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A study of antimicrobial resistance in *Staphylococcus* spp. isolated from healthy, travelling and residential, Norwegian dogs

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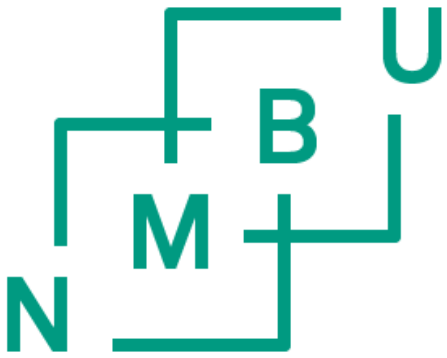

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Content

Acknowledgements

The work presented in this essay was conducted at the Institute for Food Safety and Infection Biology at the Norwegian University of Life Sciences. The work was part of a larger doctoral project on the occurrence of antimicrobial resistance in different groups of the Norwegian dog population.

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Abstract

Antimicrobial resistant bacteria are of an increasing concern in both human and veterinary medicine. In this research project, we investigated the occurrence of antimicrobial resistance in species of *Staphylococci* in different segments of the Norwegian dog population. The work presented in this research essay has a particular emphasis on dogs travelling from abroad to Norway, as well as healthy, Norwegian dogs with no signs of infectious or inflammatory disease. Samples were collected from healthy dogs arriving at the Oslo International Airport in the fall of 2016, and from healthy, residential, Norwegian dogs in the spring of 2017. A total of 72 dogs from abroad were sampled, and 62 residential, Norwegian dogs. From the samples from dogs from abroad, 39 isolates of *Staphylococci* spp. were collected. From the residential Norwegian dogs, 19 isolates were collected. Methicillin-resistance was not found in any of the isolates, using both phenotypic and genotypic methods. 23% percent of the dogs from abroad were classified as multi-resistant, being resistant to 3 or more antimicrobials, while none of the samples from residential, Norwegian dogs were multi-resistant. Resistance to penicillin and tetracyclines was more prevalent in the residential, Norwegian dogs, than those from abroad. More research is needed to conclude whether travel abroad poses a health risk for the Norwegian dog population.

Oppsummering

Antibiotikaresistens sees på som en stor trussel for både human- og dyrehelse. I dette forskningsprosjektet undersøkte vi forekomsten av antibiotikaresistens i stafylokokker i forskjellige segmenter av den norske hundepopulasjonen. I denne forskningsoppgaven er hovedfokus på klinisk friske hunder som reiser til Norge fra utlandet, og norske hunder bosatt i Norge, uten tegn på klinisk sykdom. Prøver ble samlet inn fra hunder som reise til Oslo Lufthavn høsten 2016, fra utlandet, og friske, norske hunder som ikke hadde nylig vært utenlands høsten 2017. 72 hunder fra utlandet ble prøvetatt, og 62 norske hunder. Fra de utenlandske hundene ble det isolert 39 isolater av stafylokokker, mens det fra de norske hundene ble isolert 19. Prøvene ble analysert for antibiotikaresistens ved hjelp av fenotypiske og genotypiske metoder. Ingen meticillinresistens ble påvist. 23% av isolatene fra utlandet ble klassifisert som multiresistente (resistente mot 3 eller flere antibiotikum), mens ingen av de norske isolatene viste multiresistens. Forekomsten av penicillinresistens og tetracyklinresistens var høyere blant de norske isolatene, enn blant de utenlandske. Mer forskning trengs for å konkludere hvorvidt utenlandsreiser og en åpen hundepopulasjon fører til en økt helserisiko for norske hunder.

1. Introduction

The discovery of antimicrobials has been one of the most important modern medical discoveries ever made, having saved millions of lives and cured countless life-threatening infections. However, the rapid emergence of resistance to these antimicrobials is becoming an increasingly pressing concern, threatening our ability to cure and treat common bacterial infections, as well as life-threatening and serious infections. In general, the use of antimicrobials is deemed to be the greatest driver for antimicrobial resistance. Both internationally and nationally, governments and health organizations have made antimicrobial resistance a top priority. The World Health Organization considers antimicrobial resistance to be one of the biggest threats to global health today (WHO, 2014).

