Praca oryginalna ***Original paper***

Investigation of nasal colonization by coagulase-positive staphylococci and methicillin resistance in dogs*

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Received 29.07.2021 *Accepted 25.01.2022*

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Summary

This study aimed to determine the presence, rate, and species distribution of coagulase-positive staphylococci (CPS) in nasal swab samples from dogs and to investigate phenotypic and genotypic methicillin resistance. To this end, 21 (10.5%) CPS, including 7 (33.3%) *S. aureus* and 14 (66.6%) *S. intermedius* group (SIG), were isolated from nasal swab samples from 200 dogs. A total of 14 SIG members (100%) were also identified as *S. pseudintermedius* by multiplex polymerase chain reaction (m-PCR). Phenotypic methicillin resistance was observed in 16 (76.19%) of the 21 CPS isolated from 200 dogs from 8 different clinics and shelters. To detect genotypic methicillin resistance, the presence of the *mecA* and *mecC* genes, responsible for methicillin resistance, was detected by the multiplex polymerase chain reaction method. The *mecA* gene was found in 8 (38.09%) of the 21 isolates. The *mecC* gene was not detected in any of the samples, including isolates showing phenotypic methicillin resistance. It was found that neither the presence of CPS nor the presence of the *mecA* gene was statistically correlated with age, sex, or antibiotic use in the previous year ($p > 0.05$). Furthermore, CPS and *mecA*-positive isolates were evaluated according to whether they were obtained from shelters or clinics. It was concluded that the source of the isolates was not important for our study ($p > 0.05$). Our findings suggest that phenotypic resistance detected in epidemiological studies should be confirmed by molecular methods. At the same time, the fact that *mecC* gene-positive staphylococcal isolates were not detected in our study is promising for Turkey.

Keywords: coagulase-positive Staphylococcus, dog, mecA, mecC

It is reported that the rate of antimicrobial resistance in bacteria has increased rapidly, and this increase may lead to the return to the pre-antibiotic era and incurable diseases (24).

In addition to being a standard microflora agent in humans and animals, staphylococci are pyogenic factors that cause local and systemic infections, especially infections of pathogenic, humanoid, and zoonotic character. Coagulase-positive staphylococci (CPS) have attracted considerable attention due to their resistance mechanisms against antimicrobials (11).

Since the 1960s, methicillin-resistant staphylococcal strains have been a cause of hospital-acquired infections, the rate of which is rapidly increasing across the world (26). The misidentification of methicillin-resistant staphylococci as susceptible leads to treatment failures. Likewise, the identification of methicillin-susceptible staphylococci as resistant leads to the unnecessary use of glycopeptide antibiotics. Resistance can also be heterogeneous, because factors other than penicillin-binding protein PBP 2a influence the degree to which it is expressed. In addition, bacterial strains with low-level resistance to methicillin may produce large amounts of β-lactamase and therefore not exhibit intrinsic resistance (19, 29).

Methicillin resistance develops as a result of the fact that methicillin-resistant staphylococci contain PBP. This PBP is different from PBP 1, 2, and 3 contained

* This work was supported by the Scientific Research Project Coordination Unit of Istanbul University-Cerrahpaşa. (Project no: 27790).

in normal staphylococcal strains. It is called PBP 2a and has a low affinity for cephalosporins and carbapenem-derived antibiotics. PBP 2a maintains its activity in the presence of these antibiotics and ensures the survival of bacteria by binding peptidoglycan cross-links in the cell wall. The PBP2a enzyme is encoded by the *mecA* gene or the *mecC* gene found on the chromosome. Studies support the transmission of isolates carrying the *mecC* gene from livestock to humans (1, 4, 28).

