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Original Article

Molecular Detection of Antibiotic Resistance Determinants in *Lactobacillus* Bacteria Isolated from Human Dental Plaques

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Introduction: Dental caries is a significant public health problem which destroys the calcified tissues of the teeth. The frequency of *lactobacilli* in dental caries is usually high. The objective of this study was to evaluate the incidence of these bacteria in dental caries and to identify the antibiotic resistance patterns of them. **Methods:** Specimens from dental plaque were collected from 30 patients. Phenotypic (Gram staining, cell morphology, and biochemical tests) and molecular (PCR) methods were used for identification of *Lactobacillus* bacteria. The antimicrobial sensitivity test was performed by standard Kirby-Bauer disk diffusion method to determine their resistance to chloramphenicol, penicillin, vancomycin, nitrofurantoin, cefixime, cefazolin, amoxicillin-clavulanic acid, and tetracycline. Antibiotic resistance genes were detected using multiplex PCR method as well. **Results:** Fourteen out of 30 (46/6%) samples were *Lactobacillus* positive. The *mecA* was the essential gene responsible for penicillin resistance. Also, the bla_{TEM} and bla_{OXA-1} genes had more significant role in cefazolin and cefixime resistance than bla_{SHV}, and *these* two genes had the similar role in the amoxicillin-clavulanic acid resistance. Also, tetracycline resistance was caused by both *tetK* and *tetM* genes identically. **Conclusion:** In conclusion, *Lactobacillus* bacteria had an essential role in the formation of dental plaque and showed multidrug-resistant patterns to commonly used antibiotics. *J Med Microbiol Infect Dis, 2017, 5 (3-4): 51-55. DOI: 10.29252/JoMMID.5.3.4.51*

Keywords: Dental plaque, *Lactobacillus*, Multiplex PCR, Resistance genes.

INTRODUCTION

Dental decay is a significant, prevalent chronic problem of the oral cavity [1]. Dental plaques or biofilms, which are formed on teeth surfaces are the primary cause of dental caries and consist of native oral microflora [2]. *Lactobacillus* species are among the most important organisms contributing the development of dental plaques and dental caries [3]. Cariogenic bacteria like *Lactobacillus* produce acids as a result of carbohydrate metabolism, which destructs superficial dental structures and leads to dental caries [2]. Selective pressure through administration of antibiotics for the treatment of microbial diseases of the human and animals leads to dissemination and spread of antibiotic resistance genes [4]. Earlier studies showed an overall increase of *Lactobacillus* species in saliva before the formation of carious lesions. So the *Lactobacillus* count has been widely used to determine various risk factors including the development of plaque and dental caries [5].

This study was carried out to find the prevalence of *Lactobacillus* species in dental plaques, and also to determine the antibiotic resistance patterns of the isolates using PCR-based molecular techniques.

MATERIAL AND METHODS

Study population. Thirty plaque samples were collected from the people attending the dental unit of Tabriz University of Medical Science (TUMS), Tabriz, Iran (2015). Thirty Iranian adult patients (including fifteen men and fifteen women) aged 20-40 years with dental plaque were included in this study. Informed consent was obtained from all participants, and the ethical committee approved the study (No. 2309894.)

Collection of samples. Samples were collected from along the cervical edge of the teeth and then were homogenized by vortexing in a tube containing sterile phosphate- buffered saline (PBS) [6].

Isolation and identification of *lactobacilli*. Amounts of 0.1 ml of homogenized suspensions were cultured on MRS (Man, Rogosa, and Sharpe) agar (Sigma, USA), and incubated for 48 h in a 5% CO_2 incubator at 37°C. The *Lactobacillus* isolates grown on MRS agar were identified by Gram staining, cell morphology, and biochemical tests, *e.g.*, catalase test, gas production from glucose, arginine hydrolysis, asculin hydrolysis, nitrate reduction and citrate utilization test [7]. The identified *Lactobacilli* were transferred to MRS broth containing 15% glycerol and stored at -20°C [4].

Molecular identification of *Lactobacilli*. An overnight culture of the isolates in MRS broth was used for DNA extraction, and genomic DNA was extracted using a bacterial genomic DNA extraction kit (Fermentase,

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Germany). Simply, the cultures were centrifuged for 10 min at 5000 xg, and 10-20 mg of pellets were transferred to 1.5 ml microtubes and resuspended in 200 µl of TE buffer. Amounts of 200 µl of the suspensions were mixed with 400 µl of lysis solution and incubated at 65°C for 5 min.