The purpose of this research project was to investigate the occurrence of antimicrobial resistant *Staphylococcus* spp. in dogs travelling to Norway from abroad, as well as from different healthy dog populations in Norway. The research discussed in this essay is part of a larger research project that aims to compare the antimicrobial resistance pattern of clinical MRSP isolates to clinical methicillin-sensitive *S. pseudintermedius* isolates, as well as *S. pseudintermedius* carrier isolates from healthy Norwegian dogs and from dogs traveling abroad. Only results from the carrier isolates from healthy dogs will be included in this essay. For a comprehensive overview of the results of our research project, the reader is referred to the attached article manuscript.

1.2 Staphylococcus spp.

The species of staphylococci that are of interest for this research project are gram-positive bacteria that are commonly found as commensals on the skin and mucous membranes of animals and humans. They are facultative anaerobic bacteria, with a gram-positive spherical appearance in the microscope. Staphylococci are divided into two different sub-groups, the coagulase-negative and the coagulase-positive staphylococci. In routine diagnostic laboratories, a

coagulase-test is used to differentiate between the two, with the species of interest for this project giving a typically positive result. Historically, the coagulase-positive staphylococci have been viewed as more pathogenic, with the coagulase-negative species more playing the role of opportunistic pathogens. (Becker et al, 2014).

For the purpose of this research project, the two main species of interest were the coagulase-positive *Staphylococcus aureus*, and *S. pseudintermedius*.

S. aureus is a commensal isolated most commonly from the skin and mucous membranes of humans. 30% of humans are asymptomatic carriers of *S. aureus* on the nasal mucosa (Chambers et al, 2009). The species is an opportunistic pathogen, associated with skin infections, urinary tract infections and surgical wounds, but also potentially fatal diseases such endocarditis, osteomyelitis and sepsis (Turner et al, 2019). Additionally, *S. aureus* is considered a food-borne pathogen, with the ability to produce enterotoxins.

S. pseudintermedius is a major opportunistic pathogen in dogs and cats. The species was first described in 1976, but was then known as *S. intermedius*. In 2005, the species was split into three clusters: *S. intermedius*, *S. pseudintermedius* and *S. delphini*. The bacteria are found on the skin and mucous membranes of pet animals, and are most frequently isolated from the nose and the anal region in the dog (van Duijekeren et al, 2011). Studies that examine carriage rates in dogs have quite variable results, ranging from 46 to 92% (Bannoehr et al, 2009). This might in large part be due to differences in sampling techniques and sampling sites, as well as differences in the laboratory methods used for identification and isolation of the bacteria. Studies have also shown that carriage rates are higher in dogs with atopic dermatitis than in healthy dogs (Fazakerley et al, 2009).

The bacteria belonging to the *S. intermedius*-group can be difficult to differentiate from each other using routine laboratory procedures. However, for the purpose of this research project, all bacterial isolates isolated from the dogs in our project, and identified as belonging to the *S. intermedius*-group, were considered *S. pseudintermedius*, in accordance with common

international standards. This was also confirmed using a polymerase chain reaction-restriction fragment length polymorphism test, based on a MbOI restriction site in *S. pseudintermedius,* that is not present in the other members of the SIG.

1.3 Diagnostic methods

Phenotypic methods are used to identify isolates of *S. aureus* and *S. pseudintermedius*. Both species are isolated based on their appearance when inoculated on blood agar, and with their typical gram-positive, spherical appearance when examined under a microscope after gram-staining. Further biochemical testing using coagulase-, mannitol-, catalase,- and OPNG-testing confirms the isolates as *S. aureus* and *S. pseudintermedius.*

The most common method for genotyping strains of *S. aureus* and *S. pseudintermedius* is multilocus sequencing technique. This is done by sequencing seven housekeeping genes, and assigning a sequence type (ST) to the isolate. Isolates with seven identical housekeeping genes are considered a clone, and therefore the same sequence type. If STs differ by single nucleotide polymorphism at fewer than three nucleotides, they are grouped into the same clonal complex (CC) (Chambers et al., 2009). Pulsed field gel electrophoresis can also be used to group isolates of *S. aureus* and *S. pseudintermedius.*

1.4 Antimicrobial resistance testing

In this research project, both genotypic and phenotypic methods were used to screen for antimicrobial resistance. This type of testing is carried out by clinical and diagnostic laboratories worldwide, in order to screen for resistance, and ensure appropriate antimicrobial treatment of infections. The methods are also used in research, for example in epidemiological studies that investigate the occurrence and spread of resistance. Whereas genotypic methods of resistance screen for the gene responsible for the mechanisms of resistance, phenotypic methods screen for the expression of these genes.

