Identification and Detection of Oral Pathogens Lactobacilli in Saliva Sample

ABDUBAKI MOHAMEDNAISSIR AHMED¹, DR. G. S. SHUKLA²
¹PG Scholar, Dept of MLT, SHIATS, Allahabad, India.
²Professor, Dept of MLT, SHIATS, Allahabad, India.

Abstract: Lactobacillus is a type of bacteria with multiple different species in the genus and most Lactobacillus species in humans are considered harmless. It is also reported that few Lactobacillus species are responsible for tooth decay and cavity formation in children. But in adults, the bacterium is reported to create several oral diseases and is found in connection with several diseases causing bacterium. In this study saliva samples were collected from patients reported to have any of the oral disease and equal number of samples were collected from healthy individuals who did not had any diseases and cultured for the presence of Lactobacillus. It was seen that the number of bacterium is huge in patient sample when compared with the healthy samples. One of the representative isolate was sequenced through 16s rRNA region to and identified as Lactobacillus fermentum strain IMAU32272. The isolates were molecularly characterized using RAP-D-PCR and a dendrogram constructed revealed that they were almost similar.

Keywords: DNA, RAPD-PCR, rRNA.

I. INTRODUCTION
Lactobacillus, is a genus of Gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria.[1] They are a major part of the lactic acid bacteria group. In humans they are part of the vaginal microbiota.[2] Many species in this genus have had their genome sequenced.[3] Lactobacillus is Gram-positive (they retain crystal violet dye), facultative anaerobe (they can produce energy through glycolysis and fermentation when oxygen is not present). Lactobacillus is a member of the lactic acid bacteria group (its members convert lactose and other sugars to lactic acid). Lactobacilli were the first microorganisms implicated in the development of dental caries[1]and have been shown to be associated with the dental carious process[2]. The Lactobacilli count represents the number of lactobacilli present in 1mL of saliva (CFU/mL), and is used to determine the efficiency of dietetic measures or to evaluate caries risk. It can be used alone or in combination with other associated parameters[3], [4]. Granath et al. [5] suggested that the saliva count of lactobacilli is a better criterion than that of Streptococcus mutans as the former count is strongly correlated with caries [6]–[12]. Specifically, a higher decay missing fill (DMF) index is associated with a greater number of children harboring a high Lactobacillus count [13], [14]. Although there is a strong association between lactobacilli and caries, little is known regarding the relationship at the species level because of difficulties in identifying Lactobacillus species by conventional methods [15]. The oral cavity shelters a very numerous and various microbial flora. One major actor of this complex ecosystem is the dental plaque which develops naturally on oral tissues. This biofilm shows a very complex organization that remains relatively stable with time despite regular environmental changes [16]. When the equilibrium is compromised and when an imbalance appears among the indigenous bacteria, pathologies such as dental caries or periodontitis could occur [17]. Historically, lactobacilli were the first microorganisms implicated in dental caries development [18]. They appear during the first years of a child’s life, and are present in high numbers in saliva, on the dorsum of the tongue, mucous membranes, the hard palate, in dental plaque and, in fewer numbers, on tooth surfaces [19]. 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 [20], partly erupted third molars or orthodontic devices [21].

Aims and Objectives:
- The present study is to detect the presence of Lactobacilli, from healthy and patients’ samples.
- Molecular characterization of isolated Lactobacilli using RAPD markers.
- Molecular identification of Lactobacilli isolate through 16s rRNA sequencing by Sanger’s Method.

II. MATERIALS AND METHODS
A. Source And Confirmation Of Isolates Of Lactobacilli
Strains of Lactobacilli were obtained from twenty different plaque samples 10 positive samples and 10 negative samples. The samples were collected from Oxford Dental College (Bangalore, India) Lactobacilli isolates from these individuals were originally cultured from MRS medium, 100 - 10-5 dilutions of individual sample was done and plated on MRS media. Ten isolates of Lactobacilli per individual were selected at random from primary isolation plates of MRS
medium. If differences in colony morphology were evident, efforts were made to include each of the different colony types. The colony in MRS media confirms the Lactobacilli species.

B. Genomic DNA Isolation
The method used to isolate chromosomal DNA from Lactobacilli was as follows. Cultures of Lactobacilli were initiated from pure single colonies as follows. DNA was isolated from the samples provided following the modified CTAB method. The extracted DNA was quantified using Spectrophotometer (Sartorius) for standardizing the PCR. The below mentioned protocol was implemented to extract the genomic DNA from the sample provided.

Protocol:
- The sample was centrifuged at 10,000 rpm for 10 minutes to pellet down the bacterial cells. The supernatant was discarded.
- To the pellet added 675µl of extraction buffer and incubated at 37oC for 30 min.
- To the above 75µl of SDS was added and incubated at 65oC for 2 hours followed with centrifugation at 10000 rpm for 10 min at 4oC.
- The supernatant was collected in a sterile micro centrifuge tube.
- Equal volumes of Chloroform: Isoamyl alcohol was added and centrifuged 10000 rpm for 10 min at 4oC.
- The aqueous phase was transferred to a sterile micro centrifuge tube.
- Added 0.6 volumes of isopropanol and incubated at room temperature for 1 hour.
- After incubation it was then centrifuged at 10000 rpm for 10 min.
- The pellet was washed with 500µl of 70% Ethanol and centrifuged at 10000 rpm for 10 min at room temperature.
- The pellet was air dried and dissolved in 20µl of sterile water.

