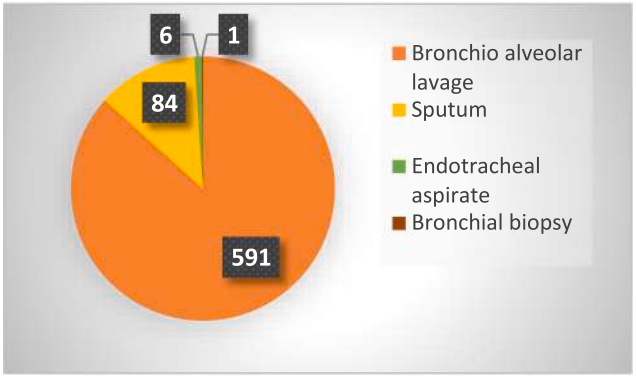

Fig. 1. Distribution of pulmonary samples.

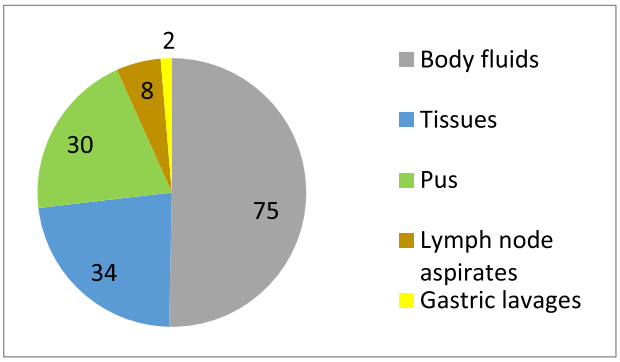

Fig. 2. Distribution of extrapulmonary samples.

Table 1
AFB smear comparison with culture.

	Culture Positive	Culture Negative
Smear Positive	69	3
Smear Negative	111	648

4. Discussion

In spite of rapid strides in diagnosis and treatment, TB still continues to be a menace in many developing countries, including India. TB is among the 10 cardinal causes of mortality across the globe. The fight against TB has definitely given notable results. In the last 17 years, about 53 million lives were redeemed from the clutches of TB, mainly through timely diagnosis and effective treatment. TB incidence and mortality rate is at 2 and 3% per year respectively [5]. The major hindrance in the combat against TB is the lack of early diagnosis and appropriate and timely treatment. Programs like RNTCP and DOTS are efficiently confronting this issue with high priority, thus decreasing complications towards a great extent. Additionally, since 2003, Foundation for Innovative Diagnostics (FIND) has been working towards betterments in TB diagnostics and has improved the access to new diagnostic tools, by all countries, impartially [6].

Our study included the assessment of performance of GeneXpert® system in detecting MTB infection among the patients in and around the district of DK in Karnataka. Such an evaluation is the first of its kind in this area and thus the comparison is mainly with other studies across India [7] or abroad [8–12]. There are a few studies which have compared the performance of CB-NAAT for both pulmonary and extrapulmonary samples [8]. Most of the studies focus either on pulmonary samples [7,9,10] or on extrapulmonary samples alone [11,12]. The positivity in males was comparatively higher to that of females, in India, as well as abroad, reflecting the fact that males are more often infected by TB than females [13]. A study based on knowledge of TB reported that men sought better healthcare while women tend to self-medicate [14]. Earlier studies also reported that, apart from socio-economic and cultural factors, there may be certain biological gender factors that give rise to this sexual bias in TB [13].

The sensitivity and PPV of the CB-NAAT in our study was lower when compared to previous studies (91.4 and 86.5% respectively) [8]. The lower sensitivity can be attributed to the 19 false negatives (16 BAL) obtained with the CB-NAAT. BAL is known to have a lower sensitivity for the detection of MTB by CB-NAAT [10]. Another reason could be the very low load of the organism in the sample, lower than the detection limit of CB-NAAT (131 CFU/mL of sample) [2]. The lower PPV can be attributed to the 33 positive cases obtained by CB-NAAT which failed to grow in culture. The inability to grow in culture and ability to be detected by CB-NAAT may have been due to the paucibacillary nature of extrapulmonary specimens. It may also be due to the treated cases where

Table 2
The sensitivity, specificity, PPV and NPV of CB-NAAT in comparison to culture.

SAMPLES (n = 831)	SENSITIVITY (%)	SENSITIVITY (%)		SPECIFICITY (%)	PPV (%)	NPV (%)
		S + C+*	S−C+#			
PULMONARY (n = 682)	82.11	97.96	65.21	94.76	73.58	96.75
EXTRA-PULMONARY (n = 149)	92.59	100	85.71	95.73	83.33	98.25
OVER ALL	84.45	98.39	70	94.93	75.73	97.02

* Smear positive and culture positive.
# Smear negative and culture positive.

even the dead bacilli were detected by the CB-NAAT or the cases where the use of NALC-NaOH treatment for decontamination of the samples proved excessively harsh resulting in no growth. The specificity and NPV were almost at par with the previous studies (93 and 95.6% respectively) [8]. In the case of pulmonary samples, the total sensitivity was similar to other studies (79.5%) and was lesser than that of certain other studies (95.7%) [9,10]. While the NPV and the sensitivity for smear positive and culture positive samples were comparable to former studies (94–98.1% and 88.9–99.2% respectively), the specificity, PPV and sensitivity for smear negative and culture positive samples were lower than previous studies (99.6–100%, 99–100% and 73.1–77.7% respectively) [9,10]. In the case of extrapulmonary samples, the total sensitivity, sensitivity for smear negative and culture positive samples, smear positive and culture positive samples and NPV were higher than that of former studies (71–83, 66, 95 and 90% respectively) [11,12]. These differences observed in terms of extrapulmonary samples can be accounted to the lower number of extrapulmonary samples included in the study. The specificity and PPV were similar to previous studies (95 and 83% respectively) [11,12].