Phenotypic methods screen isolates for antimicrobial resistance *in vitro*. Common methods of doing so include broth microdilution, as well as disk diffusion. In broth microdilution, the isolate is incubated in broth containing different dilutions of the antimicrobial agent of interest. Microdilution can be prepared manually, as well as using automated dispensing equipment. The lowest concentration that inhibits growth of the antimicrobial is known as the minimum inhibitory concentration (MIC). This MIC is useful when considering the clinical use of an antimicrobial against an isolate, as the MIC can be compared to the expected concentration of the antimicrobial in the tissue that is to be treated. If the concentration that can be achieved is higher that the MIC of the isolate, the antimicrobial agent is appropriate for use as treatment. With disk diffusion, the isolate is inoculated on a Mueller Hinton-agar plate, and tablets containing antimicrobials are placed on the agar. If the isolate is sensitive to the antimicrobial in question, growth is inhibited. When using this method, growth inhibition zones can be measured around the antimicrobial tablets, and compared with a set of standardized criteria for determining if the isolate is sensitive or resistant to the antimicrobial. In general, the smaller the growth inhibition zone, the less sensitive the isolate is to the antimicrobial in question. The resistance to the antimicrobial is then determined qualitatively, using the categories sensitive, intermediate or resistant, based on the diameter of the growth inhibition zone. The size of the zone diameter that indicates whether the bacteria is resistant or sensitive to an antimicrobial will differ from species to species of bacteria. Another phenotypic method of determining antimicrobial resistance is through the use of the gradient diffusion method. When using this method, a thin plastic strip with an antimicrobial gradient is placed on a Mueller-Hinton agar plate that has been coated with the isolate that is to be investigated, and incubated overnight. The next day, the MIC is determined by determining where the growth of the bacteria is first inhibited by the strip (Reller et al, 2009). Several guidelines exist for determining resistance using phenotypic methods. Among the most well-known are EUCAST guidelines developed by the European Committee on Antimicrobial Susceptibility Testing, as well as guidelines developed by the Clinical and Laboratory Standards Institute. In general, phenotypic methods to screen for resistance are low cost and easy to carry out, and have therefore been widely used for many years. On the other hand, many of the methods are quite time consuming. One of the problems with disk diffusion is that there are discrepancies between the guidelines used to read the results of the disk diffusion.

As mentioned, several guidelines exist, and isolates can be deemed as sensitive to an antimicrobial according to one set of guidelines, and resistant according to another.

When using results from *in vitro* methods such as disk diffusion, in a clinical setting, care must be taken when interpreting results. In some cases, *in vivo* use of the antimicrobial will yield a different result than suggested by disk diffusion results, as the antimicrobial will accumulate differently in different target tissues. Thus, a good working knowledge of the biochemical properties of an antimicrobial, and how the antimicrobial is metabolized in the body is essential for proper antimicrobial use. For example, many antimicrobials accumulate in the bladder, and isolates from urinary tract infections that show resistance to an antimicrobial when using disk diffusion can still be susceptible in the clinical setting.
Another method for determining resistance to an antimicrobial is through the use of the epidemiological cut off value, the ECOFF. The ECOFF separates a population of bacteria into wild type and those that have an acquired mechanism of resistance that is phenotypically expressed. The ECOFF is defined as the highest MIC within the wild population, where organisms lack phenotypically expressed resistance. The ECOFF does not necessarily correlate with clinical susceptibility and resistance to an antimicrobial (Brown, 2011).

