Norwegian University of Life Sciences
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Philosophiae Doctor (PhD)
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Antimicrobial resistance in companion animals and their home environments – A One Health perspective

Antibiotikaresistens i kjæledyr og deres hjemmemiljø – fra et Én helse perspektiv

Mari Røken

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“There is such a thing as the poetry of a mistake, and when you say, "Mistakes were made," you deprive an action of its poetry, and you sound like a weasel.”

— Charles Baxter

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Mari Røken

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1 Abbreviations and definitions

AMR – Antimicrobial resistance/resistant
ARG – Antimicrobial resistance gene
AST – Antimicrobial susceptibility testing
CC – Clonal complex
CLSI – Clinical and Laboratory Standards Institute
CoNS – Coagulase-negative staphylococci
ECOFF – Epidemiological cut-off
EUCAST – European Committee on Antimicrobial Susceptibility Testing
FAO – Food and Agriculture Organization
HT-qPCR – High throughput quantitative polymerase chain reaction
ICE – Integrative conjugative elements
LPSN – List of Prokaryotic names with Standing in Nomenclature
MGE – Mobile genetic elements
MIC – Minimum inhibitory concentration
MLS – Macrolide, lincosamide, streptogramin B
MRCoNS – Methicillin-resistant coagulase negative staphylococci
MRM – Methicillin-resistant mammaliicocci
MRS – Methicillin-resistant staphylococci
MRSA – Methicillin-resistant *Staphylococcus aureus*
MRSE – Methicillin-resistant *Staphylococcus epidermidis*
MRSP – Methicillin-resistant Staphylococcus pseudintermedius
MSCRAMM – Microbial surface component recognizing adhesive matrix molecule
NETs – Neutrophil extracellular traps
ONPG – O-nitrophenyl-beta-D-galactopyranoside
ORSAB – Oxacillin Resistance Screening Agar Base
PBP – Penicillin binding protein
PCR – Polymerase chain reaction
PIA – Polysaccharide intercellular adhesin
PVL – Panton-Valentine leukocidin
PSM – Phenol-soluble modulins
qPCR – Quantitative polymerase chain reaction

SCC – Staphylococcal cassette chromosome
SBS – Sequencing by synthesis
SIG – *Staphylococcus intermedius* group
ST – Sequence type
UNEP – United Nations Environment Programme
VKM – Norwegian Scientific Committee for Food and Environment
WGS – Whole-genome sequencing
WHO – World Health Organization
WOAH – World Organization for Animal Health

2 List of papers

Paper 1

Antimicrobial resistance - Do we share more than companionship with our dogs?

M. Røken, K. Forfang, Y. Wasteson, A. H. Haaland, H.G. Eiken, S. Hagen, A. M. Bjelland

Journal of Applied Microbiology, 2022, DOI: 10.1111/jam.15629

Paper 2

Transmission of methicillin-resistant *Staphylococcus* spp. from infected dogs to the home environment and owners

M. Røken, S. Iakhno, A. H. Haaland, Y. Wasteson, A. M. Bjelland

Antibiotics, 2022, DOI: 10.3390/antibiotics11050637

Paper 3

The home environment is a reservoir for methicillin-resistant coagulase-negative staphylococci and mammaliicocci

M. Røken, S. Iakhno, Y. Wasteson, A. H. Haaland, A. M. Bjelland

Submitted to *Applied and Environmental Microbiology*

3 Abstract

Antimicrobial resistance (AMR) is disseminated in all three sectors comprised by the One Health concept: Humans, animals, and environment. Yet, much is unknown regarding the reservoirs for antimicrobial resistance in our immediate surroundings, namely our companion animals and home environments. Therefore, this thesis investigates the companion animals' and home environments' roles as reservoirs for antimicrobial resistance genes (ARGs), methicillin-resistant staphylococci (MRS), and mammaliicocci (MRM). Further does the thesis investigate the transmission potential of clinical methicillin-resistant staphylococci from infected pets to their owners and surrounding environments.

Fecal samples from healthy dogs and owners were analyzed for the presence of ARGs. Humans, companion animals, and home environments from different types of households (infected pet, healthy pet, and no-pet households) were screened for MRS and MRM. Bacterial isolates were further examined phenotypically and genotypically to determine their species identity, sequence types, SCC*mec* and to characterize their resistance and virulence genes.