In the present study, CB-NAAT was found to be better than that of smear microscopy (with a difference of above 45% in terms of sensitivity), as observed by the earlier studies [15]. Adding to its advantage, this test is rapid, require minimal training of personnel and lower biosafety level (compared to culture) [16]. The CB-NAAT is used for the rapid detection of MTB in the samples. In settings where the incidence of non-tuberculous *Mycobacteria* (NTM) exceeds that of MTB, this assay may not be a success as a rapid diagnostic tool. Our study has shown a significant presence of probable NTM in cultures (58 out of 180 positive cultures). With growing incidence of NTM in many areas in India [17], CB-NAAT needs to be made more useful by including a distinguishable detection of NTM. Apart from this, the CB-NAAT is also comparatively disadvantageous in terms of its cost, shelf-life of cartridges, requirement of continued power supply and the need for the periodic servicing and calibration of the equipment [18]. This study could have been strengthened if all the mycobacteria isolates in our study were identified to species level.

5. Conclusion

This study could therefore, successfully favor the use of CB-NAAT, using the Cepheid GeneXpert® system, as a rapid method for the detection of MTB alone. Further research is required for development of a better diagnostic method that can simultaneously distinguish MTB and NTM, thus rendering to detect and treat the increasing incidence of infections caused by NTM.

Ethical Statement

The study "Validity of a CB-NAAT assay in diagnosing Pulmonary and Extrapulmonary Tuberculosis in comparison to culture: A study from an urban area of South India" was conducted after the approval of the Institutional Ethical committee. There was no patient involved. The study was conducted on the coded blinded left over sample.

CRediT authorship contribution statement

Aishwarya Raj: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing - original draft. **Shrikala Baliga:** Funding acquisition, Methodology, Resources, Supervision, Validation, Visualization, Writing - review & editing. **M. Suchitra Shenoy:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **B. Dhanashree:** Data curation, Formal analysis, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **P. Prasanna Mithra:** Conceptualization, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Smitha K. Nambiar:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Leesha Sharon:** Formal analysis, Investigation, Validation, Visualization, Writing - original draft.

References

[1] Mcnerney R, Clark TG, Campino S, Rodrigues C, Dolinger D, Smith L, et al. Removing the bottleneck in whole genome sequencing of Mycobacterium tuberculosis for rapid drug resistance analysis: a call to action. Int J Infect Dis 2016;56:130–5.
[2] Innovation C. GeneXpert® Xpert® MTB/RIF. Sunnyvale (CA): Cepheid Innovation; 2012. p. 1–24.
[3] Central TB Division. Manual for sputum smear fluorescence microscopy. New Delhi: Central TB Division; 1–16 p.
[4] Siddiqi S, Rüsch-Gerdes S. MGIT™ procedure manual. Geneva (CH): Foundation for Innovative Diagnostics; 2006. p. 1–89.
[5] World Health Organization. Global tuberculosis report 2017 [Internet]; 2017. Available from: http://www.who.int/tb/publications/global_report/en/.
[6] Foundation for Innovative Diagnostics. FIND Tuberculosis Project portfolio [Internet]. [cited 2018 Jan 15]. Available from: https://www.finddx.org/tb/.
[7] Nikam C, Kazi M, Nair C, Jaggannath M, Manoj M, Vinaya R, et al. Evaluation of the Indian TrueNAT micro RT-PCR device with GeneXpert for case detection of pulmonary tuberculosis. Int J Mycobacteriol 2014;3(3):205–10.
[8] Ioannidis P, Papaventsis D, Karabela S, Nikolaou S, Panagi M, Raftopoulou E, et al. Cepheid GeneXpert MTB / RIF assay for mycobacterium tuberculosis detection and rifampin resistance identification in patients with substantial clinical indications of tuberculosis and smear-negative microscopy results. J Clin Microbiol 2011;49(8):3068–70.
[9] Kwak N, Choi SM, Lee J, Park YS, Lee C, Lee S, et al. Diagnostic accuracy and turnaround time of the Xpert MTB / RIF assay in routine clinical practice. PLoS ONE 2013;8(10):1–6.
[10] Sharma SK, Kohli M, Raj NY, Chaubey J, Dinkar B, Vishnubhatla S, et al. Evaluating the diagnostic accuracy of Xpert MTB/RIF assay in pulmonary tuberculosis. PLoS ONE 2015;10(10):1–9.
[11] Vadwai V, Boehme C, Nabeta P, Shetty A, Alland D, Rodrigues C. Xpert MTB / RIF: a new pillar in diagnosis of extrapulmonary tuberculosis? J Clin Microbiol 2011;49(7):2540–5.
[12] Sharma S, Kohli M, Chaubey J, Yadav R, Sharma R, Singh B, et al. Evaluation of Xpert MTB/RIF assay performance in diagnosing extrapulmonary tuberculosis among adults in a tertiary care centre in India. Eur Respir J 2014;44(4):1090–3.
[13] Neyrolles O, Quintana-murci L. Sexual inequality in tuberculosis. PLoS Med 2009;6:12.
[14] Hoa N, Thorson A, Long N, Diwan V. Knowledge of tuberculosis and associated health-seeking behaviour among rural Vietnamese adults with a cough for at least three weeks. Scand J Public Health 2003;31(62):59–65.
[15] Zeka AN, Tasbakan S, Cavusoglu C. Evaluation of the GeneXpert MTB/RIF assay for rapid diagnosis of tuberculosis and detection of rifampin resistance in pulmonary and extrapulmonary specimens. J Clin Microbiol 2011;49(12):4138–41.