Genotypic methods screen for the genes responsible for antimicrobial resistance. While several methods exist, for the purpose of this essay, the use of Polymerase Chain Reaction (PCR) to screen for genes known to code for specific resistance to an antimicrobial agent is discussed. Genotypic methods have the advantage of being rapid, and in many instances, very reliable. However, they have the disadvantage that due to the fact that resistance to a specific antimicrobial often can be mediated by several different genes and mechanisms, screening for only one of several genes that can mediate resistance will not necessarily identify the resistance gene of the isolate in question. In order to mitigate this problem, several multiplex PCR methodologies have been developed, where several genes are screened for in the same PCR set-up. PCR also requires a positive and negative control, in order to validate the results of the PCR. One of the challenges is that good controls are not always available. In order to ensure that results from a PCR is valid when no control is available, an isolate that yields a band of the correct size

can be sequenced, and if confirmed by bioinformatics tools to contain the right gene, used as a control in later runs. In many cases, genotypic methods are used to confirm the results of the phenotypic methods, and to provide additional information. Genotypic methods often also require more skills on the part of the person carrying out the test.

1.5 Antimicrobial resistance

Resistance to antimicrobials in bacteria can be mediated by either natural mechanisms, genetic mutations or acquired mechanisms transferred between bacteria. The transfer of resistance genes by the use of mobile genetic elements, like plasmids, is known as horizontal gene transfer (von Wintersdorff et al, 2016). The single most important factor driving the dissemination of antimicrobial resistance is the use of antimicrobials, which places a selective pressure on the resistant bacteria.

S. aureus is a bacterium that is naturally susceptible to most antimicrobials. However, strains of *S. aureus* that are resistant to several antimicrobials have over the last decades become an increasingly large problem. The start of this problem can be traced back to the 1940s, with the emergence of strains of *S. aureus* resistant to penicillin in hospitals very soon after the start of therapeutic use of penicillin (Chambers et al 2009). In the 1960s, strains resistant to methicillin emerged in healthcare-settings, and were named healthcare-associated MRSA (HA-MRSA).

Methicillin-resistance in *Staphylococcus* spp. is mediated by the *mecA*-, or the *mecC*-genes, which encode the production of a modified penicillin-binding protein (PBP). The *mec* genes are encoded on a "Staphylococcal chromosomal cassette," known as SCCmec. This cassette is also found in coagulase-negative staphylococci. The penicillin-binding protein encoded for has a low affinity for beta-lactam antibiotics, and cell growth is therefore not prevented by the use of these antimicrobials. Community-associated MRSA strains (CA-MRSA) emerged in the 1990s, infecting healthy people with no previous history of hospitalization (Chambers et al 2009, Turner et al, 2019). Strains known as livestock-associated MRSA (LA-MRSA) are also increasingly a

threat, with the main focus being on isolates belonging to clonal complex 398. Strains of LA-MRSA trace back to livestock, and are a particularly large problem in pig farming. Isolates have also been identified in other livestock productions in Europe, for instance in dairy cattle and turkeys (Cuny et al, 2015). In Denmark, LA-MRSA is considered almost endemic in the production of swine (Flemming et al 2014). In Germany, approximately half of all pig farms are considered colonized (Cuny et al, 2015). LA-MRSA can spread to humans, and cause the same infections as other strains of MRSA (Cuny et al, 2015). In Norway, the Norwegian authorities have adopted a policy of stamping out in any pig farms where MRSA is confirmed (NORM/NORM-VET, 2015).

Methicillin-resistant *S. pseudintermedius* (MRSP) first emerged in 2006 (Damborg et al 2016). As in *S. aureus*, methicillin-resistance in *S. pseudintermedius* is also mediated by the *mec*A- or *mec*C- gene, located on the SCCmec. MRSP causes the same kind of infections in dogs and cats as methicillin-sensitive *S. pseudintermedius* (MSSP), such as skin and wound infection, ear infections and urinary tract infections. However, these infections are much more difficult to treat than infections with MSSP. The bacteria are most commonly isolated from dogs, but can also be isolated from cats. They also have a zoonotic potential, and have been isolated in cases of human disease, for example from a 65-year old patient with an infection following a bone marrow transplant (Savini et al, 2013). The occurrence of MRSP-infections in humans is likely underreported, as many laboratories simply classify all coagulase-positive staphylococci resistant to methicillin as MRSA.

