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RESEARCH ARTICLE

Streptococcus minor; Can There Be A Potential Pathogenic Bacterial Agent In Dog Bites?

Streptococcus minor in dog bites

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ABSTRACT

Dogs and humans are in constant interaction which can be in the form of close friendship, or sometimes an attack by dogs on people. Dog bite cases are common in the world and *Streptococcus* species are often isolated from these cases and most frequently isolated species is *Streptococcus canis*. *Streptococcus minor* which was described in 2004 has been isolated in dog bite cases. This research was aimed to reveal the presence of *S. minor* in canine oral flora. In this study, 19 Gram-positive cocci were isolated from 50 dog oral swab samples. Of 19 isolates, 17 isolates were catalase-negative and were typed genotypically by PCR and sequencing. Eight isolates were identified as *S. minor*. *S. minor* isolates were found to be resistant to tetracycline at a rate of 75% and susceptible to other antibiotics at various rates. Trimethoprim resistance gene was detected in one *S. minor* isolate and tetracycline resistance gene was found in one *S. minor* isolate. The results of this research, it has been shown that *S. minor* can be isolated from dogs oral flora and it can appear as a potential bacterial pathogen in dog bite cases.

Keywords: Antibacterial Drug Resistance, Dogs, Molecular Sequencing Data, *Streptococcus.*

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Streptococcus Minor; Köpek Isırıklarında Potansiyel Patojenik Bakteriyel Etken Olabilir Mi?

Köpek ısırıklarında *Streptococcus minor*

ÖZ

Köpekler ve insanlar, yakın arkadaşlık veya bazen köpeklerin insanlara saldırması şeklinde olabilen sürekli bir etkileşim halindedir. Köpek ısırık vakaları dünyada sık görülmektedir. Bu vakalardan sıklıkla *Streptococcus* türleri izole edilir ve en sık izole edilen tür *Streptococcus canis*'tir. 2004 yılında tanımlanan *Streptococcus minor* köpek ısırması vakalarında izole edilmiştir. Bu araştırmada köpek ağız florasında *S. minor* varlığının ortaya konulması amaçlanmıştır. Bu çalışmada, 50 köpek oral svap örneğinden 19 Gram pozitif kok izole edilmiştir. Ondokuz izolattan 17'si katalaz negatif olduğu belirlenmiş ve PCR ve dizileme ile genotipik olarak tiplendirilmiştir. Sekiz izolat *S. minor* olarak tanımlandı. *S. minor* izolatlarının tetrasikline %75 oranında dirençli ve diğer antibiyotiklere çeşitli oranlarda duyarlı olduğu bulunmuştur. Bir *S. minor* izolatında trimetoprim direnç geni, bir *S. minor* izolatında ise tetrasiklin direnç geni saptanmıştır. Bu araştırma sonucunda *S. minor*'un köpeklerin ağız florasından izole edilebileceği ve köpek ısırık vakalarında potansiyel bir bakteriyel patojen olarak ortaya çıkabileceği gösterilmiştir.

Anahtar Kelimeler: Antibakteriyel İlaç Direnci, Köpekler, Moleküler Dizi Verileri, *Streptococcus.*

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INTRODUCTION

Living with animals enrich the lives of humans and including pets in daily routines ensure social interaction, exercise, emotional supports and social connectedness. Dogs are the closest friends of people, they live in the same home environment with people and they feed on foods of animal origin (Bata et al. 2020). Researches have focused on the subject of microbiota and especially focused on the microbiota of the gastrointestinal tract. Studies have shown that the gastrointestinal microbiota is closely related to the oral cavity microbiota (Zarco et al. 2012). The normal oral flora of dogs contains a large number of microorganisms which includes *Porphyromonas, Fusobacterium, Streptococcus, Capnocytophaga* genera and members of the *Pasteurellaceae* and *Neisseriaceae* families (Sturgeon et al. 2013, Oh et al. 2015, Isaiah et al. 2017, Bell et al. 2020, Ruparell et al. 2020). Some of these microorganisms can form a basic health barrier together with the immune system (Marsh 1994), but some of them may be pathogenic, cause periodontitis, dental caries and systemic disease. Dogs' age, food consumption, health status, and environmental factors influence oral microbiome composition. When dogs' health deteriorates, pathogenic oral bacteria can cause systemic infections (Fowler et al. 2001). However, pathogenic bacteria can show zoonotic properties as a result of the contact of dogs with impaired health and sometimes even biting people (Chen et al. 2010). Dog bite cases seen in humans are one of the important health problems in the world. It starts with common wound infections associated with dog bite and can develop into local and systemic infections if left untreated (Tabaka et al. 2015, Goldstein et al. 2018). It is known that 3-18% wounds of dog bites are infected with the dog's oral flora (Tabaka et al. 2015, Damborg et al. 2016) and wound infections are generally an infection involving anaerobic and aerobic bacteria. *Streptococcal* species are commonly involved in canine bite wounds and infections. *Streptococcus canis* and S*treptococcus pyogenes* are the most common pathogens in dog bite cases. However, *Streptococcus minor* species, which was identified by molecular methods in 2004, has also started to be reported in dog bite cases. Infections caused by *S. minor* can be overlooked due to the facultative anaerobic nature of the organism and the difficulty of identifying α-hemolytic streptococci at the species level with current laboratory techniques and *S. minor* does not react with Lancefield groups A, C, D, F or G antisera. *S. minor* has the potential to be the primary pathogen in dog bites (Vancanneyt et al. 2004, Tre-Hardy et al. 2016).

