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Original article

MOLECULAR CHARACTERISATION OF MULTIDRUG RESISTANT *LACTOBACILLUS* ISOLATED FROM DENTAL PLAQUE OF DOGS USING A MULTIPLEX PCR ASSAY

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Summary

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Dental caries is a significant public health problem in both humans and animals worldwide. *Lactobacillus* species have been reported to be highly prevalent in both superficial and deep caries. The aim of this study was to analyse the antibiotic resistance patterns of lactobacilli isolated from dog dental plaque samples. Thirty plaque samples were collected from dog teeth. All *Lactobacillus* isolates were identified using phenotypic and genotypic methods. Then, their antibiotic susceptibility patterns and genetic determinants responsible for antibiotic resistance were determined. Total of 17 isolates were identified as belonging to the genus *Lactobacillus* by both methods. The results of antibiotic susceptibility test showed that all isolates (100%) were resistant to cefazolin and cefixime; 94% and 88% – resistant to penicillin and tetracycline; 64%, 58%, 52% and 41% of *Lactobacillus* isolates were resistant to amoxicillin-clavulanic acid, nitrofurantoin, vancomycin and chloramphenicol respectively. The results of resistance genes identification indicated that bla_{TEM} was the most important determinant responsible for cefazolin, cefixime and amoxicillin-clavulanic acid resistance. The *mecA* gene was responsible for penicillin resistance while both *tetK* and *tetM* genes were equally involved in tetracycline resistance. According to the widespread resistance patterns seen among *Lactobacillus* isolates in this study, we concluded that antibiotic therapy for oral microbial infections should be used only where extremely needed.

Key words: antibiotic resistance, dental plaque, dog, *Lactobacillus*

INTRODUCTION

Dental plaque or biofilm is an adherent deposit of microbial communities (predominantly bacteria) and their products on tooth surfaces (Al-Mudallal *et al.*, 2008). Bacterial plaques which accumulate on dental surfaces are amongst the first etiological agents of dental caries (Maripandi *et al.*, 2011). Dental decay is a microbiologic infection of the tooth which is due to the dissolution of tooth mineral

parts by acids derived from bacterial carbohydrate fermentation (Maripandi *et al.,* 2011). The combination of genetic susceptibility factors, the presence of cariogenic bacteria like *Streptococcus* and *Lactobacillus* spp. and a source of fermentable carbohydrate leads to dental caries (Niemiec, 2011). Many dog owners are unaware that their pets may suffer from dental caries. Although the incidence of caries in dogs is lower than in humans, it does occur and we must watch for its reasons. According to studies, 5.25% of adult canine patients had one or more caries lesion, usually bilaterally symmetrical. Also, pit and fissure caries are the most common types in dogs. The deep grooves on the buccal surface of the maxillary 4th premolars and on the lingual side of the mandibular 1st molars between the mesial and central cusps are among other sites at risk (Hale, 2009).

Lactobacilli usually isolated from dental caries, are amongst the pioneering microorganisms in dental decay progress (Karpinski *et al.*, 2013). As a result the salivary *Lactobacillus* count is usually used in the caries prediction tests (Badet & Thebaud, 2008). *Lactobacillus* bacteria are Gram-positive, usually non-motile, non-sporulating microorganisms that produce lactic acid as a major product of carbohydrates metabolism (Nair & Surendran, 2005). Use of antimicrobial agents to eradicate diagnosed caries bacteria like *Streptococcus mutans* and *Lactobacilli* could reduce decay (Loesche, 1996). Cultural and biochemical methods can be used for identifying *Lactobacillus* genus but they sometimes lead to ambiguous results. On the other hand molecular methods are more exact and reliable for detection process (Roman-Mendez *et al.*, 2009). Beta-lactam antibiotics including penicillins, cephalosporins and related compounds are active against many Gram-positive, Gram-negative and anaerobic bacteria. They are usually used for treatment of oral diseases (Keith *et al.*, 2000). Yet, incorrect antibiotic usage is one of the most important factors responsible for rise of bacterial resistance to commonly used antibiotics (Raum *et al*., 2007).