1.6 Antimicrobial resistance in the Norwegian animal population

The Norwegian Veterinary Institute has carried out several studies on the prevalence of antimicrobial resistant *Staphylococcus* spp. in the Norwegian dog population. In 2004, a study was conducted where samples were collected from dogs with untreated clinical ear and skin

infections at veterinary clinics in different geographic regions of Norway. In total, 91 dogs were sampled, yielding 59 isolates. Whereas 19% of the isolates showed no resistance to any of the antimicrobials included in the study, 70% showed resistance to penicillin, 49% to fucidic acid, 42% to oxytetracycline, 10% to neomycin, 10% to streptomycin, 9% to erythromycin and 9% to clindamycin. PCR showed that resistance to penicillin was mediated by the *bla*Z gene in all isolates. Resistance to tetracyclines was mediated by the *tet*M gene in all isolates, whereas resistance to erythromycin was mediated by the *erm*B gene. Only one of the five isolates that showed phenotypic resistance to fusidic acid harbored the *fus*C gene (Nordstrøm et al. 2009). The authors of this study concluded that prevalence of antimicrobial resistance in clinical isolates of *S. pseudintermedius* was relatively high.

In 2014, the Norwegian Veterinary Institute published a study on the prevalence of MRSP in healthy Norwegian dogs. In this study, a total of 189 healthy dogs visiting ten veterinary clinics for prophylactic treatment were screened for MRSP. Swabs were collected from the mouth and the perineum of these dogs. The prevalence of MRSP in this study was 2.6 % (5 out of the 189 dogs included in the study) (Kjellman et al. 2015).The study also included MRSP isolates from 49 dogs with infections. These samples were collected at the diagnostic lab at the Norwegian Veterinary Institute from July 2008 to April 2013. MRSP isolates collected from healthy carriers, as well as the clinical isolates collected by the diagnostic lab at the Norwegian Veterinary Institute were all subject to further antimicrobial resistance testing. This testing was carried out by disk diffusion, according to the recommendations made by EUCAST. The isolates were tested against the following panel of antimicrobials: Tetracycline, fusidic acid, trimethoprim/sulfamethoxazole, ciprofloxacin, erythromycin, clindamycin, gentamicin, nitrofurantoin, penicillin, ampicillin, amoxicillin/clavulanate, and cefoxitin. Of the 54 isolates of MRSP included in the study 44/54 (81%) showed multiresistance, being resistant to 3 or more antimicrobials. Resistance was most common to erythromycin (87%), clindamycin (85%) and trimethoprim/sulfamethoxazole (78%). 46% of the isolates were resistant to tetracyclines, 26% to fusidic acid, 24% to ciprofloxacin and 39% to gentamycin. None of the isolates showed resistance to nitrofurantoin.

1.7 Background for this project

Recently the Norwegian Scientific Committee for Food Safety published an opinion entitled "Assessment of the transfer of antimicrobial resistance between pets and humans."[5] The report highlighted the need for increased investigation into the prevalence of antimicrobial resistant bacteria in pet animals, as well as the risk of dissemination of these bacteria from pets to humans. Among the factors of uncertainty, and where further research is needed, was the trend of an open Norwegian dog population, with an increasing number of dogs travelling to Norway from abroad. This accounts for both dogs being imported to Norway and for Norwegian dogs accompanying their families on vacation etc. For humans, travelling to countries with a high endemic prevalence of antimicrobial resistant microbes increases the risk of becoming a carrier of these bacteria (Wasteson, 2015; Stafykolokkinfeksjoner: veileder, 2015). We would expect that the same would hold true for pets travelling from abroad would have a higher prevalence of antimicrobial resistant bacteria, and therefore pose a potential risk for the Norwegian dog population.

The aim of the research student project of this study was to map the antimicrobial resistance patterns in *S. pseudintermedius* isolates from healthy dogs travelling to Norway from abroad, as well as to study the patterns in healthy Norwegian dogs as a control group.