Amounts of 600 µl of chloroform were added to the mixtures and gently mixed by inverting and then centrifuged at 9400 xg for 2 min. The upper aqueous phase containing DNA was transferred to new tubes, 800 µl of freshly prepared precipitation solution was added, mixed gently by several inversions at room temperature for 1-2 min and centrifuged at 9400 for 2 min. The supernatants were removed, and DNA pellets were dissolved in 100 µl of NaCl solution by gentle vortexing. Amounts of 300 µl of cold ethanol were added to tubes and kept for 10 min at -20°C and then centrifuged at 9400 xg for 4 min. The ethanol was removed, and DNA pellets were washed with 70% cold ethanol and dissolved in 100 µl of sterile deionized water.

Molecular detection of *Lactobacillus* bacteria was performed by genus-specific PCR primers F: 5′-CTC AAA ACT AAA CAA AGT TTC-3′ and R: 5′- CTT GTA CAC ACC GCC CGT CA-3′ , which amplify a ribosomal RNA intragenic spacer region between 16S and 23S fragments. The reaction contents for each 25 µL PCR consisted of 10 µl Red Amp master mix 2×, 3 µl of template DNA, 1 µl of each primer and 10 µl of deionized water. The amplification was programmed in a DNA thermocycler (MWG AC BIOTECH THERMAL CYCLER, USA) for 5 min of initial denaturation at 95°C, 20 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 30 s at 72°C with a final extension at 72°C for 7 min. Amplified products were electrophoresed on 1% agarose gel at 75v for 90 min and visualized under the UV light. A 250 bp band was indicative of the presence of *lactobacillus* bacteria [4].

Antimicrobial susceptibility pattern. The standard Kirby-Bauer disk diffusion assay was used to identify the antimicrobial agent sensitivity profile of the *Lactobacillus* isolates with 8 antimicrobial agents, chloramphenicol (30 µg), penicillin (10 U), vancomycin (30 µg), nitrofurantoin (300 µg), cefixime (5 µg), cefazolin (30 µg), amoxicillin-clavulanic acid (20/10 µg) and tetracycline (30 µg).

A 15 mm Mueller-Hinton agar plate spread with MRS was inoculated with *Lactobacillus* and incubated to a turbidity of 0.5 McFarland standards. Seven commercially antimicrobial agent disks were placed on the inoculated plate and were incubated anaerobically at 37°C for 18-20 h. The diameters zone of inhibition (mm), around each disk, was measured and interpreted by referring to the performance standard for antimicrobial susceptibility testing [8].

Detection of antibiotic resistance genes by PCR. Based on the resistance phenotypes, three different PCR protocols were used to amplify seven antibiotic resistance genes with the reagents and primers described by others (Table 1). The bla_{TEM}, $bla_{SHV,}$ and bla_{OXA-1} genes were amplified for the isolates exhibiting cephalosporin (cefazolin and cefixime) resistance[9], *blaZ* and *mecA* for penicillin resistance[1], and *tetK* and *tetM* for tetracycline resistance [10].

RESULTS

Microscopy and Biochemical tests. Out of 30 samples, 14 *lactobacillus* isolates (46%) were obtained. All 14 isolates were Gram-positive rods under oil immersion microscope. They were non-motile and catalase negative, and in the nitrate reduction test, all showed negative reactions.

Table 1. *Lactobacillus* isolates antimicrobial resistant genes and primer sequences used for PCR identification

Resistance gene	Primer Sequence	Size (bp)	Annealing temperature (°C)	References
bla_{TEM}	F 5′ ATCAGCAATAAACCAGC3′ R 5′ CCCCGAAGAACGTTTTC 3′	516	54	[9]
bla_{SHV}	F 5′ AGGATTGACTGCCTTTTTG3′ R 5′ ATTTGCTGATTTCGCTCG3′	392	54	[9]
bla_{OXA-1}	F 5′ATATCTCTACTGTTGCATCTCC3′ R 5′AACCCTTCAAACCATCC3′	619	54	[9]
blaZ	F 5′ TGA CCA CTT TTA TCA GCA ACC3′ R 5′ GCC ATT TCA ACA CCT TCT TTC3′	700	58	[11]
mecA	F 5′AAA ATC GAT GGT AAA GGT TGG C3′ R 5′ AGT TCT GCA GTA CCG GAT TTG C3′	532	58	[11]
tetK	F 5′ GTA GCG ACA ATA GGT AAT AGT 3′ R 5′GTA GTG ACA ATA AAC CTC CTA3′	360	55	[12]
tetM	F 5′ AGT GGA GCG ATT ACA GAA3′ R 5′ CAT ATG TCC TGG CGT GTC TA3′	158	55	[12]

Table 2. Antimicrobial susceptibility pattern of *Lactobacillus* isolates obtained from dental plaques

Antimicrobials	Total number of isolates tested	Percentage (%) of resistant strains
Vancomycin	14	64
Chloramphenicol	14	78
Penicillin	14	100
Amoxicillin-Clavulanic acid	14	100
Nitrofurantoin	14	71
Cefixime	14	100
Cefazolin	14	100
Tetracycline	14	92

Molecular identification of *Lactobacillus* genus. All the 14 biochemically identified isolates yielded a 250 bp amplicon indicating the DNA of *Lactobacillus* genus (Fig. 1) [4].