By analyzing the fecal samples, we documented high rates of aminoglycoside resistance genes and *mecA*, the gene responsible for methicillin resistance, in the canine fecal samples. At the group level, dogs and owners carried many of the same resistance genes. However, only a moderate proportion of the ARGs were simultaneously present in dogs and owners from the same household, indicating a low level of transmission. Interestingly, older dogs had more fecal ARGs in common with their owners.

Furthermore, we have documented that methicillin-resistant coagulase-negative staphylococci (MRCoNS) and MRM were close to ubiquitous in the home environments, regardless of type of household or human/pet carriage of MRCoNS/MRM. In addition to harboring the *mecA* gene, the majority of isolates were multidrug-resistant, expressing resistance to three or more classes of antimicrobials. Despite the vast presence of MRCoNS and MRM in their home environments, none of the healthy companion animals tested positive for methicillin-resistant bacteria. In contrast, several cases of human and infected-pet

carriage of MRCoNS were documented, of which some isolates of the same sequence type and resistance profiles were present in the home environment.

Clinical MRS were primarily recovered from the infected dogs and locations in direct contact with dogs. Nonetheless, in half of the households, clinical MRS were present in locations out of reach for the dogs, indicating an indirect transmission. In terms of zoonotic transmission, clinical MRS were recovered from the noses of two owners. In addition, several cases of owners testing positive for methicillin-susceptible *Staphylococcus pseudintermedius* (MSSP) was documented. In all the cases, MSSP was not recovered the following day, indicating that the human carriership was temporary. The resilience of clinical MRS was demonstrated by their presence in the home environment, despite infection recovery, cleaning measures, and the euthanization of one dog.

In summary, the findings in this thesis have illuminated our immediate surroundings as reservoirs for AMR. The study contributes to a broader knowledge base for studies and risk assessments regarding AMR transmission in the interface between humans, companion animals, and home environment.

4 Norsk sammendrag

Antibiotikaresistens er utbredt i alle sektorer innebefattet av Én Helse-begrepet: Mennesker, dyr og miljø. Allikevel er det mye man ikke vet om reservoarer for antibiotikaresistens i våre nærmeste omgivelser. Denne avhandlingen undersøker derfor kjæledyrs og hjemmemiljøets rolle som reservoar for antibiotikaresistensgener, meticillinresistente stafylokokker (MRS) og mammalikokker (MRM). I tillegg fokuserer avhandlingen på overføringspotensialet til MRS fra kjæledyr til eiere og deres omgivelser.

Avføringsprøver fra friske kjæledyr og eiere har blitt undersøkt for antibiotikaresistensgener. I tillegg har mennesker, kjæledyr og hjemmemiljøet fra ulike typer husholdninger blitt screenet for meticillinresistente stafylokokker og mammalikokker. Videre ble bakterieisolater fenotypisk og genotypisk analysert for identifisering av art, sekvenstype, SCC*mec*-kassett, samt virulens- og resistensgener.

Gjennom analysen av hundeavføringsprøvene avdekket vi en høy frekvens av aminoglykosidresistensgener og *mecA*, genet som gir meticillinresistens. Hunder og eiere bar på mange av de samme resistensgenene på gruppenivå, men innad i husholdningene var det kun en moderat andel av resistensgener til felles, noe som tyder på et lavt overføringsnivå mellom hund og menneske. Interessant nok, fant vi at eldre hunder hadde flere resistensgener til felles med eierne sine enn yngre hunder.

Studien har videre dokumentert at meticillinresistente koagulasenegative stafylokokker (MRKNS) og MRM nærmest er ubikvitære i hjemmemiljøer uavhengig av type husholdning og bærerskapsstatus hos menneske/kjæledyr. Flertallet av miljøisolatene var multiresistente. Til tross for den høye forekomsten av MRKNS og MRM i hjemmemiljøet var det ingen av de friske kjæledyrene som testet positivt for MRKNS eller MRM. I stedet avdekket vi flere humane tilfeller av bærerskap, samt flere hunder som i tillegg til å ha en pågående infeksjon med meticillinresistente *Staphylococcus pseudintermedius* bar på MRKNS.

Kliniske MRS ble hovedsakelig funnet på hundene med pågående infeksjon og på steder disse hundene hadde direkte kontakt med. Vi fant også kliniske MRS på steder utenfor rekkevidde for hundene, noe som tyder på at det har forekommet indirekte overføring av bakteriene innad i husholdningen. Når det gjelder zoonotisk overføring, avdekket vi to tilfeller av humant bærerskap av kliniske MRS. I tillegg registrerte vi flere tilfeller der eiere testet positivt for meticillinsensitive *S. pseudintermedius* (MSSP). Vi kunne imidlertid ikke påvise noen tilfeller av humant bærerskap av MSSP over flere dager på rad, noe som indikerer at bærerskapet kun var midlertidig. De kliniske MRSenes motstandsdyktighet manifesterte seg ved at de kunne påvises i hjemmemiljøet flere uker uten kliniske symptomer eller tilstedeværelse av hund.