2 Materials and Methods

2. 1 Collection of isolates

The isolates from dogs travelling from abroad were collected at the Oslo International Airport, in the fall of 2016. Dogs that are normally resident in Norway but had been abroad for varying time periods, as well as foreign dogs travelling to Norway were sampled. All samples were

collected by the author. Our original goal was to sample 500 dogs, a task we believed would be achievable, as more than 9000 dogs arrive at the Oslo International Airport every year (personal communication with the Norwegian Food Safety Authority). Non-clinical isolates from Norwegian dogs were collected in the spring of 2017, from healthy dogs visiting veterinary clinics in Norway for prophylactic treatment (vaccines, etc), as well as healthy dogs belonging to students and employees at the Norwegian University of Life Sciences. Samples were taken by swabbing the corner of the mouth and the perineum of the dog. Owners of sampled dogs completed a questionnaire regarding traveling status, health status, antibiotic treatment in the last 14 days, as well as breed, age and sex. Samples from dogs that had been treated with antibiotics in the last 14 days, or had any clinical signs of infection as stated by the owner, were excluded from the study. After collection the swabs were placed in a coal medium, and analyzed within 48 hours. The swabs were placed in 9 mL of Mueller Hinton broth with added 5 % NaCl, and incubated overnight at 37 °C. The samples were then inoculated on two different agar plates, standard blood agar and Mannitol Salt agar with oxacillin for detection of MRSP/MRSA, and incubated at 37 °C for 24, and 48 hours. Presumptive isolates of *S. pseudintermedius* were identified phenotypically using the biochemical tests mannitol, catalase and ONPG, as well as Gram-staining. One isolate from each sample was subjected to further testing.

All *S. pseudintermedius* isolates were confirmed by using a PCR-restriction fragment length polymorphism approach (Bannoehr et al. 2009).

2.2 DNA extraction

Bacterial DNA was extracted using the following procedure: One or two colonies of each isolate was mixed with 3 mL Brain Heart Infusion broth (BHI) and incubated at 37 °C overnight. 100 µL of each bacterial suspension was centrifuged at 13200 rpm for 3 min. The supernatant was removed and the pellet was resolved with 50 µL lysostaphin (50 µg/mL). The tubes were heated at 37 °C for 10 min before adding 50 µL proteinase K (100 µg/mL) and 150 µL 0,1 M Tris (pH 7,5), and then heated at 37 °C for 10 min and 95 °C for 5 min. The extracted DNA was stored at -

20 °C prior to analysis. For detection of the *ermB* gene DNA was isolated using Qiagen DNeasy Blood and Tissue Kit (qiagen.com).

2.3 Antimicrobial resistance testing

Antimicrobial resistance was determined by disk diffusion according to the methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). The following antimicrobial agents were included in the test panel: fucidic acid, ampicillin, amoxicillin with clavulanic acid, clindamycin, erythromycin, gentamicin, penicillin, enrofloksacin, nitrofurantoin, cefoxitin, sulfonamide and trimethoprim and tetracycline. These antimicrobials where chosen because they overlap with antimicrobials tested for in other publications of interest, thus allowing for comparison of rates of resistance. Resistance to oxacillin was used for determination of phenotypic methicillin resistance. Categorization of the isolates as resistant or susceptible towards the panel of antimicrobial agents, as well as oxacillin, was based on clinical breakpoints determined by EUCAST. Antimicrobial susceptibility testing of all isolates collected from dogs that had been abroad were carried out by the author, whereas testing of the Norwegian isolates were carried out by technicians at the routine microbiology laboratories at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences

2.4 Detection of resistance genes

PCR for detection of methicillin resistance genes *mecA* and *mecC* was carried out by previously described methods. Two positive controls were also included in the PCR, one for *mecA* and one for *mecC*. An isolate from the routine microbiology laboratory at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, was used as positive control for *mecA*. The isolate had previously been confirmed by DNA sequencing of the PCR product. NTCT 13552 was used as positive control for *mecC*. Isolates were categorized as beta-lactam resistant (methicillin resistant) based on the presence of the *mecA* or the *mecC* genes.

PCRs for detection of a number of additional resistance genes were also carried out. The resistance genes included *bla*Z (betalactam), *dfr*G (trimethoprim), *tet*M (tetracycline), *aacA-aph*D (aminoglycoside) and *erm*B (macrolides). Genes, primer sequences, amplicon size and annealing temperatures are described in detail in the attached article manuscript. Positive controls for the additional resistance genes were not available, hence amplified PCR products were sent for DNA sequencing and the isolates were then used as positive controls. PCR was for the most part carried out by the author, with some assistance from laboratory engineers at the Department of Food Safety and Infection Biology at the Norwegian University of Life Sciences.