Antibiotic sensitivity results. Antibiotic susceptibility results of 14 *Lactobacillus* isolates are shown in Table 2.

The highest resistance rates among the isolates were against penicillin, cefixime, cefazolin, amoxicillin-clavulanic acid (100%) followed by tetracycline (92%). All the *Lactobacillus* isolates had resistance to 4 antibiotics, and 57% showed resistance to all the antibiotics tested. The isolates showed the least resistance rate (64%) to vancomycin.

Occurrence of antibiotic resistance genes. Among 14 penicillin-resistant isolates, multiplex PCR analysis showed that 14 (100%) had a 532 bp band indicative of *mecA* gene and 1 (2.4%) had a 700 bp band indicative of *blaZ* gene (Fig. 2). All the 14 *Lactobacillus* isolates resistant to amoxicillin-clavulanic acid, cefazolin, and cefixime had 516 bp and 619 bp bands relating to bla_{TEM} and bla_{OXA-1} genes. One isolate (2.4%) showed a 392 bp band indicative of bla_{SHV} gene (Fig. 3). Among 13 tetracycline-resistant *lactobacilli*, all showed 360 bp and 158 bp bands relevant to *tetK* and *tetM* genes (Fig. 4). Thus, the results of the multiplex PCR analysis matched with the results of the phenotypic antibiotic resistance patterns.

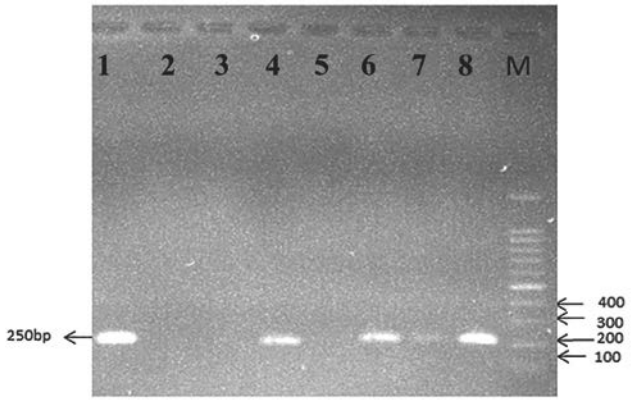

Fig. 1. PCR identification of *Lactobacillus* genus. M, 100 bp DNA marker; lane 1, positive control (*Lactobacillus casei* ATCC393); lanes 2 and 3, negative control (*Staphylococcus aureus* ATCC 12600); lanes 4 to 8, plaque samples

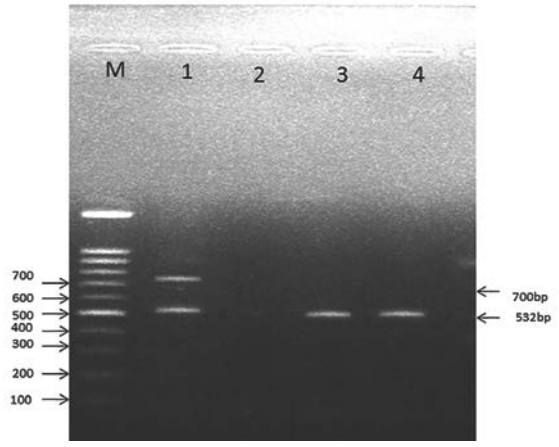

Fig. 2. Agarose gel electrophoresis of products from the first multiplex PCR assay. Lane M, 100 bp DNA marker; lane1, *mecA* (532 bp) and *blaZ* (700 bp), lane 3, *mecA* (532 bp); lane 4, positive control (*S. aureus mecA* isolate)

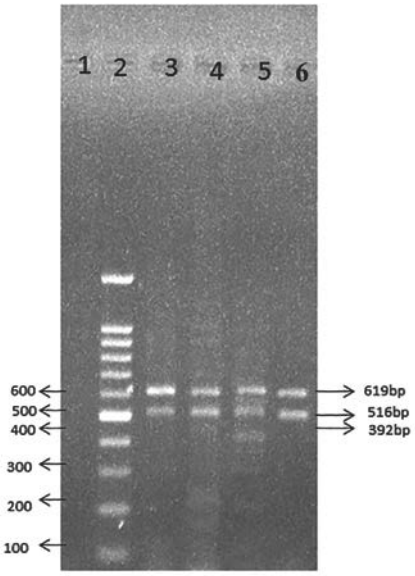

Fig. 3. Agarose gel electrophoresis of products obtained by the second multiplex PCR assay. Lane 1, negative control (*Escherichia coli* J53); lane 2, 100 bp DNA marker; lane 3, positive control (mixture of *E. coli* bla_{OXA-1} (ECO71) and *E. aerogenes* bla_{TEM} (EAE01) isolates); lanes 4 and 6, bla_{TEM} (516 bp) and bla_{OXA-1} (619 bp); lane 5, bla_{TEM} (516 bp), bla_{OXA-1} (619 bp) and bla_{SHV} (392 bp)

