Original article

MOLECULAR DETECTION OF ANTIBIOTIC RESISTANCE GENES IN MULTIDRUG-RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES FROM DOG DENTAL PLAQUE

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Summary

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Multidrug resistant *Staphylococcus aureus* strains are a major health care problem both in humans and animals. In this work we described three multiplex PCR assays for detection of clinically relevant antibiotic resistance genes in *S. aureus* isolated from dog dental plaques. Thirty dental plaque samples were collected; then cultural, biochemical and molecular tests performed for isolation and identification of *S. aureus* from samples. The antibiotic susceptibility of the isolates were checked by Kirby Bauer disc diffusion method and the prevalence of antibiotic resistance genes determined using multiplex PCR assay. As a result *S. aureus* was isolated from 18 dog plaque samples. Fifteen of these isolates were resistant to penicillin. The *mecA* gene was more prevalent than *blaZ* among penicillin-resistant bacteria. Ten of the isolates were resistant to tetracycline. The percentage of *tetM* was higher than *tetK* among them. Also, 10 of the isolates were resistant to cefazolin among them bla_{TEM} detected in higher rate than bla_{SHV} and bla_{OXA-1}. Hence multiplex PCR assay is a suitable method for detection of antibiotic resistance patterns of *S. aureus* isolates.

Key words: antibiotic resistance, *Staphylococcus aureus*, dental plaque, dog

INTRODUCTION

Dental plaque is the biofilm on the teeth with different types of microbes. It is an important agent of dental caries (Altayyar *et al.*, 2015). Oral disorders are prevalent in dogs, causing pain and usually leading to tooth loss (Elliott *et al.*, 2005). Dental caries results from bacterial decay of tooth structures. Oral bacteria which accumulate in dental plaque, ferment carbohydrates, and as a result, release acids on the tooth surface (Hale, 2009).

Periodontal disease is an important disorder of the oral cavity in dogs. Soft diet which is very popular among dog owners and the lack of hygiene causes accumulation of dental plaque colonised with bacteria (Kyllar & Witter, 2005).

Periodontal disease occurs in two steps. Gingivitis is the first stage in which gum inflammation is induced by dental plaque bacteria. Periodontitis (inflammatory disease of the periodontal ligament and alveolar bone) is the second stage caused by mixed-species bacterial biofilm. These disorders are not only detrimental to the oral cavity but also affect the general health of the animal (Zambori *et al.*, 2012).

From human dental plaques, Gram positive bacteria are isolated more frequently than Gram negative ones (Rozkiewicz *et al.*, 2006). The bacterial composition of canine plaque was shown to be widely different from that of humans with only 16.4% of taxa shared. So, alternative mechanisms may play an important role during canine oral biofilm development. For example in humans, bacteria of the genus *Streptococcus* are necessary for initiate biofilm formation and the transition from health to disease is detected by a change from predominance of Gram positive species to mainly Gram negative ones. This situation is however reversed in canine plaque (with health associated species mainly belonging to the Gram negative group). On the other hand streptococcal species don't play an important role in canine plaque biofilms initiation and their prevalence is low. In one study, the most prevalent species among canine oral microbiome were *Bergeyella zoohelcum, Neisseria shayeganii* and *Moraxella* spp. (Holcombe *et al.*, 2014).

S. aureus is an opportunistic pathogen colonising the teeth of patients, in the form of biofilm (Ansari *et al.*, 2011). Antibiotic usage for treatment of periodontal disease or other oral infections may result in the increase of the number of staphylococci in the oral cavity. These bacteria easily become resistant to antibacterial drugs and this may lead to super infection (Loberto *et al.*, 2004). Multidrug-resistant *S. aureu*s constitute an important health care problem, so using suitable and specific tools the exact detection of antimicrobial resistance in these bacteria has become a significant concern in clinical diagnosis (Perez-Roth *et al.*, 2001).

There are several phenotypic methods for detection of microorganisms' antimicrobial susceptibility patterns, which however are not discriminating enough. So, it is essential to use more rapid and accurate methods like PCR for identification of staphylococci and their resistance patterns (Pournajaf *et al.*, 2014).

In this study, a Multiplex PCR assay was used to find out the prevalence of antibiotic resistance genes among different *S. aureus* strains isolated from dog dental plaque.