Today, molecular methods, especially PCR-based ones are preferentially used to determine antimicrobial resistance determinants (Leski *et al.*, 2013). Clinical resistance of organisms to β-lactam antibiotics is associated with reduced permeation of the drugs through the outer cell membrane, inactivation of the compounds by β-lactamases, and the inability of the compounds to bind to target penicillin-binding proteins that have been changed (Moosdeen, 1997). Cephalosporin resistance in bacteria is often mediated by TEM- and SHV-type beta-lactamases. TEM-type and OXA-1 enzymes have the major role in amoxicillin-clavulanic acid resistance (Colom *et al.*, 2003). Also, *blaZ* and *mecA* genes are specific for penicillin and oxacillin-like β-lactam antibiotic resistance (Kang *et al.*, 2014).

There are many studies on dental caries in humans, but this problem is poorly studied in dogs. So the purpose of this study was to detect *Lactobacillus* prevalence in canine dental plaques and to identify their antimicrobial resistance genes.

MATERIALS AND METHODS

Collection of samples

Thirty plaque samples were collected from 4–8 years old German shepherd dogs, referred to Dr Onsori pet clinic, Urmia, Iran (2015). All samples were placed in sterile tubes containing 2 mL normal saline and homogenised (Al-

Mudallal *et al.*, 2008). Then homogenised samples were cultured on MRS agar (Sigma, USA) and incubated in 5% CO_2 at 37 ^{o}C for 48–72 h (Nair *et al.*, 2005).

Phenotypic identification of the genus Lactobacillus

The Gram reaction characteristics and cell morphology of all the isolates were examined using standard staining method. After confirming the Gram reaction, each isolate was further identified by biochemical tests like catalase, motility and nitrate reduction.

Genotypic characterisation of Lactobacillus

For molecular identification of lactobacilli to the genus level, all *Lactobacillus* isolates were cultured on MRS broth (Sigma, USA). The overnight cultures were applied for DNA extraction using Fermentase DNA extraction kit (Fermentase, Germany). Then extracted DNA was used as a template for identifying *Lactobacillus*. The PCR reaction was performed in a 25 µL reaction mixture using DNA thermo-cycler (MWG AC BIOTECH THERMAL CYCLER, USA). A primer pair specific for 16s/23s ribosomal RNA intergenic spacer region of *Lactobacillus* was used in the PCR reaction. Primer sequences were as followed: F: 5′-CTC AAA ACT AAA CAA AGT TTC-3′ and R: 5′-CTT GTA CAC ACC GCC CGT CA-3′. 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 deionised water. Cycling conditions comprised an initial denaturation step for 5 min at 95^{o}C, amplification: 20 cycles of 30 s at 95 ^{o}C, 30 s at 55 ^{o}C and final 30 s at 72 ^{o}C. The final extension step was for 7 min at 72 ^{o}C. The reaction products were resolved on a 1% agarose gel. A 100 bp DNA ladder (Fermentase, Germany) was run on each gel as a size reference (Gad *et al.*, 2014).

Antimicrobial susceptibility testing

Antibiotic susceptibility studies were performed by the standard disc diffusion method using the following antibiotics: tetracycline (30 µg), chloramphenicol (30 µg), penicillin (10 units), vancomycin (30 µg), nitrofurantoin (300 µg), cefixime (5 µg), cefazolin (30 µg), amoxicillin-clavulanic acid (20/10 µg). The results were interpreted as described in National Committee for Clinical Laboratory Standards guidelines (Ozgumus *et al.*, 2007). Then the antibiotic resistance pattern of each isolate and the percentage of multiresistant bacteria were determined.