Tab. 1. Sequences of the primers used and amplicon sizes

Target gene	Sequence	Primer	Amp (bp)	Source
S. intermedius	CATGTCATATTATTGCGAATGA AGGACCATCACCATTGACATATTGAAACC	in-F in-R3	430	Sasaki et al., 2010
S. pseudintermedius	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	pse-F2 pse-R5	926	Sasaki et al., 2010

Tab. 2. Sequences of the *mecA/mecC* gene primers and amplicon sizes

Target gene	Sequence	Amp (bp)	Source
mecA	5'-TCCAGATTACAACTTCACCAGG-3' 5'-CCACTTCATATCTTGTAACG-3'	162	Stegger et al., 2012
mecC	5'-GAAAAAAAGGCTTAGAACGCCTC-3' 5'-GAAGATCTTTTCCGTTTTCAGC-3'	138	Stegger et al., 2012

This study aimed to determine the carriage and species distribution of CPS in the nasal cavity of dogs. Furthermore, it also aimed to investigate methicillin resistance in staphylococci isolates and to investigate methicillin resistance at both phenotypic and molecular levels (the presence of the *mecA* and *mecC* genes) in isolates containing CPS.

Material and methods

Samples and culture. Nasal swab samples were collected from 200 dogs from 4 clinics and 3 shelters in the Istanbul province (88 samples from 4 clinics and 112 samples from 3 shelters). One swab was used for the two nares of the same animal. Antibiotic usage history was determined by asking the veterinarians. If antibiotics had been used, samples for the study were collected at least two weeks after antibiotic usage. Among the 200 dogs, there were 102 males and 98 females, 62 young animals and 138 adults, 38 dogs treated with antibiotics and 162 animals without a history of antibiotic use. The samples delivered to the laboratory were inoculated in Mueller-Hinton broth medium (Merck, 1.05437.0500) containing 6.5% NaCl and left for incubation for 24 hours at 37°C. Then they were inoculated on the ORSAB agar medium (Oxoid, CM1008) and incubated at 37°C for 24 hours. Gram staining was first performed from the blue-colored suspected colonies of staphylococci. Isolates identified as Gram-positive cocci were spread on slides, and 3% H_2O_2 (Sigma, H3410) was dropped on them. Gas bubble-forming, catalase-positive bacteria were recognized as staphylococci (20). Finally, isolates previously purified by incubating at 37°C for 24 hours on blood agar medium (Himedia, M001) were identified using an automated system (Phoenix 100, BD Diagnostic Systems, USA). Slide and tube coagulase tests (Bactident) were made to confirm species identified as CPS in the automated system.

***Staphylococcus intermedius* group distinction.** The BIO-RAD InstaGene Matrix DNA Isolation (K732-6030) kit was used for extraction. A standard and common PCR Mix was prepared for amplification of *S. intermedius* and *S. psuedintermedius* gene regions. Field strains identified by a previous study and belonging to the laboratory were used for positive control (25).

5 µl template DNA, 1.5 mM $MgCl_2$, 1X PCR buffer, 0.2 mM dNTP mix (Thermo Scientific, R0192), 0.5 U Taq polymerase (Thermo Scientific, EP0402), 0.4 µM forward primer, and 0.4 µM reverse primer (presented on Tab. 1), and then DNase and RNase-free distilled water (Thermo Scientific, 10977035) was added so that the total volume would be 50 µl. According to the PCR protocol determined by Sasaki et al. (32), pre-denaturation was performed at 95°C for 2 minutes. The initial denaturation was followed by 30 cycles of denaturation at 90°C for 30 sec, the binding of primers at 56°C for 30 sec, and synthesis at 72°C for 1 min. The last synthesis step was carried out at 72°C for 2 min.

Phenotypic determination of methicillin resistance. To investigate methicillin resistance in CPS, oxacillin and cefoxitin susceptibilities were examined by the disk diffusion method (Kirby-Bauer method). Inoculation was performed on Mueller-Hinton agar media (Oxoid 1.05404) from suspensions based on McFarland 0.5 prepared in physiological saline from pure cultures. In the evaluation of the disk diffusion tests, the resistant zone diameter was assumed to be $\leq$ 10 mm for oxacillin, while the inhibition zone diameter for cefoxitin was assumed to be $\leq$ 21 mm (6).

Genotypic determination of methicillin resistance. Amplification was also performed to investigate the presence of the *mecA* and *mecC* genes in the genomic DNA obtained. The *mecA* gene amplification in isolates was performed by PCR using primers encoding the 162 bp fragment on the gene according to the method specified by Stegger et al. (34), and the presence of the *mecC* gene was determined with primers encoding the 138 bp fragment. The primers used are presented in Table 2.