MATERIALS AND METHODS

Sample collection

Thirty dental plaque samples were collected from dogs undergoing routine dental treatment at Dr Onsori Pet Clinic, Urmia, Iran in 2016. A small scraping from the dental plaque was taken using a sterile curette. Each sample was immediately placed in phosphate-buffered saline and processed within 2–3 h from collection (Elliott *et al.*, 2005).

Isolation and identification of Staphylococcus spp.

After homogenising the samples, they were inoculated on blood agar and manitol salt agar (Sigma, USA) for *Staphylococcus* isolation. The plates were incubated at 37 °C for 24 h (Devapria *et al.*,

2013). Gram staining, catalase and nitrate tests were also used as biochemical examinations to confirm *Staphylococcus* genus (Zambori *et al.*, 2012).

Molecular identification of S. aureus

Total genomic DNA from all isolates suspected as *Staphylococcus* was extracted from overnight cultures at 37 °C in nutrient broth using DNA extraction kit (Fermentase, Germany). DNA concentrations were determined using spectrophotometer and the samples were stored at –20 °C until used for PCR assay. The standard single PCR assay using a DNA thermo cycler (MWG AC BIOTECH THERMALCYCLER, USA), targeting *S. aureus* specific sequence was developed. Primers used in this study were: sau1: 5$^{/}$ AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG 3$^{/}$ and sau2: 5$^{/}$ CGT AAT GAG ATT TCA GTA GTA AAT ACA ACA3$^{/}$. PCR amplification was performed in a 25 µL reaction mixture consisted of 10 µL Red Amp master mix 2×, 3 µL of template DNA, 10 µL of deionized water and 1 µL of each primer. Reaction conditions were optimized to be 94 °C for 30 min as initial denaturation, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension step at 72 °C for 4 min. Amplification products were electrophoresed in 1.5% agarose gel containing 1×TBE at 75 V for 1.5 h and visualised under UV light. Amplification of 107 bp band indicated *S. aureus* (Strommenger *et al.*, 2003).

Antibiotic susceptibility test

Antimicrobial susceptibility testing was performed for bacterial isolates using Kirby-Bauer disk diffusion method on Mueller Hinton agar plates. Test cultures were swabbed on the top of the media and allowed to dry for 10 min. The antimicrobial agents tested were: tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), penicillin (10 units), vancomycin (30 µg), azithromycin (10 µg) and cefazolin (30 µg). The zone of inhibition diameters (mm) around the disks were measured and interpreted by referring to the performance standard for antimicrobial susceptibility testing, as described by the Clinical and Laboratory Standards Institute (CLSI) guidelines (2015).

Multiplex PCR for the detection of antibiotic resistance genes

Three multiplex PCR assays were performed to identify antibiotic resistance determinants. In the first assay for beta lactamase (penicillin) resistance genes *mecA* and *blaZ* were targeted. The *mecA* and *blaZ* specific primer pairs for amplification of 532 and 700 bp fragments were: *mecA* : F5′ AAA ATC GAT GGT AAA GGT TGG C3′ and R5′ AGT TCT GCA GTA CCG GAT TTG C3′, *blaZ*: F5′ TGA CCA CTT TTA TCA GCA ACC3′ and R5′ GCC ATT TCA ACA CCT TCT TTC3′. Thermal profile initiated with 95 °C for 240 s (initial denaturation), followed by 30 cycles of 95 °C for 60 s (denaturation), 58 °C for 60 s (annealing) and 72 °C for 60 s (extension). The final extension step was at 72 °C for 420 s. The PCR products were electrophoresed on 2% agarose gel for 1.5 h at 75 V and visualised under UV light (Kang *et al.*, 2014).

For tetracyclines (tetracycline), *tetM* and *tetK* genes fragments were targeted. The following primers were used: *tetK*: F5′ GTA GCG ACA ATA GGT AAT AGT 3′ and R5′ GTA GTG ACA ATA AAC CTC CTA3′, *tetM:* F 5′ AGT GGA GCG ATT ACA GAA3′ and R 5′ CAT

ATG TCC TGG CGT GTC TA3´. Initial denaturation which occurred at 94 °C for 3 min, was followed by 30 cycles of amplification using the following parameters: 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 4 min. The PCR products were analysed by electrophoresis on a 1.5% agarose gel. Detection of the 158 bp fragment of the *S. aureus tetM* and a 360 bp amplicon of tetk gene, was taken as indicative of the presence of the *tetM* and *tetK* genes, respectively (Strommenger *et al.*, 2003).