Molecular detection of antibiotic resistance genes

Genotypic analysis of antibiotic resistance was done through three multiplex PCR assays using universal primers (Table 1) following the procedures described below.

Isolates that were resistant to penicillin were subjected for PCR-base detection of *mecA* and *blaZ* genes. PCR amplification was carried out as follows: one cycle at 95 ^{o}C for 240 s, 30 cycles 95 ^{o}C for 60 s, 58 ^{o}C for 60 s and 72 ^{o}C for 60 s with a final extension period at 72 ^{o}C for 420 s. After amplification, the PCR products were analysed on 2% agarose gel by electrophoresis and stained with safe dye for visualization (Kang *et al.*, 2014).

Isolates that were resistant to cephalosporins and amoxicillin-clavulanate were subjected for PCR-base detection of bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes. The PCR reaction consisted of initial denaturation at 94 ^{o}C for 5 min, followed by 32 cycles at 94 ^{o}C for 30 s, 30 s of

Table 1. Primer sequences used for PCR identification of *mecA*, *blaZ*, *tetK*, *tetM*, *bla TEM, bla SHV* and *bla OXA-1* genes

Primer	Gene	Sequences
Forward	*mecA*	5′- AAA ATC GAT GGT AAA GGT TGG C- 3′
Reverse	*mecA*	5′- AGT TCT GCA GTA CCG GAT TTG C- 3′
Forward	*blaZ*	5′- TGA CCA CTT TTA TCA GCA ACC- 3′
Reverse	*blaZ*	5′- GCC ATT TCA ACA CCT TCT TTC- 3′
Forward	*bla* $_{TEM}$	5′- ATC AGC AAT AAA CCA GC- 3′
Reverse	*bla* $_{TEM}$	5′- CCC CGA AGA ACG TTT TC- 3′
Forward	*bla* $_{SHV}$	5′- AGG ATT GAC TGC CTT TTT G- 3′
Reverse	*bla* $_{SHV}$	5′- ATT TGC TGA TTT CGC TCG- 3′
Forward	*bla* $_{OXA-1}$	5′- ATA TCT CTA CTG TTG CAT CTC C- 3′
Reverse	*bla* $_{OXA-1}$	5′- AAA CCC TTC AAA CCA TCC- 3′
Forward	*tetK*	5′- GTA GCG ACA ATA GGT AAT AGT- 3′
Reverse	*tetK*	5′- GTA GTG ACA ATA AAC CTC CTA- 3′
Forward	*tetM*	5′- AGT GGA GCG ATT ACA GAA- 3′
Reverse	*tetM*	5′- CAT ATG TCC TGG CGT GTC TA- 3′

annealing at 54 °C, 1 min of extension at 72 °C with final extension step at 72 °C for 10 min. Amplified samples were submitted to 2% agarose gel electrophoresis and stained by safe dye (Colom *et al.*, 2003).

Finally the isolates that were resistant to tetracycline were subjected for PCR-base detection of *tetK* and *tetM* genes which were responsible for tetracycline resistance. The PCR program was as followed: 3 min of initial denaturation, 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s (annealing) and 72 °C for 30 s. The 4-min final extension was carried out at 72 °C. The PCR products were analysed by electrophoresis on 1.5% agarose gel (Strommenger *et al.*, 2003).

RESULTS

Isolation and identification of Lactobacillus bacteria

A total of 17 *Lactobacillus* (56.6%) isolates were recovered from the 30 dog dental plaques samples. Using Gram staining, all 17 isolates were purple coloured Gram positive rods under light microscopy. They were non-motile, catalase negative bacteria with no ability to reduce nitrate.

The molecular identification of *Lactobacillus* to the genus level was done using PCR assay. When a DNA from the *Lactobacillus* isolates was used as a template, a 250 bp band was obtained on agarose gel under UV light (Fig. 1). By combination of both biochemical and molecular identification, it was found that bacteria belonging to *Lactobacillus* genus wererecovered from 56.6% of dog dental plaque samples.