Kort oppsummert har studien belyst våre nærmeste omgivelsers rolle som reservoar for antibiotikaresistens. Funnene i studien bidrar til et bedre kunnskapsgrunnlag for utredning av risiko for overføring av resistens i krysspunktet mellom menneske, kjæledyr og hjemmemiljø.

5 Synopsis

5.1 Introduction

5.1.1 One Health

With a continuously growing human population and increasing globalization, humans, animals, and environments worldwide are connected directly and indirectly through international travel and trade. Alongside the expanding human population, the number of domesticated animals and the use of natural resources have increased, resulting in overpopulation, habitat destruction, and loss of biodiversity (Evans and Leighton, 2014). Consequently, diseases are more easily spread as humans, animals, and wildlife are connected closer together. Although the link between human, animal and environmental health was recognized already by the ancient Greeks, it took another couple of thousand years before the modern concept of "One World, One Health" was developed into a framework in 2008 (WHO and UNICEF, 2008). The framework has been variably interpreted and implemented as many definitions and approaches has been circulating in different sectors (Villanueva-Cabezas, 2022). Therefore, the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the World Organization for Animal Health (WOAH), and the United Nations Environment Programme recently agreed to promote a unifying operational definition of One Health. The new definition proclaims that: "One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems. It recognizes that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent. The approach mobilizes multiple sectors, disciplines, and communities at varying levels of society to work together to foster wellbeing and tackle threats to health and ecosystems while addressing the collective need for clean water, energy, and air, safe and nutritious food, taking action on climate change and contributing to sustainable development" (WHO, 2021).

5.1.1.1 Zoonotic diseases

Zoonotic diseases are a central part of the One Health approach. The term "zoonosis" was coined in the 19th century by the German physician and pathologist Rudolf Virchow to indicate infectious diseases transmitted from animals to humans (Schultz, 2008). Today, when referring to the term, it also comprises infectious diseases transmitted from humans to animals, sometimes referred to as "reverse zoonoses," "zooanthroponoses," or "anthroponoses" (Messenger *et al.*, 2014). Zoonotic infectious agents comprise bacteria, viruses, parasites, and prions. They can be directly transmitted through physical contact between humans and animals, or indirectly, by vectors such as mosquitos, or through contaminated food and water. Approximately 75 % of recently emerging infectious diseases in humans are of animal origin, and 60 % of all human pathogens are zoonotic (Bueno-Marí *et al.*, 2015). Bacterial zoonoses comprise a wide span of infectious agents. Some of the major bacterial zoonotic diseases are listed in Table 1.

Table 1. List of selected bacterial zoonotic diseases, agents, and some of their main host spectra. Based on the European Union One Health 2020 Zoonoses Report (European Food Safety et al.*, 2021).*

Disease	Agent	Animal hosts
Anthrax	*Bacillus anthracis*	Bison, domestic ruminants, deer, dogs, mink, pigs
Brucellosis	*Brucella* spp.	Domestic ruminants, dogs, pigs
Campylobacter enteritis	*Campylobacter jejuni, Campylobacter coli*	Domestic ruminants, cats, chickens, dogs, ferrets, pigs, mink
Hemorrhagic colitis/hemolytic uremic syndrome	Shiga toxin-producing *Escherichia coli*	Domestic ruminants
Leprosy	*Mycobacterium leprae*	Monkeys, rats, mice, cats
Leptospirosis	*Leptospira interrogans*	Wild and domestic animals, dogs
Listeriosis	*Listeria monocytogenes*	Domestic ruminants, birds
Salmonellosis	*Salmonella enterica*	Birds, cats, dogs, domestic animals
Tuberculosis	*Mycobacterium bovis, Mycobacterium caprae*	Domestic ruminants, pigs, wild boars, deer
Tularemia	*Francisella tularensis*	Wild animals including hares and rodents
Yersiniosis	*Yersinia enterocolitica*	Pigs, domestic ruminants, wild animals and birds

5.1.2 Antimicrobials and antimicrobial resistance

Closely linked to human and animal health are the advances in modern medicine. The introduction of antimicrobial medication laid the foundation for these advances by enabling physicians and veterinarians to treat infections that earlier caused high mortality rates. The reduction of bacterial pneumonia-related deaths from eighteen to less than one percent between World War I and World War II was a striking manifestation of the revolutionizing effect the introduction of antimicrobials had on human health (Markel, 2013). Today, antimicrobials are among the most prescribed medicines globally, and consumption continues to increase (Klein *et al.*, 2018; Sriram *et al.*, 2021). The use and misuse of antimicrobials have resulted in the

emergence of antimicrobial-resistant (AMR) bacteria, rendering the antimicrobials ineffective against bacterial infections. As antimicrobial resistance exists in all sectors of One Health, it is a central issue that needs to be addressed with a One Health approach.