An attempt was also made by the author to determine MIC for the isolates, using Sensititre plates from ThermoFisher Scientific. All isolates collected from the dogs that had been abroad were screened with companion animal Sensititre Plates. However, this plates proved to be inappropriate for the species of *Staphylococcus* spp. included in our study, as the MIC cut-off for several of the antimicrobials on the plate were higher than the values included on the plate.

3. Results

In total, samples were taken from 72 dogs arriving at the Oslo International Airport in the fall of 2017. Samples were taken from dogs arriving from 21 countries in Europe, as well as Singapore, Russia, Turkey, Mexico, USA and Canada. Samples were collected from dogs that had never before been to Norway, but were imported for residence, as well as Norwegian dogs that had been abroad with their owner for varying lengths of time. Of the dogs that were imported from abroad for residence, the majority were young dogs under the age of 1. The results of antimicrobial resistance testing for these dogs are presented collectively under the category “dogs from abroad,” regardless of the duration of their stay. A total of 62 samples were collected from local Norwegian dogs. 14 of these were collected from dogs belonging to student and employees at the Norwegian University of Life Sciences. These dogs were sampled by their owners. The remaining 48 samples were collected with the help of small animal clinics in the Oslo area, who sampled patients presented to the clinic for routine procedures, such as vaccinations and deworming.

3.1 Isolation of *Staphylococcus* spp.

Staphylococcus spp. was isolated from 39 of the 72 samples collected from dogs travelling to Norway from abroad (54 %). From the 62 isolates collected from local Norwegian dogs, 19 *Staphylococcus* spp. were isolated (30%).

3.2 Phenotypic resistance

Isolates showed resistance to a variety of antimicrobials when tested using disk diffusion. These results are presented in table 1. No resistance was seen towards oxacillin, indicating no methicillin-resistance was present. None of the isolates showed any resistance to nitrofurantoin. Most common was resistance to penicillin. Of the 39 isolates from dogs travelling to Norway, 9 were multi-resistant (resistant to three or more different classes of antimicrobials). None of the 19 isolates from healthy Norwegian dogs showed any multi-resistance.

3.3 Genotypic resistance

None of the isolates harbored the *mec*A- or the *mec*C -gene, supporting the results from phenotypic testing that none of these isolates were methicillin-resistant. The *bla*Z gene which mediates penicillin-resistance was found in 25 of the 39 isolates from dogs travelling from abroad, and 14/19 of the local Norwegian dogs. The gene mediating resistance to trimethoprim sulfa, *dfr*G, as well as the gene mediating resistance to erythromycin, *erm*B, was found in a small number of the dogs travelling from abroad, but not in isolates from local Norwegian dogs. None of the isolates harbored the *aacA-aph*D-gene that mediates resistance to gentamycin. A small number of isolates in both groups tested positive for the *tet*M-gene, mediating resistance to tetracyclines.

Antimicrobial ***Resistance gene***	**Group 1** **(Arriving from abroad)** N_{tot}=39	**Group 2** **(Norwegian)** N_{tot}=19
Penicillin	27	13
Ampicillin	27	13
blaZ	*25*	*14*
Amoxicillin/clavulanic acid	2	0
Oxacillin	0	0
mecA	*0*	*0*
mecC	*0*	*0*
Trimethoprim/ sulfamethoxazole	2	0
dfrG	*3*	*0*
Tetracycline	7	4
tetM	*8*	*4*
Erythromycin	5	0
ermB	*5*	*0*
Gentamicin	1	0
aacA-aphD	*0*	*ND*
Fucidic acid	1	0
Enrofloxacin	2	0
Clindamycin	8	0

Table 1 Presence of antimicrobial resistance in isolates of S. pseudintermedius in dogs from abroad and Norwegian dogs. ND=not done.