The concentration of chromosomal DNA was determined spectrophotometrically (260nm) by standard procedures and its quality was assessed by the 260:280 nm absorbance ratio and by electrophoresis on agarose gel.

C. 16s rDNA PCR For Species Identification
Quantification of Isolated DNA: The quantity of the isolated DNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany). From the stock 1µl DNA was mixed with 49-µl sterile distilled water to get 50 times dilution. The A260/A280 ratio was recorded to check the purity of DNA preparation.

PCR Amplification: Reagents and the optimal PCR reaction mixture PCR amplification of 16s region was done in 20 µl of reaction mixture containing PCR buffer, 1X (Kappa, SA); MgCl2, 3 mM; dNTP mix, 0.25 mM; TaqDNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease free water is used as negative control.

Agarose Gel Electrophoresis Of PCR Products: After the DNA was isolated from the sample the quality was analyzed in an Agarose gel as follows:
- 2 gram of Agarose powder was added to a 250 ml conical flask containing 100 ml TAE (1X) buffer and mixed properly.
- The Agarose suspension was melted in a microwave or hot water bath until clear.
- The solution was cooled to about 50-55°C swirling the flask occasionally.
- 1-2 µl of Ethidium Bromide solution was added to the melted agarose gel and mixed well.
- The open ends of the casting tray were sealed with a cellophane tape.
- The combs were placed in the gel casting tray.
- Melted agarose was poured into the casting tray without forming air bubbles and was cooled until solid.
- The combs were carefully pulled out and removed the tape and the gel was placed in the electrophoresis chamber.
- Sufficient amount of 1X TAE Buffer was added so that there is about 2-3 mm of buffer over the gel.
- 6 µl of 6X Sample Loading Buffer was added to each 25 µl of PCR sample.
- Each sample/ DNA ladder was carefully pipetted into separate wells in the gel.
- Electrode wires were connected to the power supply, making sure the positive (red) and negative (black) are correctly connected.
- Power supply was turned on to about 100 volts (should not exceed 5 volts/cm between electrodes).
- After sufficient run turn off the power pack, the gel was removed by using gloves.
- The amplified DNA in the gel was visualized with UV light, photographed and documented using UV transilluminator.

DNA Elution From Agarose Gel (Credora Gel Elution Kit):
- PCR fragment was excised from the agarose gel using a sterile scalpel.
Identification and Detection of Oral Pathogens Lactobacilli in Saliva Sample

- The gel slice was transferred to 1.5 ml or 2 ml tube.
- 650 µl Gel Solubilizer was added.
- Sample was incubated for 10 min at 50°C to dissolve slice and vortexed.
- 50 µl Binding Optimizer was added and vortexed.
- 750 µl of sample was transferred to Spin Column A placed in Collection Tube.
- Sample was centrifuged at 10000 g for 1 min. Filtrate was discarded and collection tube was reused.
- Any residual solution was loaded and step 7 was repeated.
- 700 µl Wash Buffer C was added.
- Sample was centrifuged at 10000 g for 1 min. Filtrate was discarded and Collection Tube was reused.
- Steps 9 and 10 were repeated.
- Sample was centrifuged at Max speed for 2 min and Collection Tube was discarded.
- Spin Column A was placed in 1.5 ml Elution Tube.
- 30-50 µl Elution Buffer was added directly to Spin Column membrane.
- Sample was incubated at room temperature for 1 min.
- Sample was centrifuged at 6000 g for 1 min to elute DNA.

Sequencing: The eluted amplicons were sequenced at Scigenome, Cochin using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer’s instructions.

D. Genetic Diversity Analysis

The diversity analysis was done using 05 RAPD primers for all the positive isolates following the below mentioned procedure.

<table>
<thead>
<tr>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>94°C</td>
<td>37°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td>5 min</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>40 cycles</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

After the RAPD PCR the amplified products were run on a 2% Agarose gel as below

Agarose Gel Analysis Of RAPD PCR (2% Agarose):
- 2 gram of Agarose powder was measured and added to a 250 ml conical flask.
- To the flask 100 ml TAE (1X) buffer was added and mixed properly.
- The agarose was melted in a microwave or hot water bath until the solution became clear.
- The solution was allowed to cool to about 50-55°C with swirling of the flask occasionally to cool evenly.
- 1-2 µl of Ethidium Bromide solution was added to the melted agarose gel and mixed well.
- Ends of the casting tray were sealed and combs were placed.

The 05 RAPD markers resulted in different banding pattern ranging from 100 – 2000 bp. For construction of a dendrogram, presence and absence of bands were scored as 1 and 0 respectively. Using the PAST statistical software diversity between the samples were analyzed using Paired group algorithm with Jaccard distance matrix.