5.1.2.1 Antimicrobials

The healing effects of mushrooms, beer yeast, and molds were known to be effective in treating infected wounds, already around 1500 BC (Duckett, 1999). However, it was not until the discovery of new antimicrobials in the late nineteenth century and the following upscaling of the production that antimicrobials revolutionized medicine. Infections could now be prevented and treated, thus facilitating the development of modern medicine as we know it today.
The terms antimicrobials, antibiotics, and antibacterial agents are often used interchangeably. However, their definitions differ slightly from each other. Antibiotics such as penicillins are natural derivatives produced by microorganisms that possess antibacterial activity at low concentrations. In contrast, antibacterial agents, e.g., sulfonamides and fluoroquinolones, are synthetic compounds designed for the same purpose (Bryskier, 2005). Antimicrobials are a broader term, referring to any substance that kills or inhibits microorganisms (Purssell, 2019). For the sake of ease, "antimicrobials" is used when referring to antibiotics and antibacterial agents in this thesis.

Antimicrobials comprise a wide variety of chemical compounds with different properties and targets. They disrupt essential processes in bacterial cells, causing either bacterial cell death or preventing cell reproduction, and are classified by their chemical structure. Hence, antimicrobials with similar structures affect similar targets. Antimicrobials can be divided into bactericidal and bacteriostatic substances (Finberg *et al.*, 2004). The bactericidal antimicrobials' action mechanisms include inhibition of cell wall- or DNA synthesis, which cause irreparable damage and bacterial cell death. Beta-lactams are the most widely used substances in this group. Other bactericidal agents include quinolones, nitrofurans, and glycopeptides. Bacteriostatic antimicrobials stop the bacteria from reproducing by, for instance, inhibiting the bacteria's protein- or folic acid synthesis. The reduced reproduction of bacteria aids the host immune system in eliminating the bacteria. Examples of bacteriostatic antimicrobials are tetracyclines, macrolides, lincosamides, and sulfonamides. Aminoglycosides are sometimes referred to as bacteriostatic antimicrobials as they target the bacteria's protein synthesis.

However, at higher concentrations, aminoglycosides display a bactericidal effect. The most widely used antimicrobial classes and their targets in the bacteria are shown in Figure 1

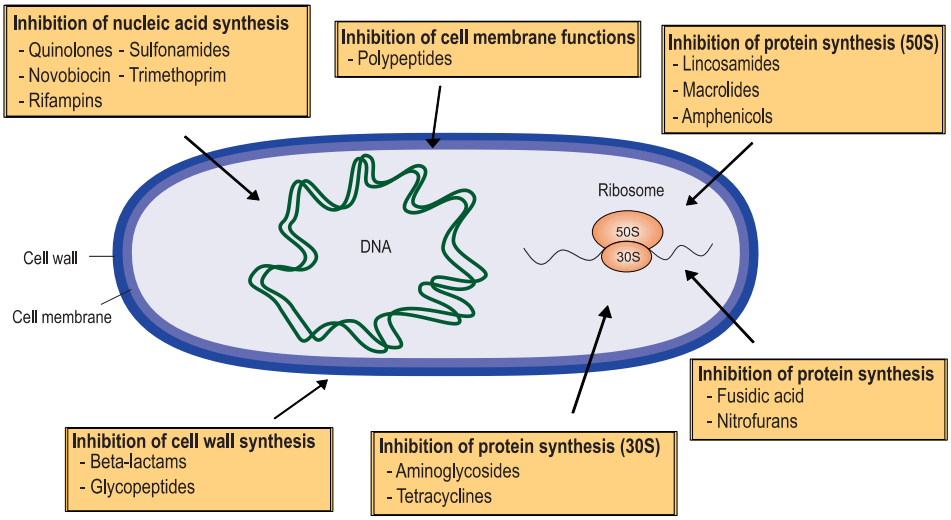

Figure 1. Modes and sites of action for antimicrobial drugs.