In this study, it was aimed to reveal the presence of *S. minor* species, which has recently gained importance in dog bite cases, in canine oral flora and its antibiotic susceptibility.

MATERIAL AND METHODS

Sample Collection

Samples were taken with cotton swabs from oral cavities of randomly selected 50 dogs. The oral swab samples were transported at +4°C to the microbiology laboratory.

Phenotypic Identification

Each swab sample was plated on 5-7% Columbia Blood Agar. Plates were incubated for 24-48 h at 37°C microaerophilic condition. After the incubation, plates were examined and small, smooth, translucent and alpha-hemolytic colonies were subcultured to the Triyptic Soy Agar to obtain of pure cultures. When pure colonies were obtained, each colony was isolated according to the Gram staining microscopy, and catalase tests. Gram-positive cocci and catalase-negative isolates were determined and were recorded as suspected *Streptococcus sp* (Razali et al. 2020).

Genotypic Identification

DNA Extraction

DNA extraction were performed from isolates as *Streptococcus sp.* recommended by the manufacturer using the Genomic DNA Purification Extraction Kit (Thermo Fisher Scientific™) for use in PCR. The DNA samples were stored in cryotubes at -20°C up to the PCR.

16S rRNA PCR for *Streptococcus sp.*

For molecular identification of the 16S rRNA genes were amplified using universal primers (27F and 1492R) by SimpliAmp Thermal Cycler Applied Biosystems (Thermo Fisher Scientific™). PCR amplicons were electrophoresed on 2% agarose gel and were visualized on UV transilluminator (Vilber Lourmat). 16S rRNA gene specific bands at 1450 bp were considered positive (Lane 1991).

Purification and Sequencing of PCR Product

PCR amplicons were purified with enzymatic purification kit for sequencing. Purified PCR products concentrations were prepared ~50ng for sequencing PCR. PCR products were sequenced with 1492R PCR primers (3.2 pmol) using the Big Dye Terminator Ready Reaction Mixv 3.1. Nucleotide sequences were run on an ABI Prism 310 Genetic Analyser (Applied Biosystems). The nucleotide sequences of PCR products was analysed using Standard Nucleotide BLAST® NCBI Genomic Reference Sequences. The results obtained were compared electronically with the NCBI Blast® nucleotide sequences and the percent similarity rates were determined (Turner et al. 1999).

Determination of Antimicrobial Susceptibility

For the determination of antibiotic susceptibility pattern of the *Streptococcus minor* isolates were used the Kirby-Bauer disc diffusion method (CLSI 2016). Antibiotic discs were used comprising ampicilline (10µg), streptomycin (300µg), vancomycin (30µg), eritromycin (15µg), florfenicol (30µg), cefotaxime (30µg), cefepime (30µg), trimethoprim (20µg), methicillin (5µg), tetracycline (30µg), sulfamethoxazole-trimethoprim (25µg), amoxicillin-clavulanic acid (30µg), penisilin (10 IU) (Oxoid, Hampshire, England).

Determination of Antibiotic Resistance Genes

For detection of antibiotic resistance genes, PCR protocols were examined by list of references in Table 1. PCR master mix were prepared a total volume of 25 µl; including of 5µl 10X PCR Buffer, 2.5 mM MgCl2, 200 µM dNTP's, 0.5 µM of each primer (F & R), 2U Taq DNA polymerase, 3µl template DNA. The amplification conditions were as follow; an initial denaturation step at 94°C for 8 min; by 32 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 80 s and elongation at 72°C for 2 min; 1 cycle of final elongation at 72°C for 10 min. (Randall et all. 2002, Toro et al. 2005, Mammeri et al. 2005, Van et al. 2008). PCR products were electrophoresed on 2% agarose gel and were performed on Vilber Lourmat UV transilluminator. The PCR product bands were evaluated on target gene product size (Table 1).