[16] Suleiman K. An activist's guide to Tuberculosis diagnostic tools. (NY): Treatment Action Group [Internet]; 2017. Available from: http://www.treatmentactiongroup.org/tb/diagnostic-tools.
[17] Umrao J, Singh D, Zia A, Saxena S, Sarsaiya S, Singh S, et al. Prevalence and species spectrum of both pulmonary and extrapulmonary nontuberculous mycobacteria isolates at a tertiary care center. Int J Mycobacteriol 2016;5(3):288–93.
[18] Raizada N, Sachdeva KS, Sreenivas A, Vadera B, Gupta RS, Parmar M, et al. Feasibility of decentralised deployment of Xpert MTB/RIF test at lower level of health system in India. PLoS ONE 2014;9(2):1–9.

IJPSR (2017), Vol. 8, Issue 6 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 21 November, 2016; received in revised form, 16 January, 2017; accepted, 02 February, 2017; published 01 June, 2017

EFFECT OF LACTIC ACID BACTERIA ON BIOFILM FORMATION BY *STREPTOCOCCUS MUTANS*: AN *IN VITRO* STUDY

A. Raj, P. Bhati and R. Bhadekar *

Department of Microbial Biotechnology, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University, Pune – Satara Road, Katraj, Pune - 411046, Maharashtra, India.

Keywords:

Biofilm, Dental plaque, Glucosyltransferase, Lactic Acid Bacteria, *Streptococcus mutans*

Correspondence to Author:
R. Bhadekar

Associate Professor
Department of Microbial Biotechnology, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University, Pune – Satara Road, Katraj, Pune - 411046, Maharashtra, India.

E-mail: neeta.bhadekar@gmail.com

ABSTRACT: Dental biofilms are majorly developed by *Streptococcus mutans*, a cariogenic microorganism and are one of the major effects of glucosyltransferases (Gtf) produced by it. This study was conducted to determine the antagonistic potential of probiotic bacteria against *S. mutans* biofilm formation. We studied the anti-biofilm formation ability of three *Lactobacillus* species viz *L. rhamnosus, L. acidophilus and L. plantarum*. Inhibition of biofilm formation was checked on a cell culture cluster. The samples with robust inhibitory activity were selected to check their effect on Gtf activity by estimating the changes in the amount of glucan synthesized, the Gtf catalyzed reaction product. The results showed that, the cell free broth of *L. plantarum* and *L. acidophilus* has significant inhibitory and displacement activity on biofilm formation by the cariogenic organism when employed individually and in combination. Further, the interference (80% reduction) in glucan synthesis observed by applying these lactic acid bacteria (LAB) samples suggested their possible role in inhibition of glucosyltransferase (insoluble) [Gtf-I]. In conclusion, our studies demonstrated repressive activities of two LAB species, on the expression of *S. mutans* virulence genes to reduce its biofilm formation which may be associated with Gtf-I enzyme. Thus the observations would help to develop a potent strategy to combat dental plaque.

INTRODUCTION: Regardless of all the advancements in oral health sciences, one of the worst global health concerns affecting humans of all ages (especially children) is that of plaque related and caries related diseases. Dental caries is an annihilation of the dental hard tissues. Their uncritical quality and omnipresence have decreased their importance in the overall human health. Nonetheless the global encumbrance regarding the care of these dental issues can be astounding.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(6).2533-38

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (6).2533-38

Cariogenic bacteria, fermentable carbohydrates, a susceptible tooth and host and time are the predominant etiologic agents. The most potent and highly caries-associated bacteria were found to be *Streptococcus mutans* [1].

Fermentable carbohydrates, especially sucrose [2], are utilized by *S. mutans* and other cariogenic organisms. This leads to the excessive production of acids. With the accumulation of acids, only cariogenic organisms prevail on the dentine surface decreasing the occurrence of other bacteria and thus leading to the formation of a protective biofilm. Therefore a diseased condition persists on the tooth surface until the biofilms are either mechanically or chemically treated for. One important feature of *S. mutans* is its possession of *gtf-B*, that codes for Glucosyltransferase (insoluble)

[Gtf-I]. This particular enzyme is required for the production of insoluble glucans, which is one of the most important exo-polysaccharides involved in dental biofilms, and hence mediating the firm adherence of *S. mutans* to the tooth surface [3, 4].