Antibiotic susceptibility results

From 17 *Lactobacillus* isolates, 16 (94%) were multiresistant, all of them resistant to cefazolin and cefixime, 16 (94%) were resistant to penicillin, 15 (88%) were tetracycline-resistant, 11 (64%) of the *Lactobacillus* were amoxicillin-clavulanate resistant. Ten (58%), 9 (52%) and 7 (41%) isolates were resistant to nitrofurantoin, vancomycin and chloramphenicol, respectively.

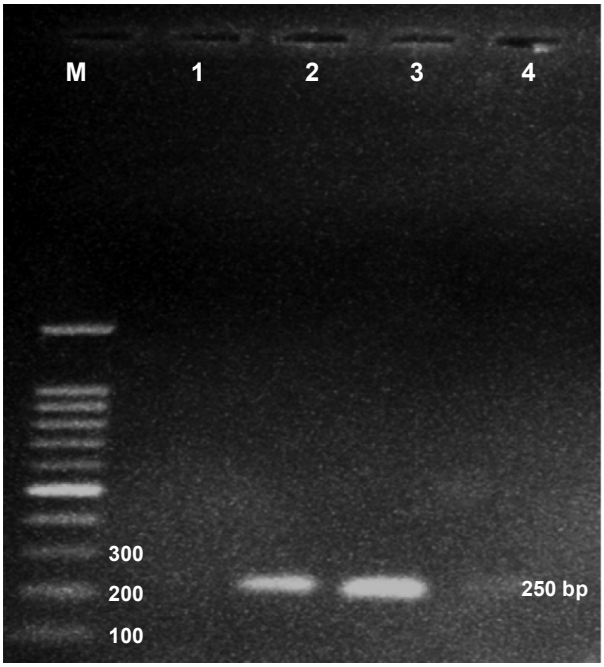

Fig. 1. 16s/23s rRNA intergenic spacer region gene found at 250 bp on 1% agarose gel after PCR amplification. Lane M – 100 bp ladder marker; lane 1 – negative control; lane 2 – positive control; lane 3 – *Lactobacillus* 16s/23s rRNA intergenic spacer region gene found at 250 bp.

Resistance determinant identification

All 16 penicillin-resistant lactobacilli, had the 532 bp band indicative of *mecA* gene. None of the isolates exhibited the *blaZ* gene. Therefore, the *mecA* determinant was the major gene responsible for penicillin resistance (Fig. 2).

Among the cefazolin and cefixime resistant lactobacilli, all generated fragments of 516 bp, 11 (64%) generated 619 bp and 6 (35%) had 392 bp bands on agarose gel electrophoresis, which were indicative of bla_{TEM} (516 bp), bla_{OXA-1} (619 bp) and bla_{SHV} (392 bp). So the bla_{TEM} gene was the most important determinant responsible for cefazolin and cefixime resistance (Fig. 3). Also, 58% of *Lactobacillus* isolates were resistant to amoxicillin-clavulanate and bla_{TEM} was probably the first important gene about this resistance pattern, followed by bla_{SHV} gene (Fig. 3).

All 15 tetracycline-resistant lactobacilli had 360 and 158 bp bands indicative of *tetK* and *tetM* genes confirming that both genes had the same role in tetracycline resistance (Fig. 4).

DISCUSSION

Dental plaque or biofilm which develops on oral tissues is a complex organisation which remains stable with time despite regular environmental changes. Whenever the balance among indigenous bacteria is compromised, dental caries could appear (Badet & Thebaud, 2008). *Lactobacillus*