Table 1. Antibiotic resistance gene primer sequences

Primers	Sequences (5'-3')	Size of Product (bp)	Target gene	References
aadA1-F	TATCCAGCTAAGCGCGAACT	447	Streptomycin resistance	Randall et al. 2004
aadA1-R	ATTTGCCGACTACCTTGGTC			
tetA-F	GGTTCACTCGAACGACGTCA	577	Tetracycline resistance	Randall et al. 2004
tetA-R	CTGTCCGACAAGTTGCATGA			
tetB-F	CCTCAGCTTCTCAACGCGTG	634	Tetracycline resistance	Randall et al. 2004
tetB-R	GCACCTTGCTGATGACTCTT			
dfrA1-F	GGAGTGCCAAAGGTGAACAGC	367	Trimethoprim resistance	Toro et al. 2005
dfrA1-R	GAGGCGAAGTCTTGGGTAAAAAC			
Qnr-F	GGGTATGGATATTATTGATAAAG	670	Floroquinolone resistance	Mammeri et al. 2005
Qnr-R	CTAATCCGGCAGCACTATTTA			
aac[3]-IV-F	CTTCAGGATGGCAAGTTGGT	286	Gentamicin resistance	Van et al. 2008
aac[3]-IV-R	TCATCTCGTTCTCCGCTCAT			
Sul1-F	TTCGGCATTCTGAATCTCAC	822	Sulfonamide resistance	Van et al. 2008
Sul1-R	ATGATCTAACCCTCGGTCTC			
blaSHV-F	TCGCCTGTGTATTATCTCCC	768	Cephalothin resistance	Van et al. 2008
blaSHV-R	CGCAGATAAATCACCACAATG			
CITM-F	TGGCCAGAACTGACAGGCAAA	462	Ampicillin resistance	Van et al. 2008
CITM-R	TTTCTCCTGAACGTGGCTGGC			
ereA-F	GCCGGTGCTCATGAACTTGAG	419	Erytromycin resistance	Van et al. 2008
ereA-R	CGACTCTATTCGATCAGAGGC			

RESULTS

Phenotypic and Genotypic Identification

In this study, 19 (38%) Gram-positive, cocci were isolated from 50 oral swab samples of dogs. The catalase test was performed on 19 Gram-positive isolates; 2 (10.5%) isolated found to be catalase-positive and 17 (89.5%) isolates found to be catalase-negative. Gram-positive, catalase-negative 17 (89.5%) isolates were evaluated *Streptococcus sp.* 17 (89.5%) *Streptococcus sp.* suspected isolates were passaged on Tryptic soy agar plates and DNA extractions were performed. PCR analysis was performed on obtained DNA using universal primers. All *Streptococcus* PCR products (n=17) were visualised at 1450 bp bands in gel image analysis.

The 17 PCR products showing the band on 1450 bp were subjected to Sanger sequencing. As a result of Sanger sequence analysis, 8 (47%) of 17 isolates were identified as *Streptococcus minor* and other 9 (53%) *Streptococcus* isolates could not be typed by the Sanger sequencing method. Of the 5 (66%) *Streptococcus* isolates were 97% similarity to *Streptococcus minor* strain

B-5-2 AP strain (Accesion Number MT510388.1) and the other 3 (44%) *Streptococcus* isolates were 97% similarity to *Streptococcus minor* strain B-3-MS-7- AP strain (Accession Number MT492055.1).

It was found that the antibiogram results of *Streptococcus minor* isolates were 100% susceptible to ampiciline, vancomycin, cefotaxime, cefepime, sulfamethoxazole-trimethoprim, amoxicillin-clavulanic acid; 87.5% susceptible to streptomycin, florfenicol, trimethoprim, methicillin; 75% sensitive to erythromycin and penicillin; 75% resistant to tetracycline (Table 2).