Thus Gtf-I is considered as the fundamental virulence factor of *S. mutans* [5]. Besides this, *gtf-D* codes for glucosyltrasferase (soluble) [Gtf-S] and *gtf-C* codes for Gtf-I and Gtf-S enzymes together [3]. Gtf-S catalyzes a reaction, of which the end product is soluble glucan, which has the ability to decrease the production of biofilms [6].

Some important mechanisms involved in protection of microbes within biofilms are antibiotic resistance, quorum sensing, latency during inhospitable conditions [7], exo-polysaccharides of the biofilms, altered micro-environment within the biofilm, altered gene expression by organisms within a biofilm etc [8]. Most of the available therapeutic agents are directed towards the inhibition of growth of cariogenic organism, but they are usually ineffective due to the biofilms formed. Besides, traditional therapeutic agents that may directly destroy the resident oral microflora and thus suppress even the beneficial aspects of those microbes. Hence application of agents that can affect the activity of Gtf enzymes thereby preventing the production of insoluble glucans seem to be much more appealing for prevention of dental plaque formation. Hence the option of the use of natural therapeutic agents (plant-derived substances, like polyphenols, including extracts of miswak [9], tea tree oil [10], green tea [11], manuka honey [12], etc) is of prime importance.

Among the natural inhibitors, probiotics are comparatively less studied for their beneficial effects on oral health. They are living microorganisms, principally bacteria that are safe for human consumption and when ingested in sufficient quantities, have beneficial effects on human health, beyond basic nutrition as defined by the United Nation's Food and Agricultural Organization (FAO) and the World Health Organization (WHO) [13, 14]. They exhibit various mechanisms of activity in the oral cavity such as – prevention of pathogen adhesion on dentine surfaces, modulation of the oral environmental conditions, production of agents that inhibit the growth of cariogenic organisms, involvement in the substrate metabolism, etc [15, 16]. These mechanisms are mainly attributable to the antimicrobial agents reported from lactic acid bacteria (LAB) that principally include organic acids, peptides, hydrogen peroxide, bacteriocins, adhesion inhibitors, etc [17]. Considering this, the present study is focused on the evaluation of anti-biofilm activity of LAB and the possible mechanism thereof.

MATERIALS AND METHODS:

Bacterial strains: *Streptococcus mutans* MTCC 497, the cariogenic organism and *Lactobacillus rhamnosus* MTCC 1408, were purchased from Microbial Type Culture Collection (MTCC); Chandigarh, India. *Lactobacillus acidophilus* NCIM 2285, *Lactobacillus plantarum* NCIM 2083 were purchased from National Collection of Industrial Microorganisms (NCIM) Resource Centre, National Chemical Laboratory; Pune, India.

Media, Chemicals and Reagents: Brain Heart Infusion (BHI) agar was used for cultivation and maintenance of *S. mutans*, whereas, deMan, Rogarosa, Sharpe (MRS) agar was used for the culture and maintenance of all LAB cultures. A Special Medium was used for the initial growth of *S. mutans* before the Gtf assay [18]. All media were purchased from Himedia, India. Other chemicals and reagents were purchased from Merck, India and are of AR grade.

Culture, Purity testing and Maintenance of bacterial strains: For all experiments *S. mutans* was cultured in BHI broth aerobically at 37 ^{0}C for 48h, while the LAB species were cultured in MRS broth at 37 ^{0}C for 24h. The cultures were stored on respective media at 4 ^{0}C until use. Sub-culturing of the cultures was done once in two weeks. In the whole study, each LAB culture (OD_{600}=1) was divided into two parts – (a) a cell free supernatant (CFB), and (b) neutralized (pH 7.0) cell free supernatant (N-CFB). These are prepared fresh, just prior to each experiment and filtered through 0.4µ millipore filter (Himedia, India).

Biofilm formation assay: *S. mutans* culture was used in the assay for the formation of biofilms as per the method described by Loo *et al* [19], using BHI with 2mM sucrose. Absorbance was measured at 575nm after 0, 12, 24, 36 and 48h of incubation

using ELISA plate reader (BioTek, USA). A negative control of the media alone was set..

Inhibition of biofilm formation: Assay for inhibition of the formation of biofilm was carried out as per the method described by Ahn *et al.,* [20] with certain modifications. The CFB and N-CFB of the LAB cultures were added to 0, 12, 24, 36 and 48h old biofilms of *S. mutans* individually or in combinations (*L. acidophilus* and *L plantarum, L. plantarum* and *L. rhamnosus, L. rhamnosus* and *L. acidophilus* or all the three together – in equal proportion). The samples were incubated for 48h after this addition and the absorbance was measured as described above. The LAB samples exhibiting favorable results were chosen to examine their effect on glucan synthesis.

Glucan synthesis estimation: The activity of *S. mutans* Gtf-I enzyme can be co-related to the amount of the glucan produced. Glucan estimation experiments were performed following the procedure described by Wenham *et al* [18], with certain modifications. The cell free broth of 48h grown *S. mutans* (medium composition per liter - 9g casein hydrolysate, 6g yeast extract, 5g peptone, 2g KH_2P0_4 and 1g Na_2SO_4) was precipitated using 70% ammonium sulphate [21].