III. RESULTS

A. Confirmation of Isolates Of Lactobacilli

The presence of specific colony on MRS agar media confirms the Lactobacilli colonies different dilutions were done but only in 100 and 10-1 dilutions showed the presence of colonies. It was observed the numbers of bacterial colonies were more in the patients sample than that seen in the healthy volunteers.

![Fig.1. Patient Samples.](image-url)
B. Genomic DNA Extraction

The DNA extracted from all the bacterial isolates were of good quality and could be used in all the downstream reactions.

C. 16s rRNAPCR For Species Identification

Quantification of DNA: The isolated genomic DNA from all the positive isolates were quantified spectrophotometrically and tabulated as follows. It was observed that all the samples yielded in good quality and sufficient quantity of genomic DNA.

<table>
<thead>
<tr>
<th>SL. NO.</th>
<th>SAMPLE</th>
<th>Quantity (ng/µl)</th>
<th>SL. NO.</th>
<th>SAMPLE</th>
<th>Quantity (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Rajeswari</td>
<td>138</td>
<td>11</td>
<td>Kiran</td>
<td>224</td>
</tr>
<tr>
<td>2.</td>
<td>Bhagy01</td>
<td>212</td>
<td>12</td>
<td>Lakshmi</td>
<td>89</td>
</tr>
<tr>
<td>3.</td>
<td>Lokeshwari</td>
<td>154</td>
<td>13</td>
<td>Ranya</td>
<td>139</td>
</tr>
<tr>
<td>4.</td>
<td>Padma</td>
<td>312</td>
<td>14</td>
<td>Ananya</td>
<td>140</td>
</tr>
<tr>
<td>5.</td>
<td>Swati</td>
<td>118</td>
<td>15</td>
<td>Atri</td>
<td>286</td>
</tr>
<tr>
<td>6.</td>
<td>Deepa</td>
<td>96</td>
<td>16</td>
<td>Remaya</td>
<td>228</td>
</tr>
<tr>
<td>7.</td>
<td>Jayamma</td>
<td>153</td>
<td>17</td>
<td>Sulaya</td>
<td>103</td>
</tr>
<tr>
<td>8.</td>
<td>Padmalakha</td>
<td>201</td>
<td>18</td>
<td>Nauthad</td>
<td>111</td>
</tr>
<tr>
<td>9.</td>
<td>Mahan</td>
<td>115</td>
<td>19</td>
<td>Antony</td>
<td>80</td>
</tr>
</tbody>
</table>

A set of specific primers were used to amplify the 16s ribosomal RNA region and on running the amplified PCR product on the gel it was observed that the primers could excise the expected region with a bandwidth of 1500bp. The gel picture is shown below –

This amplicon was eluted from the gel as mentioned in the procedure and was sequenced in an Applied Biosystem Sequencer and revealed the nucleotide sequences which was of 1489 bp in length. The above sequences obtained were searched for similar sequences in the National Center for Bioinformatics (NCBI) gene database, which showed 97% similarity with the Lactobacillus fermentum strain IMAU32272.

D. Genetic Diversity Analysis Using RAPD Markers

Five different RAPD markers were used to randomly amplify the genomic DNA of the positive isolates. The 05 RAPD markers resulted in different banding pattern ranging from 100 – 3000bp. For construction of a dendrogram with 128 bands scored for presence and absence as 1 and 0 respectively. Using the PAST statistical software diversity between the samples were analyzed using Paired group algorithm with Euclidean distance matrix.
Identification and Detection of Oral Pathogens Lactobacilli in Saliva Sample

IV. DISCUSSION

It is difficult, from the literature, to have an exact idea of the relationship between lactobacilli and dental plaque whether on the quantitative level or on the qualitative level. In effect, the numerous studies were carried out on different populations (age, DMF index, fluoride intake) and moreover, the sampling methods were also very varied. In our study we have isolated Lactobacillus from the patients who had some oral diseases and from the healthy individuals for whom no oral diseases were reported. It was observed that the number of colonies were very huge in the case of patient samples though there were few colonies appeared in the healthy samples too. Even if lactobacilli have been reported to be less detectable in the dental plaque than in saliva, this study performed in gave interesting results. In the present study an identification-detection system by using PCR was performed with specific primers designed on the basis of previously published L.delbrueckii subsp. bulgaricus prolineiminopeptidase (pepLP) gene sequence data and used this system for differentiation of Lactobacillus species. The sequencing results revealed that the isolated bacterium was Lactobacillus fermentum strain IMAU32272. The applicability of the different typing methods has been demonstrated by different studies, but the identification patterns may be difficult to repeat in different tests and laboratories. In addition, there is a need to maintain a substantial comparison data file of identification patterns. To overcome these problems the RAPD technique was chosen as a suitable means by which to reveal the needed polymorphism, hence, RAPD-PCR was used to confirm subspecies identities and to evaluate intraspecific genetic diversity among the isolates. RAPD-PCR amplification with the primer usedrevelled genomic variability among the strains of the Lactobacillus because of the presence of very intense polymorphic bands. After numerical analysis no subspecies-specific clusters were obtained. Therefore, this primer was useful for characterization of individual strains but not for subspecies identification.

V. REFERENCES