Also *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA-1}$ genes fragments were targeted for cephalosporins (cefazolin). The *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA-1}$ specific primer pairs used for amplification of 516, 392 and 619 bp fragments were as followed: *bla*$_{TEM}$: F5´ATC AGC AAT AAA CCA GC3´ - and R5´ CCC CGA AGA ACG TTT TC3´; *bla*$_{SHV}$: F5´ AGG ATT GAC TGC CTT TTTG 3´ and R5´ CGC TCG 3´; *bla*$_{OXA-1}$: F5´ ATA TCT CTA CTG TTG CAT CTC C3´ and R5´ AAA CCC TTC AAA CCA TCC3´.

The thermal cycling protocol for PCR comprised 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 1 min. Final step was at 72 °C for 10 min. The PCR products were analysed by electrophoresis on 2% agarose gel (Colom *et al.*, 2003).

RESULTS

Out of 30 collected plaque samples, 18 were suspected as staphylococci based on colony morphology and Gram staining (Gram positive cocci). All these isolates were catalase and nitrate positive. They had β-haemolytic colonies on blood agar plates and yellow coloured colonies on mannitol salt agar.

The PCR confirmed all 18 isolates as *S. aureus* as an amplified product of 107 bp band was observed in each case without amplification in negative control (Fig. 1).
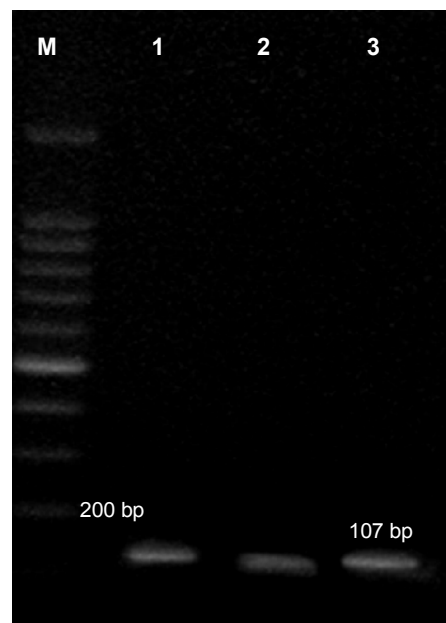

Fig. 1. Gel electrophoresis of the PCR products of *S. aureus* specific sequence. Lane M: 100 bp ladder marker; lane 1: positive control; lanes 2, 3: *S. aureus* specific sequence at 107 bp.

The results of antimicrobial sensitivity testing of the 18 *S. aureus* isolates using the disc diffusion method showed that 12 isolates had phenotypic resistance to at least two antimicrobial drugs (Table 1). With this method 15 of the isolates showed resistance to penicillin, 10 were resistant to tetracycline and 10 were resistant to cefazolin. Eight strains were resistant to vancomycin, 8 to erythromycin, 7 to azithromycin and chloramphenicol; only 6 of the isolates were resistant to gentamicin.

Table 1. Number of multiple resistance patterns in *S. aureus* isolates

Pattern No.	Antimicrobial resistance against:	Number of resistant *S. aureus* isolates
1	cefazolin, gentamicin, vancomycin, azithromycin, tetracycline, chloramphenicol, erythromycin, penicillin	4
2	cefazolin, vancomycin, azithromycin, tetracycline, chloramphenicol, erythromycin, penicillin	1
3	cefazolin, tetracycline, erythromycin, penicillin,	2
4	cefazolin, erythromycin, penicillin	1
5	chloramphenicol, azithromycin, tetracycline, chloramphenicol, E, penicillin	1
6	cefazolin, tetracycline, penicillin	1
7	cefazolin, tetracycline, chloramphenicol, erythromycin, penicillin	1
8	penicillin, azithromycin	1