The precipitate was dissolved in 0.2M phosphate buffer (pH 6.0) [crude enzyme solution] and then used for further analysis. To 1ml of this sample 0.1% sucrose was added and the reaction mixture was incubated for 48h at 37^0C [22]. The concentration of polysaccharide formed was estimated using phenol sulphuric acid method [23]. A negative control of *Staphylococcus aureus* was used.

Inhibition of Glucan Synthesis: The LAB samples selected on the basis of the results obtained in anti-biofim activity assay were used to examine their effect on glucan synthesis. The samples were added at concentrations of 5, 10, 20 and 30% (v/v) to the crude enzyme solution and the assay was performed as described previously.

Statistical Analysis: One-way ANOVA and two-way ANOVA were performed on the obtained data to confirm their statistical significance. Multiple comparisons were made at a level of $P < 0.05$.

RESULTS:

Bacterial strains and maintenance: The bacterial cultures were maintained on respective media and incubated for 48h and 24h respectively for *S. mutans* and LAB species.

Biofilm formation assay: The assay was performed to determine the formation of biofilm by *S. mutans*. Absorbance measured at an OD of 575 nm indicated the biofilm formed at different time intervals. The observations revealed increase in the amount of biofilm formed with increase in time (**Fig. 1**).

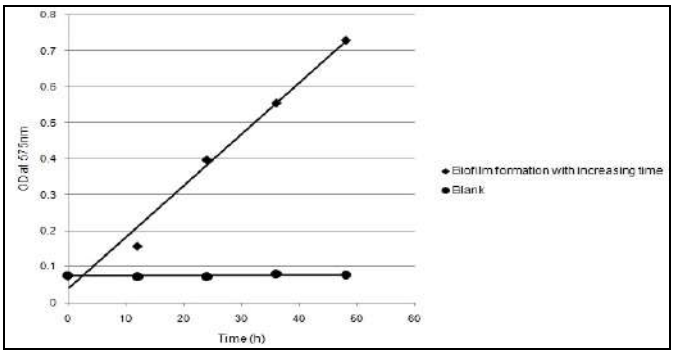

FIG. 1: BIOFILM FORMATION BY *S. MUTANS*

Inhibition of biofilm formation: In order to examine the inhibitory effect of LAB on *S. mutans* biofilm formation, the CFB and N-CFB of each LAB culture were tested individually and in combination as mentioned earlier. We observed inhibitory property when the CFB of the LAB cultures were added to growing biofilms ($P < 0.05$; two-way ANOVA). The effect was significant in the case of *L. plantarum* and *L. acidophilus* individually and also in combination ($P < 0.05$; one-way ANOVA) (**Fig. 2** and **Fig. 3**).

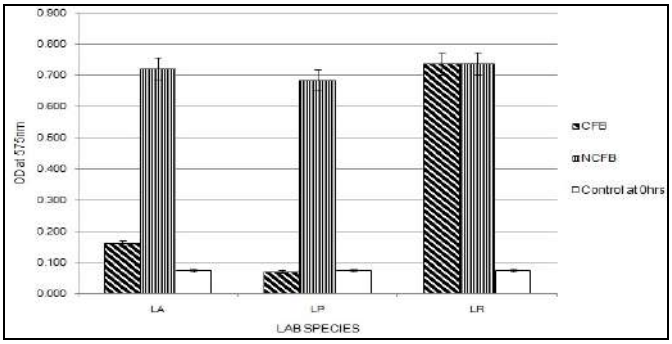

FIG. 2: INHIBITION OF *S. MUTANS* BIOFILM FORMATION BY INDIVIDUAL LAB SPECIES WHEN ADDED TO 0H OLD BIOFILM. CELL FREE BROTH (CFB) AND NEUTRALIZED CELL FREE BROTH (NCFB) OF LAB SPECIES WERE USED. (LA – *L. acidophilus*, LP – *L. plantarum*, LR – *L. rhamnosus*)

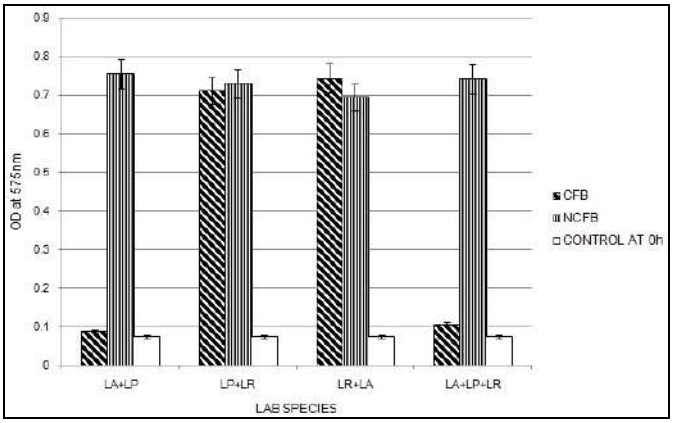

FIG. 3: INHIBITION OF *S. MUTANS* BIOFILM FORMATION BY COMBINATION OF LAB SPECIES WHEN ADDED TO 0H OLD BIOFILM. CELL FREE BROTH (CFB) AND NEUTRALIZED CELL FREE BROTH (NCFB) OF LAB SPECIES WERE USED. (LA – *L. acidophilus*, LP – *L. plantarum*, LR – *L. rhamnosus*)

The ability of LAB cultures to displace the biofilms was examined by adding the samples to 12, 24, 36 and 48h old biofilms. In this experiment also we recorded significant results in case of CFB of *L. plantarum* and *L. acidophilus*, both individually and in combination ($P < 0.05$; two-way ANOVA). In both the cases (inhibition and displacement of biofilms) the maximum inhibitory effect was observed using *L. plantarum*, followed by the combination of *L. plantarum* + *L. acidophilus* and *L. acidophilus* individually (**Fig. 4** and **Fig. 5**).