Positive controls. Reference strain ATCC 53678 was used for phenotypic and genotypic determination of methicillin resistance.

Statistical analysis. The chi-square (χ^2) test from the Statistical Package for Social Sciences (SPSS) 13.0 was used to compare the species distribution of the isolates and the resistance rates determined genotypically according to the age groups and sex of the animals from which isolation was carried out, the use of antibiotics in the previous three months, and the source of the isolates (shelter or clinic).

Results and discussion

CPS were isolated from 21 out of 200 nasal swab samples examined (10.5%): 10 out of 112 samples collected in shelters and 11 out of 88 samples collected in

clinics. Thirteen of those 21 samples originated from male dogs and 8 from females, 5 were obtained from young animals and 16 from adults. Three of those 21 samples were collected from dogs that had been treated with antibiotics in the previous year, whereas the other 18 samples came from animals without a history of antibiotic usage. Fourteen (66.6%) of the samples were identified as the *Staphylococcus intermedius* Group (SIG) and 7 (33.3%) as *S. aureus*. All 14 SIG members were identified as *S. pseudintermedius* by PCR performed with specific primers. Methicillin resistance was phenotypically observed in 16 (76.1%) of the 21 isolates: in 11 (78.5%) of the 14 *S. pseudintermedius* isolates and in 5 (71.4%) of the 7 *S. aureus* isolates. As a result of PCR amplification, the *mecA* gene was detected in 8 (38.09%) of the 21 CPS samples. Of these, 7 were identified as *S. pseudintermedius* and 1 was *S. aureus*. The *mecC* gene was not detected in any of them. The *mecA*-positive PCR results are shown in Figure 1.

It also shows phenotypic methicillin resistance in the single genotypically resistant *S. aureus* sample. All 7 genotypically resistant *S. pseudintermedius* isolates were also phenotypically methicillin-resistant. In other words, no genotypic resistance was found in 4 phenotypically resistant *S. aureus* and 4 phenotypically resistant *S. pseudintermedius* isolates. Statistical analysis showed that differences between the species distribution and genotypic resistance rates of the isolates were not significant according to the sex, age groups, and antibiotic history of the animals from which nasal swab samples were taken ($p > 0.05$). CPS and *mecA*-positive isolates were also evaluated according to the sources from which they were taken. The difference between the percentage of CPS and *mecA*-positive isolates obtained from shelters and clinics was not significant for our study ($p > 0.05$).

The diagnosis and treatment of staphylococcal infections in dogs have become very important with the emergence of evidence that staphylococci develop resistance against various antibiotics and cause zoonotic infections by being transmitted from infected or carrier dogs to humans (10, 24, 35). *S. aureus* is a CPS species found in the canine skin flora and can cause purulent infections (22). *S. intermedius* was also reported as the most common species obtained from canine skin infections and healthy canine skin (4, 18). The agent is also isolated from the external auditory canal, anal mucosa, and nasal cavity (7, 30). *S. pseudintermedius* was first identified as a CPS species different from *S. intermedius*. However, studies using gene technology have shown in recent years that isolates identified as *S. intermedius* actually consist of three different species, *S. intermedius*, *S. pseudintermedius*, and *S. delphini*, which have been reported as SIG. Thus it became evident that the common pathogen in infections is not *S. intermedius*, but *S. pseudintermedius*.