The PCR based detection of different antimicrobial resistance determinants showed that 7 of penicillin-resistant isolates had the *blaZ* and 18 had the *mecA* gene (Fig. 2, 3).
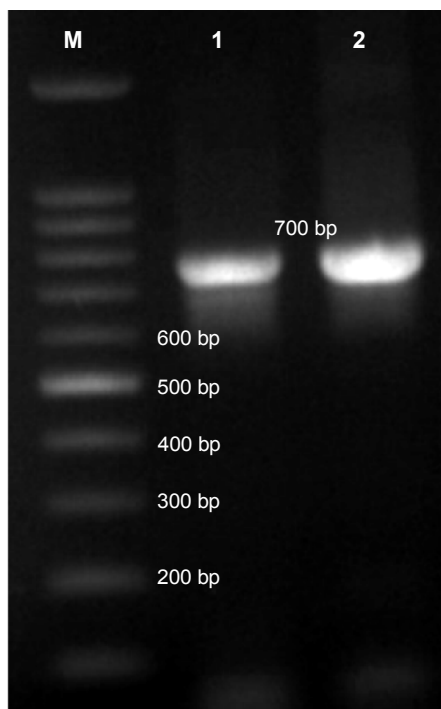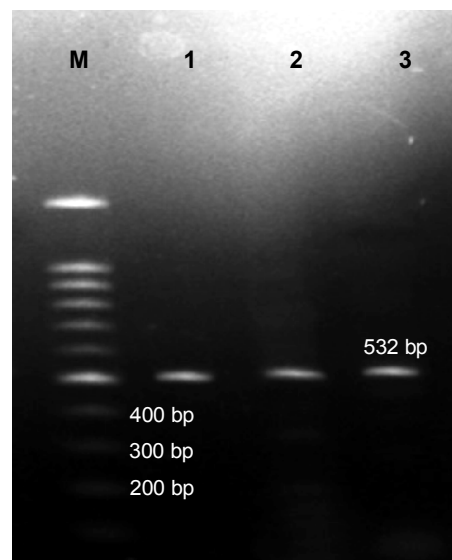

Fig. 2. PCR identification of *blaZ* with 700 bp on 2% agarose gel. Lane M: 100 bp ladder marker; lane 1: positive control; lane 2: *blaZ* gene at 700 bp.

Fig. 3. *mecA* genes at 532 bp on agarose gel after PCR amplification. Lane M: 100 bp ladder marker; lane 1: negative control; lane 2: positive control; lanes 3: *mecA* gene at 532 bp.
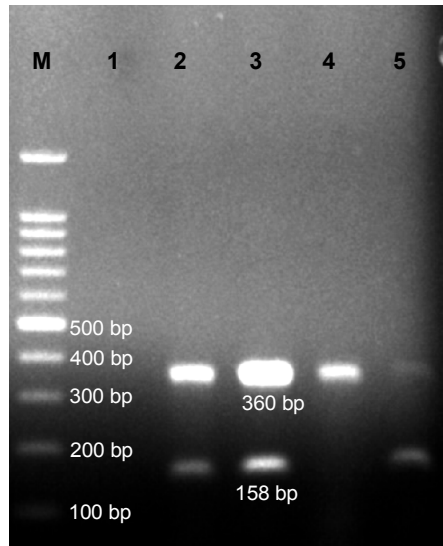
Fig. 4. Gel electrophoresis for PCR product of *tetK* and *tetM* markers in *S. aureus* isolates. Lane M: 100 bp ladder marker; lane 1: negative control; lane 2: positive control; lanes 3,5: *tetK* gene at 360 bp and *tetM* gene at 158 bp; lane 4: *tetK* gene at 360 bp.
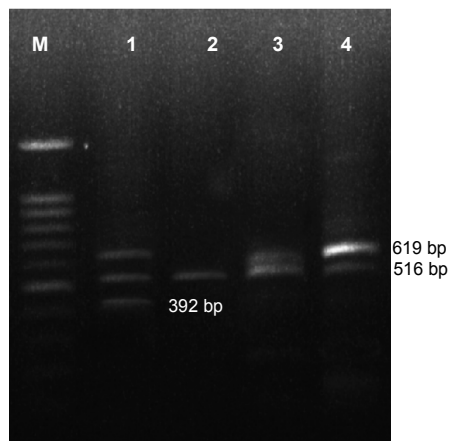

Fig. 5. bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes at 392, 516 and 619 bp on agarose gel after PCR amplification. Lane M: 100 bp ladder marker; lane 1: 2 bla_{TEM} and bla_{OXA-1} genes at 516 and 619 bp; lane 3: bla_{TEM} gene at 516 bp; lane 4: bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes at 516, 392 and 619 bp.

DISCUSSION

Biofilm is a microbial community in which cells are attached to a substratum or to each other and are embedded in a matrix of extracellular polymeric materials (Zambori *et al.*, 2012). Dental biofilm (plaque) is an important factor in development of dental caries (Altayyar *et al.*, 2015). In the oral cavity teeth have enough humidity and adherent surfaces for formation of dental plaque. Aerobic bacteria like *Staphylococci* are usually isolated from the oral cavity (Zambori *et al.*, 2012).