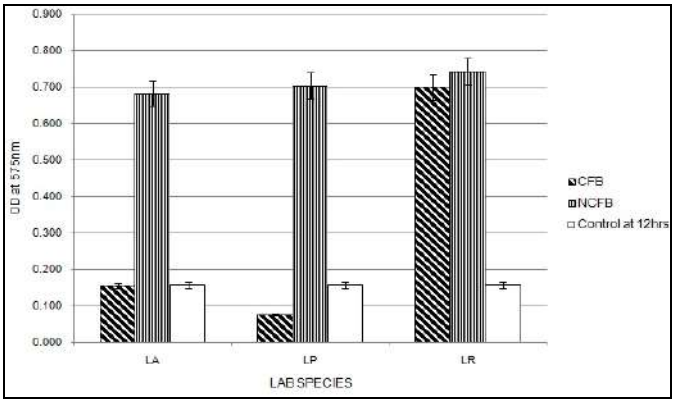

FIG. 4: INHIBITION OF *S. MUTANS* BIOFILM FORMATION BY INDIVIDUAL LAB SPECIES WHEN ADDED TO 12H OLD BIOFILM. CELL FREE BROTH (CFB) AND NEUTRALIZED CELL FREE BROTH (NCFB) OF LAB SPECIES WERE USED. (LA – *L. acidophilus*, LP – *L. plantarum*, LR – *L. rhamnosus*)

Similar results were obtained when the experiments were performed on 24, 36 and 48h old biofilms (data not shown).

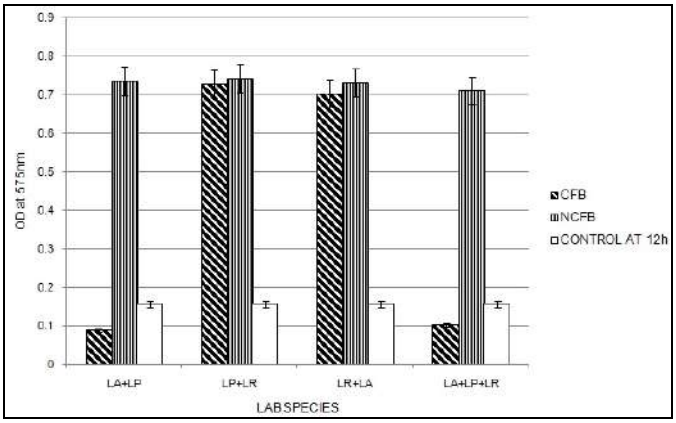

FIG. 5: INHIBITION OF *STREP. MUTANS* BIOFILM FORMATION BY COMBINATION OF LAB SPECIES WHEN ADDED TO 12H OLD BIOFILM. CELL FREE BROTH (CFB) AND NEUTRALIZED CELL FREE BROTH (NCFB) OF LAB SPECIES WERE USED. (LA – *L. acidophilus*, LP – *L. plantarum*, LR – *L. rhamnosus*)

Glucan Synthesis: The amount of insoluble glucan formed was measured using the phenol sulphuric acid method using glucose as standard. The resultant amount of glucan obtained was considered as 100% for further analysis. The inhibitory effect of LAB on glucan synthesis was checked by incubating the crude enzyme solution in the presence of the selected LAB samples. The amount of glucan synthesized was found to decrease with increase in concentration of LAB samples up to 20% ($P < 0.05$; two-way ANOVA). Maximum reduction (80%) in insoluble glucan synthesized was observed when the CFB of *L. plantarum* and *L. acidophilus* were used in combination at a concentration of 20% (v/v). This was followed by 73% reduction in glucan synthesis using CFB of *L. plantarum* (73%) (**Fig. 6**).

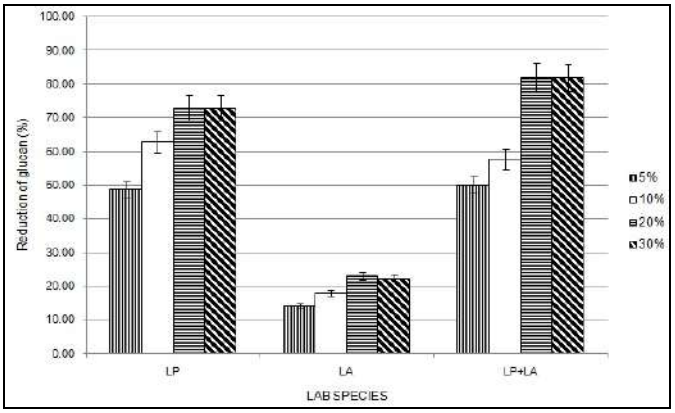

FIG. 6: EFFECT OF CFB OF LAB SPECIES ON GLUCAN SYNTHESIS. THE SAMPLES WERE ADDED AT CONCENTRATION OF 5, 10, 20 AND 30%. (LA – *L. acidophilus*, LP – *L. plantarum*, LR – *L. rhamnosus*)

DISCUSSION: Anti-biofilm activity of certain phytochemicals [24] and other bacterial metabolites [25] had been reported against *S. mutans.* However we came across very few studies on the effect of LAB on the biofilm formation of *S. mutans* and the pertinent mechanism thereof. Therefore our work focused on this aspect using *L. acidophilus*, *L. plantarum* and *L. rhamnosus* is significant.