Table 2. *S. minor* isolates antimicrobial susceptibility profile

S. minor **Isolates**	**AMP (10µg)**	**S (300µg)**	**V (30µg)**	**E (15µg)**	**FFC (30µg)**	**CTX (30µg)**	**CFP (30µg)**	**TMP (20µg)**	**M (5µg)**	**T (30µg)**	**SXT (25µg)**	**AMC (30µg)**	**P (10 IU)**
1	S	S	S	R	S	I	S	S	S	R	S	S	S
2	S	S	S	S	S	S	S	S	S	R	S	S	S
3	S	S	S	R	R	S	S	S	S	R	S	S	S
4	S	R	S	I	S	S	S	S	S	R	S	S	S
5	S	S	S	S	S	S	S	S	S	R	S	S	R
6	S	S	S	S	S	S	S	R	S	R	S	S	S
7	S	S	S	S	S	S	S	S	S	I	S	S	S
8	S	S	S	S	S	I	S	S	R	S	S	S	R
	100% S	87.5% S	100% S	75% S	87.5% S	100% S	100% S	87.5% S	87.5% S	75% R	100% S	100% S	75% S

AMP: Ampicilline, S: Streptomycin, V: Vancomycin, E: Eritromycin, FFC: Florfenicol, CTX: Cefotaxime, CFP: Cefepime, TMP: Trimethoprim, M: Methicillin, T: Tetracycline, SXT: Sulfamethoxazole-trimethoprim, AMC: Amoxicillin-clavulanic acid, P: Penisilin

In the antibiotic resistance gene analyzes, tetracycline resistance gene was found in the one S. minor isolate and the trimethoprim resistance gene was found in the one S. minor isolate. The antibiotic resistance genes were not detected on the other S. minor (n=6) isolates.

DISCUSSION

Dogs come into contact with the environment, there are also dog-to-dog differences in their oral microbiome. Generally, P*roteobacteria, Firmicutes, Bacteroidetes* and *Fusobacteria* are commonly reported in the oral bacterial composition. In addition, *Porphyromonas, Fusobacterium, Streptococcus, Capnocytophagae* and *Pasteurella* species are also found in canine oral microbiomes at varying rates (Sturgeon et al. 2013, Bell et al. 2020, Ruparell et al. 2020).

Oral microbiota is related to the oral health of dogs, but there is an increase in the number of pathogen Gram-positive and Gram-negative bacteria with periodontal diseases. Bacteria found in the oral cavity of dogs appear as potentially dangerous agents in dog bites in humans. *E.coli, Streptococcus, Staphylococcus* and *Klebsiella, Pasteurella* species are among these pathogens. The most common species isolated from dog bites is *Pasteurella* species (50%), while *Streptococcus* species (46%) is the second causative agent (Abrahamian and Golstein 2011). *Streptococcus* species can cause septicemic infections, especially by passing through bite wounds into the circulation. *Streptococcus* species play an important role in infections such as endocarditis, septic arthritis, pharyngitis and cellulitis. *Streptococcus canis* is one of the most important species isolated from bite wounds (Stefanopoulos and Tarantzopoulou 2005). Ohtaki et al. (2013) identified *Streptococcus canis* from the femur fracture site of a 91-year-old woman. Researchers reported that the dog lived in the same house with its owner. It is noteworthy that *Streptococcus canis* was isolated from the wound site, although there were no bite cases. There are literatures about *Streptococcus canis*, which causes bacteremia and ulcers on the skin, such as this case (Bert and Lambert 1997, Takeda et al. 2001, Lam et al. 2007). Takeda et al. (2001) reported that they isolated *Streptococcus canis* from septicemia that occurred 2 weeks after the dog bite in a 75-year-old woman.

In recent years, with the development of molecular diagnostic methods, identification of new *Streptococcus* species has begun. Vancanneyt et al. (2004) were identified *Streptococcus minor* for the first time in canine tonsils. *Streptococcus minor* is also included in the oral *Streptococcus* species. Then, Tre-Hardy et al. (2016) identified *Streptococcus minor* from the bite wound of a 51-year-old woman. Thus, *Streptococcus minor* was isolated for the first time as a wound infection agent originating from dog bite.

CONCLUSION

In this study, it was concluded that with the development of molecular diagnostic techniques, *Streptococcus minor* will play an important role in dog bite cases like other *Streptococcus* species. For this reason, it was important to investigate whether *Streptococcus minor* species exist in canine oral flora. For this purpose, 8 (16%) *Streptococcus minor* identifications out of 50 oral swab samples were made using sequence-based diagnostic methods. In the antibiogram analysis, it was determined that most of the isolates were sensitive to antibiotics, but resistance to tetracycline was 75%. Tetracycline and trimethoprim resistance genes were found to be in only two of these isolates.

As a result, *Streptococcus minor* species have an important potential to become a zoonotic pathogen in dog bite cases in the coming years. In the diagnosis of *Streptococcus minor* infections, it should be investigated whether there is dog contact or not. Septicemic and ulcerative infections can develop within about 2 weeks after dog bites. In these cases, it is recommended that the identification of *Streptococcus* species in isolation from wound infections should be made by molecular methods and that *Streptococcus minor* species, which may be the primary pathogen should be taken into consideration.

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