Zambori *et al.* (2015) tested the biofilm formation capacity of planktonic bacterial strains. They used microbiological examination and concluded that from all 75 isolated bacterial strains 10 were from the *Staphylococcus* genus (30.3%). In our study the percentage of *Staphylococcus* genus in dog dental plaque samples was higher (60%).

Altayyar *et al.* (2015) isolated and identified aerobic bacteria from human dental plaque biofilms using conventional methods, and revealed that 21% of the isolates were *Staphylococcus*. In this study we isolated 18 *Staphylococcus* strains from 30 clinical samples which indicates the more important role of *S. aureus* in canine dental plaque formation than in human one.

The prevalence of *Streptococcus mutans* and *Streptococcus sobrinus* in canine dental plaque was previously studied (Nouri Gharajalar & Hassanzade, 2017). These bacteria were isolated from 40% and 10% of tested samples, respectively. Also most of them showed multidrug resistance patterns toward eight tested antibiotics. In comparison with this study, it seems that *Staphylococcus* genus was more frequent than *Str. mutans* in canine dental biofilms. Both were resis-

tant to the most tested antibiotics. The molecular characteristics of multidrug resistant *Lactobacillus* isolated from canine dental biofilms were investigated in a previous study of ours. According to the results, the prevalence of these bacteria was 56.6% which was almost equal to the frequency of *Staphylococcus* in this study. Additionally, the presence of multidrug resistance patterns among tested *Lactobacillus* was in line with the present study on *Staphylococcus* (Nouri Gharajalar, 2017).

To improve the diagnosis of *S. aureus* and its potential antibiotic resistance in dog dental plaques, a single PCR assay was developed. All phenotypically identified isolates were also confirmed by PCR method. Also, three multiplex PCR assays were used for detection of penicillin, cefazolin and tetracycline resistance genes.

The relation between the phenotypic antibiotic susceptibility patterns and the antibiotic resistance genes among methicillin resistant *S. aureus* isolates was evaluated (Adwan *et al.*, 2014). It was concluded that multiplex PCR can be used for confirmation of the results obtained by the disc diffusion test, in accordance with our findings.

Trzcinski *et al.* (2000) studied the expression of resistance to tetracyclines in methicillin resistant *S. aureus* strains. They reported that the *tetK* genotype was detected in Polish isolates heterogeneously resistant to methicillin but the *tetM* genotype was observed in isolates homogeneously resistant to methicillin. In the present experiment, we also identified the prevalence of *tetK* and *tetM* genes in tetracycline-resistant *S. aureus* isolates and concluded that the *tetM* gene was more prevalent than *tetK* among our isolates.

A PCR assay for detection of nine antibiotic resistance genes of *S. aureus* was described. Among 30 *Staphylococcus* isolates, 10 were resistant to oxytetracycline. They carried either *tetK* or *tetM* resistance genes (Strommenger *et al.*, 2003).

PCR based identification of methicillin resistant *S. aureus* strains and their antibiotic resistance patterns were evaluated by Pournajaf *et al.* (2014). Among antibiotics used in their study, penicillin showed the least activity against *S. aureus* and vancomycin was the most effective. In the PCR assay, 45% of the isolates had the *mecA* gene. Also, Asfour *et al.* (2011) detected both *mecA* and *blaZ* genes in *Staphylococcus* strains isolated from bovine mastitis. They reported that 80% of the beta-lactam resistant staphylococci had the *mecA* gene and 68% had a *blaZ* determinant. In our study *mecA* gene was also more common than *blaZ* among *S. aureus* isolates.

For determining cefazolin resistance genes, three bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes were targeted by PCR assay. As a result the most prevalent genotype was for bla_{TEM} gene followed by bla_{OXA-1} and bla_{SHV} genes. In the study of Colom *et al.* (2003), a simple and reliable PCR assay was used for detection of bla_{TEM}, bla_{SHV} and bla_{OXA-1} genes. Their results was similar to ours.

In conclusion, this study demonstrated that *S. aureus* strains are amongst the most prevalent bacteria isolated from dog dental plaques. It was also observed that 66.6% of these bacteria had multidrug resistant patterns to tested antibiotics especially to cefazolin, tetracycline, and penicillin which were mainly caused by *bla TEM*, *tetM*, and *mecA* genes.

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