Some of the *in vitro* studies on *S. mutans* include i) antibacterial activity of certain fluoride compounds and herbal toothpastes against *S. mutans* [26], ii) inhibition of adherence on saliva treated beads using ginkgoneolic acid [27] and probiotic lactobacilli [28], iii) anti-biofilm activity of *L. acidophilus* [29]. iv) inhibitory effect of *L. salivarius* on *S. mutans* in a contact independent manner [30].

We have observed significant anti-biofilm activity of the cell free broth of *L. plantarum*, followed by *L. acidophilus*. Absence of inhibitory activity during the use of neutralized CFB indicated acidic pH might be one of the contributory factors for the inhibition of the formation of biofilms and/or for the destruction of the established biofilms. Further studies on other contributory factors are necessary.

It is well known that the exo-polysaccharides produced by *S. mutans* play a key role in biofilm formation [31]. The polysaccharides mainly comprise of insoluble glucans [3, 5] and Gtf-I is identified as the key enzyme for glucan synthesis.

However, to our knowledge correlation between anti-biofilm activity and interference in glucan synthesis owing to its effect on Gtf-I activity is rarely documented in literature. Apigenin, a natural product derived from certain plants, exhibited the ability to reduce the amount of insoluble glucans and enhance the soluble glucan content of the polysaccharide matrix – thus inhibiting the activity of Gtf-I *in vitro* [6]. Our experiments to affirm the role of Gtf-I as a key enzyme for glucan synthesis are in line with the previous reports [18]. Effect of LAB individually and/or in combination showed decrease in glucan synthesis up to 80% which may be due to inhibition of Gtf-I activity.

CONCLUSION: From this study it can be concluded that *L. plantarum* and *L. acidophilus* are the best probiotic candidates with respect to biofilm inhibitory activity of *S. mutans*. More studies on Gtf-S enzyme that produces soluble glucans and thus directly decrease the biofilm formation are under way. Further, *in vivo* studies to evaluate the exact effect of probiotic peptides on the biofilm formation capability of *S. mutans* and the exo-polysaccharides involved in real-time caries would help to develop a safe product for good oral health.

ACKNOWLEDGEMENTS: This work was supported by Bharati Vidyapeeth Deemed University, Pune, Maharashtra, India.

CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

REFERENCES:

1. Loesche WJ, Rowan J, Straffon LH and Loos PJ: Association of *Streptococcus mutans* with human dental decay. Infection and Immunity 1975; 11:1252-1260.
2. Keyes PH: Research in dental caries. The Journal of the American Dental Association 1968; 76:1357-1373.
3. Loesche WJ: Role of *Streptococcus mutans* in human dental decay. Microbiological Reviews 1986; 50:353-380.
4. Moye ZD, Zeng L and Burne RA: Fueling the caries process: carbohydrate metabolism and gene regulation by *Streptococcus mutans*. Journal of Oral Microbiology 2014; 6.
5. Fears KP, Gonzalez-Begne M, Love CT, Day DE and Koo H: Surface-induced changes in the conformation and glucan production of Glucosyltransferase absorbed on saliva-coated hydroxyapatite. American Chemical Society 2015; 31:4654-4662.
6. Koo H, Nino de Guzman P, Schobel BD, Vacca Smith AV and Bowen WH: Influence of cranberry juice on glucan-mediated processes involved in *Streptococcus mutans* biofilm development. Caries Research 2006; 40:20-27.
7. Balebroux ZD, Svensson SL, Gaynor SL and Swanson MS: ppGpp conjures bacterial virulence. Microbiology and Molecular Biology Reviews 2010; 74:171-199.
8. Sadykov MR and Bayles KW: The control of death and lysis in staphylococcal biofilms: a coordination of physiological signals. Current Opinion in Microbiology 2012; 15: 211-215.
9. Jeon JG, Rosalen PL, Falsetta ML and Koo H: Natural products in caries research: current (limited) knowledge, challenges and future perspective. Caries Research 2011; 45:243-263.
10. Ramage G, Milligan S, Lappin DF, Sherry L, Sweeney P, Williams C, Bagg J and Culshaw S: Antifungals, cytotoxic, and immunomodulatory properties of tea tree oil and its derivative components: potential role in management of oral candidiosis in cancer patients. Antimicrobials, Resistance and Chemotherapy 2012; 3:116-123.
11. Xu X, Zhou XD and Wu CD: The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. Antimicrobial agents and Chemotherapy 2011; 55:1229-1236.
12. Carter DA, Blair SE, Cokcetin NN, Bouzo D, Brooks P, Schothauer R and Harry EJ: Therapeutic manuka honey: no longer so alternative. Frontiers in microbiology 2016; 7.