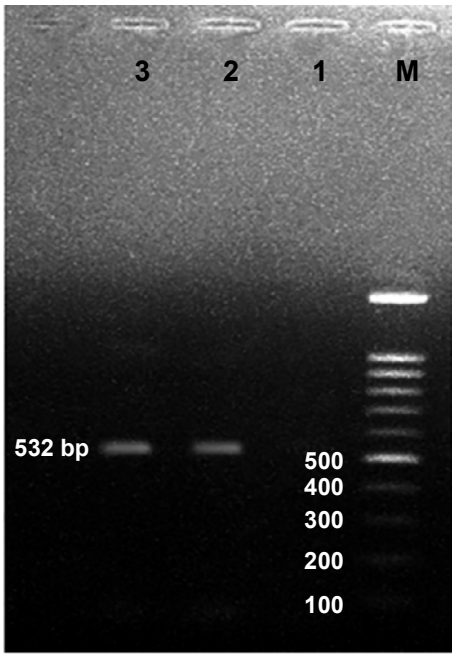

Fig. 2. Agarose gel of PCR product from *Lactobacillus* isolates, using primer group for *mecA* and *blaZ*. Lane M – 100 bp ladder; lane 1 – negative control; lane 2 – positive control; lane 3 – *mecA* gene found at 532 bp.

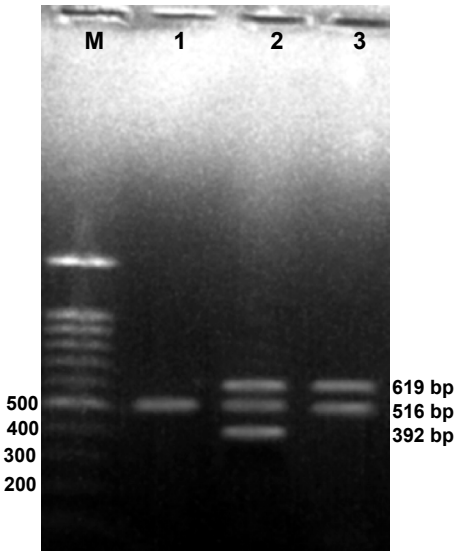

Fig. 3. Gel electrophoresis of the PCR products of bla_{TEM}, bla_{SHV} and bla_{OXA-1} resistance determinants. Lane M – 100 bp ladder; lane 1 – bla_{TEM} (516 bp); lane 2 – bla_{TEM} (516 bp), bla_{OXA-1} (619 bp) and bla_{SHV} (392 bp); lane 3 – bla_{TEM} (516 bp) and bla_{OXA-1} (619 bp).

species have been consistently associated with dental decay and are one of the most important secondary pathogens in dental carries (Daniyan & Abalaka, 2011). The ecology of *Lactobacilli* in the oral cavity was studied (Badet & Thebaud, 2008). According to their literature, lactobacilli are the first microorganisms implicated in dental caries development. Some authors have also noticed an increase in the percentage of *Lactobacillus* before the onset of carious lesions. Our study results also indicated that lactobacilli had important role in dental plaque which could lead to dental caries.

Identification of *Lactobacillus* bacteria according to phenotypic methods like culture and biochemical tests is difficult because sometimes needs determination of bacterial characteristics beyond those of popular tests (Dickson *et al.*, 2005). Molecular methods are generally more reliable in *Lactobacillus* identification process. A novel species-specific PCR assay was used for identifying *Lactobacillus fermentum* in human supragingival plaque (Dickson *et al.*, 2005). They concluded that PCR assay provides more rapid and sensitive alternative to culture methods in *Lactobacillus* identification. In this study we also used both phenotypic and genotypic methods for *Lactobacillus* detection. All phenotypically detected isolates were also further confirmed using PCR based assay.

Selective pressure of antibiotics usage in both human and veterinary treatments and also spreading of antibiotic resistant microorganisms has aggravated acquisi-

tion and dissemination of resistant genes. The prevalence and susceptibility patterns of bacterial isolates from human dental caries was studied (Daniyan *et al.*, 2011). All *Lactobacillus* strains in their study were resistant to chloramphenicol, nitrofurantoin and tetracycline but only 41% of our *Lactobacillus* isolates were resistant to chloramphenicol, 58% were resistant to nitrofurantoin and 88% were tetracycline resistant. On the other hand the most frequent resistance pattern was against cefazolin and cefixime antibiotics (100%).