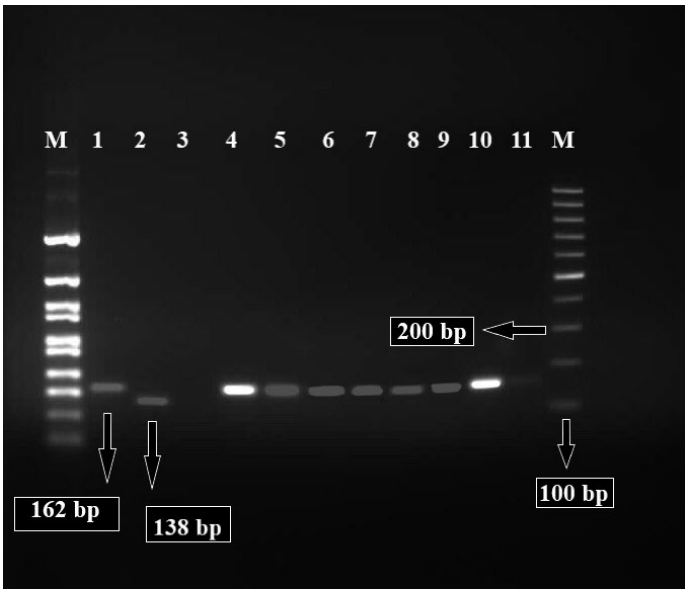

Fig. 1. m-PCR results for the *mecA* and *mecC* genes

In 2005, Sasaki et al. (31) managed to distinguish *S. intermedius* from the other SIG members by biochemical methods, but they could not identify phenotypic markers required to distinguish *S. pseudintermedius* from *S. delphini*.

In pet animals, pathogenic species, such as *S. aureus*, *S. pseudintermedius*, and *S. intermedius*, are isolated from the nose, skin, and anal regions, and these agents are thought to be transmitted to the skin or other regions, especially from the nose and mouth (5, 39).

In a study investigating the presence of *Staphylococcus* species in the skin of healthy animals, CPS was isolated from the back skin of 10 out of 25 healthy domestic dogs (11). In a study conducted in 2006, Daaloul-Jedidi M. et al. (8) reported that 22.2% of nasal swab samples from dogs contained CPS. In 2013 Petrov et al. (28) reported that they isolated coagulase-positive staphylococci from 193 dogs (45.2%) with otitis externa. In another study conducted in 2013, Henry et al. (16) obtained 116 isolates from dogs and reported that most of the Gram-positive bacteria isolated were CPS. In the present study, *Staphylococcus* sp. was isolated from 31 (17.5%) nasal swab samples collected from 200 dogs, and 21 (10.5%) of those 31 isolates were CPS. These results support the findings of previous studies and the idea that the nasal region is a reservoir for CPS, as also reported by Walther et al. (38) in 2012.

Biberstein et al. (4) investigated CPS in different animal species and found that 91.8% of 195 staphylococcal isolates obtained from different body parts of dogs (urinary tract, respiratory tract, skeletal system, reproductive system, skin, and blood) were *S. intermedius*, and 8.2% were *S. aureus*.

Hoekstra and Paulton (17), who investigated the antibiotic susceptibilities of 867 *S. aureus* isolates and 1339 *S. intermedius* isolates obtained from different body parts (nose, eye, ear, genital area, abscess, skin, and throat) of 2206 dogs of different ages and both

sexes, reported that 153 (47.07%) of 325 nasal isolates from dogs with skin lesions were *S. aureus* and 172 (52.92%) were *S. intermedius.*

Epstein et al. (9) isolated methicillin-resistant *S. intermedius* from 6 out of 36 dog nasal swabs in 2009.

In 2014 Hariharan et al. (14) obtained 116 isolates from 66 dogs and reported that staphylococci were the most isolated Gram-positive bacteria. Among 29 *S. intermedius* isolates, identified by the API method, they found 27 *S. pseudintermedius*, 1 *S. aureus*, and 1 *S. schleiferi subsp. coagulans* by the multiplex PCR method. They applied the M-PCR gene sequencing method to a strain that had previously been phenotypically identified as *S. intermedius* and found that it was actually *S. pseudintermedius*. Likewise, in our study, all 14 isolates identified as SIG by the BD Phoenix automated system were identified as *S. pseudintermedius* when re-examined by the Multiplex PCR method.

Similarly, in this study, 14 of the isolates (66.6%) were identified as SIG, and 7 (33.3%) were identified as *S. aureus*. All 14 SIG members were identified as *S. pseudintermedius* by PCR performed with specific primers. It was concluded that *S. pseudintermedius* was dominant in the nasal flora of the dogs, compared to *S. aureus* and *S. intermedius*, and samples identified as *S. intermedius* in the past might actually be *S. pseudintermedius*.