13. Bhadekar R and Parhi P: Non-dairy functional foods: Potential of probiotics. In: Garg N, Abdel-Aziz SM and Aeron A eds. Microbes in food and health. Springer International Publishing, Switzerland, 2016: 1-27.
14. Dixit G, Samarth D, Tale V and Bhadekar R: Comparitive studies on potential probiotic characteristics of *Lactobacillus acidophilus* strains. EurAsian Journal of Biosciences 2013; 7:1-9.
15. Krishnappa S, Srinath S, Bhardwaj P and Mallaya CH: Role of probiotics in prevention of dental caries: a review. International Journal of Advances in Medicine and Dental Sciences 2013; 1:86-91.
16. Kalra M, Bhaskar DJ, Punia H, Singh V and Jinghala V: Probiotics and oral health. Journal of contemporary dentistry 2015; 5:104-106.
17. Donelli G: Activity of probiotics on biofilm-growing pathogens of the oral cavity. Microbial Ecology in Health & Disease 2013; 24.
18. Acharya S: Probiotics: current knowledge update. International Journal of Pedodontic Rehabilitation 2016; 1:79-83.
19. Loo CY, Corliss DA and Ganeshkumar N: *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. Journal of Bacteriology 2000; 182:1374-1382.
20. Ahn SJ, Ahn SJ, Wen ZT, Brady L and Burne RA: Characteristics of biofilm formation by *Streptococcus mutans* in the Presence of Saliva. Infection and Immunity 2008; 76:4259-4268.
21. Hamada S, Horikoshi T, Minami T, Kawabata S, Hiraoka J, Fujiwara T and Ooshima T: Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. Infection and Immunity 1991; 59:4161-4167.
22. Chaudhuri B, Rojek J, Vickerman MM, Tanzer JM and Scannapieco FA: Interaction of Salivary alpha-Amylase and Amylase-Binding-Protein A (AbpA) of *Streptococcus gordonii* with Glucosyltransferase of *S. gordonii* and *Streptococcus mutans.* BioMed Central Microbiology 2007; 7:60.
23. Dubois M, Gilles KA, Hamilton JA, Rebers PA and Smith F: Calorimetric method for determination of sugars and related substances. Analytical Chemistry 1956; 28:350-356.
24. Murata RM, Branco-de-Almeida LS, Franco EM, Yatsuda R, dos Santos MH, de Alencar SM, Koo H and Rosalen PL: Inhibition of *Streptococcus mutans* biofilm accumulation and development of dental caries *in vivo* by 7-epiclusianone and fluoride. Biofouling 2010; 26:865-872.
25. Jansen R, Irschik H, Huch V. Schummer D, Steinmetz H, Bock M, Schmidt T, Kirschning A and Müller R: Carolacton: a macrolide ketocarbonic acid that reduces biofilm formation by the caries-and endocarditis-associated bacterium *Streptococcus mutans*. European Journal of Organic Chemistry 2010; 7:1284-1289.
26. Randall JP, Seow WK and Walsh LJ: Antibacterial activity of fluoride compounds and herbal toothpastes on *Streptococcus mutans*: an *in vitro* study. Australian Dental Journal 2015; 60:368-374.
27. He J, Wang S, Wu T, Cao Y, Xu X and Zhou X: Effects of ginkgoneolic acid on the growth, acidogenicity, adherence, and biofilm of *Streptococcus mutans in vitro*. Folia Microbiologica 2013; 58:147-153.
28. Vestman NR, Timby N, Holgerson PL, Kressirer CA, Claesson R, Domellof M, Ohman C, Tanner ACR, Hernell O and Johansson I: Characterization and *in vitro* properties of oral lactobacilli in breastfed infants. BMC Microbiology 2013; 13:193-204.
29. Ciandrini E, Campana R, Casettari L, Perinelli DR, Fagioli L, Manti A, Palmieri GF, Papa S and Baffone W: Characterization of biosurfactants produced by Lactobacillus spp. and their activity against oral streptococci biofilm. Applied Microbilogy and Biotechnology 2016; 15:6767-6777.
30. Wu CC, Lin CT, Wu CY, Peng WS, Lee MJ and Tsai YC: Inhibitory effect of *Lactobacillus salivarius* on *Streptococcus mutans* biofilm formation. Molecular Oral Microbiology 2015; 30:16-26.
31. Falsetta ML, Klein MI, Colonne PM, Scott-Anne K, Gregoire S, Pai C-H, Gonzalez-Begne M, Watson G, Krysan DJ, Bowen WH and Koo H: Symbiotic relationship between *Streptococcus mutans* and *Candida albicans* synergizes virulence of plaque biofilms *in vivo*. Infection and Immunity 2014; 85:1968-1981.

How to cite this article:
Raj A, Bhati P and Bhadekar R: Effect of lactic acid bacteria on biofilm formation by *Streptococcus mutans*: an *in vitro* study. Int J Pharm Sci Res 2017; 8(6): 2533-38.doi: 10.13040/IJPSR.0975-8232.8(6).2533-38.