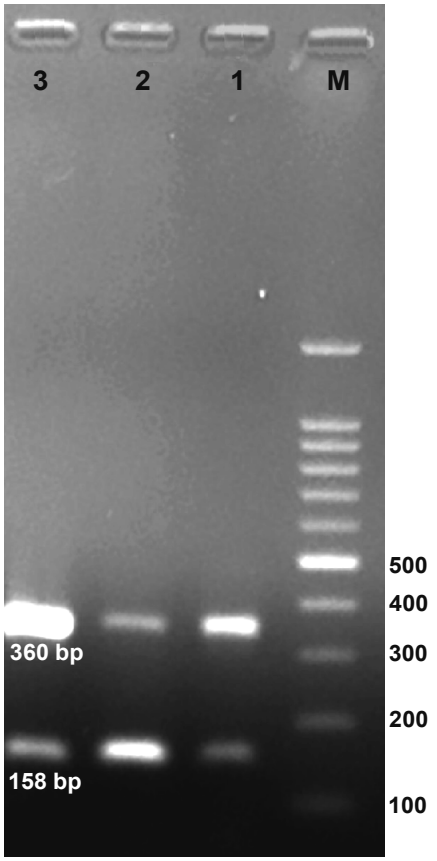

Fig 4. PCR detection of *tetK* and *tetM* markers in *Lactobacillus* isolates. Lane M – 100 bp ladder marker; lane 1 –positive control; lanes 2,3 – *tetK* (360 bp) and *tetM* (158 bp).

Studies on targeted isolation of bacterial species associated with canine periodontal health or disease from dental plaque were performed by Davis *et al.* (2014). They used quantitative polymerase chain reaction approach for bacterial screening process and concluded that their approach could be applied to any uncultured bacterial species where knowledge about their environmental requirements is low.

Fayaz *et al.* (2014) also determined prevalence and antibiotic susceptibility patterns of dental biofilm forming bacteria in humans. All *Lactobacillus* species isolated in their study were resistant to chloramphenicol, tetracycline, and gentamicin. In our research, however, the commonest resistance pattern was against cephalosporins followed by penicillin. Resistance to tetracycline was on the third place and that to chloramphenicol was the least common pattern identified among our *Lactobacillus* isolates.

Tetracycline-resistant bacteria constituted an average of 11% of all cultivable oral microflora (Villedieu *et al.*, 2003). The most common identified *tet* gene was *tetM* but the frequency of *tetK* gene was low. In this study, the prevalence of both *tetK* and *tetM* genes among the isolates was very high.

Binta & Patel (2016) screened β-lactamase producing oral anaerobic bacteria and the presence of *cfxA* and bla_{TEM} genes that are responsible for resistance to β-lactam antibiotics. Fifty one percent of the isolates carried *cfxA* while none carried bla_{TEM} gene. In this study bla_{TEM} was the most important determinant responsible for resistance to β-lactam antibiotics. Also, Koukos *et al.* (2016) studied the prevalence of bla_{TEM} and *nim* resistance genes in isolates from the oral cavity of Greek subjects and established that bla_{TEM}

gene was found in 36% of the isolates but the *nim* gene was not detected in any of the samples. According to our results 64% of isolates carried the bla_{TEM} gene. Antibiotic resistance genes in *Staphylococcus aureus* isolated from the oral cavity of Tunisian children (Zmantar *et al.*, 2012) demonstrated that the frequency of *blaZ* gene among strains was 100%. On the contrary, in our study, none of the isolates had the *blaZ* gene

According to the results of this study, we concluded that antibiotics should be used when extremely needed for control and treatment of infections. They inappropriate use results in persistence and dissemination of multidrug resistant bacteria in human and animal hosts.

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