Although studies on methicillin-resistant staphylococci in Turkey have focused on livestock, the number of studies increases every year, and the increase in isolation rates draws attention. In most veterinary diagnostic laboratories, phenotypic methods are used to detect methicillin resistance in staphylococci. Oxacillin or cefoxitin are often used to detect methicillin resistance because they are more sensitive and reliable. To this end, tube dilution and disk diffusion tests are widely used. The disk diffusion test is the preferred method in clinical microbiology (27). In 2000, Sünbül et al. (33) found that the sensitivity, specificity, and basic compatibility of the disk diffusion test were higher than those of the other tests. In 1994, Razlighi and Derbentli (29) found the microdilution and agar screening method to be 100% compatible, while the sensitivity of the disk diffusion test was 100% and the specificity was 97.5% compared to the two methods.

In the present study, methicillin resistance was determined phenotypically by the disk diffusion test according to standards recommended by CLSI 2015 (6).

Kaynarca (21) determined by the disk diffusion method that 71 strains isolated from cows with mastitis in Aydın province and identified as *S. aureus* were 93% methicillin-resistant.

It has been reported that the isolation of methicillin-resistant staphylococci has been increasing (23). In studies on methicillin resistance in raw milk from cows with mastitis in Turkey, Uçan and Aslan (37) reported methicillin resistance in 1 out of 75 *S. aureus* isolates, whereas Hadimli et al. (13) reported it in 14 out of 78 *S. aureus* isolates.

In a study conducted by Aydın and Gültekin (2) in 2001 on 274 staphylococcal strains isolated from humans by the Kirby-Bauer method, methicillin resistance was found in 10.9% of the isolates. In a study conducted by Bağcıgil et al. (3) in Denmark in 2007, methicillin resistance was detected in 13% of coagulase-negative staphylococcal isolates obtained from the nose of dogs.

In the present study, antibiogram tests performed to determine phenotypic resistance profiles revealed methicillin resistance in 16 (76.19%) of the 21 isolates. Eleven of the resistant isolates were *S. pseudintermedius*, and 5 of them were *S. aureus*. It was concluded that the methicillin resistance rate detected by the phenotypic method was relatively high.

The presence of heterogeneous methicillin resistance in staphylococci and the fact that this resistance is affected by environmental factors are still unresolved problems. Until these issues have become clear, researchers suggest that, under conditions in which molecular analysis is impossible, staphylococcal strains should be tested simultaneously with different antibiotic disks that induce the *mec*A gene to detect methicillin resistance, and if incompatible results are obtained, methicillin resistance should be determined by molecular methods in reference laboratories. Due to the heterogeneous expression of the mecA gene, the phenotypic demonstration of methicillin resistance still poses a problem (27). The reason why 8 isolates in which methicillin resistance was detected did not have the *mecA* gene is not fully understood. It is thought that there may be other genes responsible for phenotypic resistance in bacteria that do not contain the relevant gene (15). Although there is resistance in phenotypic tests in cases such as point mutations in existing PBPs and excessive production of another PBP, *mecA* negativity can be observed (12, 36).

It was determined that the differences between the species distribution and genotypic resistance rates of the isolates were not significant according to the sex, age groups, and known history of antibiotic use of the sampled animals in lifetime ($p > 0.05$). The fact that the data on antibiotic use in the previous year did not have sufficient significance suggests that the information obtained from the centres was incorrect. According to the statistical analysis, the source of the 21 CPS isolates (clinics or shelters) was not significant for our study.

CPS colonization was detected in the nasal mucosa of 21 (10.5%) of the 200 animals examined, and the dominant species causing nasal germ carriage was *S. pseudintermedius*. Methicillin resistance was phenotypically observed in 16 (76.1%) of the 21 isolates. The *mecA* gene was detected in 8 of those 16 strains. These findings suggest that phenotypic resistance detected in epidemiological studies should be confirmed

by molecular methods. Methicillin resistance, which was detected at a relatively high rate (38.09%), is genotypically important. These results also show that dogs with nasal colonisation by methicillin-resistant CPS may serve as a source of infection for humans. Detailed studies are needed especially on pathogenicity. The fact that *mecC* gene-positive staphylococcal isolates were not detected in our study may be considered promising for Turkey. We think, however, that it is important to continue epidemiological studies on the presence of the *mecC* gene, which may appear in the future.

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