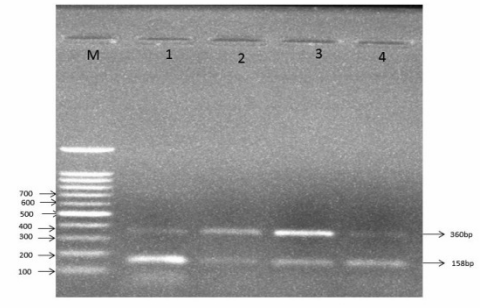

Fig. 4. Agarose gel electrophoresis of products obtained by the third multiplex PCR assay. Lane M, 100 bp DNA marker; lane 1, positive control (*S. aureus* ATCC 694.01); lanes 2 to 4, *tetK* (360 bp) and *tetM* (158 bp)

DISCUSSION

The bacteria belonging to *Lactobacillus* genus have long been known to correlate with dental decay [13]. Dental caries is caused by irreversible solubilization of tooth mineral by acids byproducts of certain bacteria like members of the genus *Lactobacillus* that adhere to the tooth surface and form dental plaques [10]. In this study, the incidence of *Lactobacillus* genera in dental plaque samples was studied. We identified *Lactobacillus* bacteria in 46% of the dental plaques based on microscopy, biochemical tests, and PCR. In a similar study, Maripandi *et al.* (2011) didn't report any *Lactobacillus* from dental caries which contradict with our findings.

Byuan *et al.* (2004) analyzed the diversity of *Lactobacillus* species present in progressive dental decays by real-time PCR and found 18 different isolates [14], among which 12 showed a relation with the known *Lactobacillus* species and only about half of them were similar to the species previously isolated from the human dental caries. The rates they reported are in agreement with our findings, but considerably higher than previous works in which culture-based methods were used [14].

In another study by Daniyan and Abalaka, on frequency and susceptibility profile of bacterial isolates of dental decays, only 6**.**1% of the isolates were *Lactobacillus*, but all the strains were resistant to all the antibiotics used in their trial. In our study, the percentage of *Lactobacillus* species isolated from dental caries was higher than their results, but all the *Lactobacillus* isolates showed multidrug-resistant patterns which are similar to their findings [15].

In a study, Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) identified 88.6% of the *lactobacilli* isolates, as confirmed by 16S rRNA sequencing, in colonies grown from the saliva of the individuals with caries on Rogosa agar media. Their findings confirm the significant role of *Lactobacillus* in the development of dental caries which is in agreement with our findings [16]. Also, Nouri *et al.* (2017) studied the prevalence of cariogenic bacteria in dog dental plaques and identified *Streptococcus mutans* and *Streptococcus sobrinus* in 8 (40%) and 2 (10%) of samples, respectively. Most of the isolates displayed multidrug resistance patterns to the antibiotics used in the study [17].

The conventional phenotypic analysis for determination of bacterial susceptibility to antimicrobials is now completed by molecular biology assays [4]. In the study of Nouri Gharajalar and colleagues (2017), all the 17 *lactobacilli* isolated from 30 dental plaque samples showed a multidrug resistance pattern [18]. In this study, we used both phenotypic and genotypic methods for detection of antibiotic resistance genes. All the *Lactobacillus* isolates had *mecA* gene, whereas only 2.4% possessed *blaZ* gene. These two genes are responsible for penicillin and β-Lactam antibiotic resistance. So, *mecA* was the essential gene responsible for penicillin resistance among the *lactobacilli* isolates. Analyzing of cephalosporin-resistant isolates showed the presence of bla_{TEM} and $bla_{OXA\text{-}1}$ in all of them, but only 2.4% had bla_{SHV} gene. The β-Lactamases such as the TEM and SHV types belong to the so-called extended-spectrum β-Lactamases (ESBLs), which can inactivate extended-spectrum Cephalosporins [19]. Here, the bla_{TEM} and $bla_{OXA\text{-}1}$ genes had higher activity against cefazolin and cefixime. Meanwhile, TEM and OXA-1 enzymes were the most important-Lactamases responsible for amoxicillin-clavulanic acid resistance. According to our results, both genes had the same role in amoxicillin-clavulanic acid resistance.

In conclusion, *Lactobacilli* have an essential role in dental plaques and showed multidrug-resistant patterns which could be screened rapidly using multiplex PCR method.

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ETHICAL APPROVAL

Here, we declare all ethical standards were respected in preparation of this article.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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