4. Discussion

The aim of this research project was to investigate the occurrence of antimicrobial resistant *Staphylococcus* spp. in dogs travelling to Norway from abroad, as well as from different healthy dog populations in Norway. The project had a particular emphasis om methicillin-resistant *Staphylococcus* spp. No methicillin-resistance was found in any of our sampled dogs, when using either phenotypic or genotypic methods. In the study from the Norwegian Veterinary Institute in 2014, methicillin-resistance was found in 2,6% of the sampled dogs. While this is more resistance than found in this research project, the prevalence is still low. This is in accordance with similar studies done in other countries, where the prevalence of MRSP in healthy carriers varies from none (Wedley et al 2014) to 4,6% (Gòmez-Sanz et al. 2011). This suggests that the rates of carriage of methicillin-resistant *Staphylococci spp.* in healthy dogs globally seems to be quite low.

Our original goal of collecting 500 samples was unfortunately not met. There seems to be a seasonal variation in when these dogs arrive, with likely most dogs arriving during major holiday periods, and with fall perhaps being a quiet period. In addition, with the EU regulations currently in place that allow for relatively free travel of dogs within Europe, with no requirement for any notification to the authorities prior to travelling with a dog to another country, the author had to wait in the customs area for dogs to show up, without any prior knowledge of whether or not any dogs would be arriving that day. In hindsight, this approach was time-consuming, and arguably, inefficient. The small sample size of our study means that the study should be viewed as a pilot study, and care should be taken not to overemphasize the significance of the findings.

Methicillin-susceptible *Staphylococci* ssp. were collected from 54% of the dogs that had been abroad, and 30% of the Norwegian dogs sampled. These numbers were expected when considering that other studies have found carriage rates for *Staphylococci* ssp. to vary from 46% to 92% (Bannoehr et al, 2009). Studies have shown that dogs with atopic skin disease are much more likely to be carriers of *S. pseudintermedius*. One study found carriage rates in dogs with AD to be over 90%, whereas *S. pseudintermedius* was only isolated from a little over 30% of the healthy dogs included in the same study (Fazakerley et al 2009). In our study, all dogs with any

signs of atopic skin disease were excluded. One can perhaps further speculate that the reason for a lower number of *Staphylococcus* spp. isolated from the local Norwegian dogs compared with the dogs travelling from abroad is due to human errors when collecting the samples, as all of these isolates were collected by owners or veterinarians not directly involved with the project.

The isolates in our study showed varying rates of resistance to other antimicrobials. 23% (9/39) of the isolates collected from dogs travelling to Norway where categorized as multi-resistant, whereas none of the Norwegian isolates where multi-resistant. In contrast, in the study carried out by the Norwegian Veterinary Institute in 2014, where 54 isolates of MRSP were studied, 81% (44/54) of the isolates showed multi-resistance. While the antimicrobial panel used in this study was not the same as in our study, this still suggests that isolates that are methicillin-resistant are more likely to carry other resistance genes as well, suggesting perhaps that resistance genes accumulate together. An interesting observation is that resistance to penicillin and tetracyclines was more prevalent in isolates from the dogs that had not been abroad, when using both phenotypic and genotypic methods to screen for resistance.

We chose to use phenotypic and genotypic methods to determine rates of resistance. While there is good correlation between the results of the phenotypic and genotypic methods, the correlation is not perfect. In cases where genotypic resistance is found, but no phenotypic resistance, one should consider the possibility that the bacterial isolate does not express the resistance gene. In the event where phenotypic resistance is proven, but not genotypic, other mechanisms or resistance genes may be relevant for consideration.

5. Conclusion

While travel increases the likelihood of becoming a carrier of MRSA in humans, results from our study do not indicate a similar trend in dogs. MRSA and MRSP was not found in the dogs that had been abroad, or the dogs that had not travelled. While a higher percentage of isolates from

dogs that had been abroad showed multi-resistance, rates of resistance against several antimicrobials where higher in Norwegian dogs when compared to those that had been abroad. In addition, our research indicates the presence of lower rates of resistance to other antimicrobials in methicillin-susceptible *Staphylococci*, when compared with those that are methicillin-resistant.

Further research is needed to answer the questions raised by the Norwegian Scientific Committee for Food Safety on how travel and an open dog population affects the spread of antimicrobial resistance. While our research has examined the presence of antimicrobial-resistance in a segment of the skin microflora, several other bacteria that are part of the microflora of a dog, for example the intestinal flora, should be examined. We would further urge that the potential for new and exotic diseases related to pathogenic bacteria, parasites and viruses should be considered when examining potential health risks posed by travelling with dogs.

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