The golden age of antimicrobial discovery lasted between the 1940s and 1970s when more than 20 classes of natural and synthetic antimicrobials were discovered and put into use. The discovery of daptomycin in 1986 marks the preliminary end of developing new antimicrobials, as no new classes have been discovered since then (Durand *et al.*, 2019). At the same time, resistant bacteria have been selected and disseminated worldwide. Consequently, physicians and veterinarians are left with an ever-decreasing number of alternatives when choosing appropriate medication for treating infections.

5.1.2.2 Antimicrobial resistance

AMR is the ability of microorganisms to survive and grow in the presence of antimicrobials (Balaban *et al.*, 2019). It is not a human-made phenomenon. On the contrary, it is an ancient property that likely has been present for millions of years due to the microorganisms' evolutionary strategy to survive and compete for resources in the presence of other microbes (Holmes *et al.*, 2016). One strategy is to produce antimicrobial compounds to outcompete neighboring organisms. Another is to defend themselves against the toxic compounds produced by others. Although

AMR is not a consequence of human activity on its own, the excessive human- and agricultural use of antimicrobials has escalated the selection of resistant bacteria. Bacteria have proven to be able to adapt to environmental threats rapidly. Following the introduction of new antimicrobials to the market, resistance to nearly all antimicrobials has been detected (Ventola, 2015) (Figure 2). In fact, the level of resistance has become such a significant problem that the WHO has listed AMR as one of the top ten threats to global health (WHO, 2019). In 2019, an estimated 1.27 million human deaths were attributable to AMR bacteria, and this number is expected to increase to as many as 10 million by 2050 (Murray *et al.*, 2022).

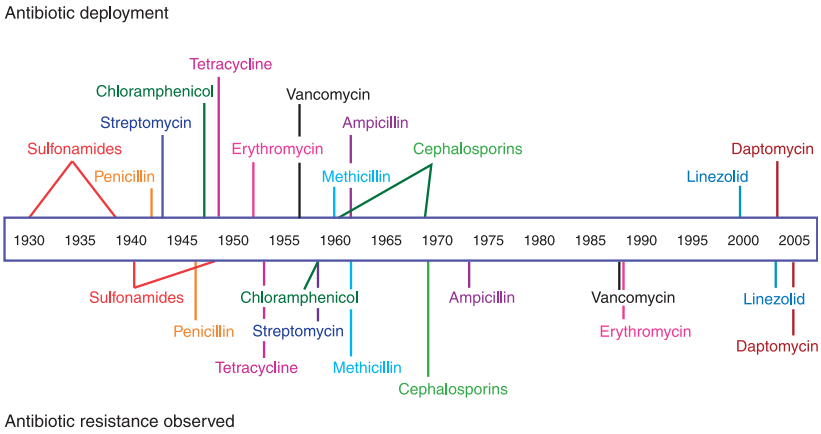

Figure 2. Timeline of antimicrobial deployment and the evolution of antimicrobial resistance. The year the antimicrobials were deployed is indicated above the timeline, and the year resistance to each antimicrobial first was observed is indicated below the timeline. Reprinted by permission from Springer Nature, Nature Chemical Biology, (Clatworthy et al., *2007).*

5.1.2.3 Resistance mechanisms

The mechanisms responsible for AMR may be innate or acquired and includes five main resistance mechanisms (Figure 3). Firstly, preventing the antimicrobials from reaching their target by reduced permeability is a widespread mechanism in Gram-negative bacteria. The outer membrane of Gram-negative bacteria provides a physical barrier that the drugs need to penetrate. In addition, the outer membrane contains porins which can be reduced in numbers, replaced, or altered structurally to prevent drug molecules from entering (Delcour, 2009). A second strategy is the active elimination of antimicrobials through drug efflux pumps, denying the antimicrobials access to their targets. Bacteria may possess different types of efflux pumps, some drug-specific and some capable of transporting a variety of compounds, namely multidrug efflux pumps (Reygaert, 2018). Target modification is a third strategy that involves altering of bacterial components, thus blocking or reducing the drugs' ability to bind to their target molecules (Reygaert, 2018). A fourth strategy is target protection, a mechanism involving a protein physically associating with an antimicrobial target to inhibit the binding of the drug molecule (Wilson *et al.*, 2020). The final mechanism involves drug inactivation, which comprises either actual enzymatic degradation of drugs or transfer of a chemical group to the drug molecules, thereby diminishing the amount of drug available to bind to their target. (Reygaert, 2018)

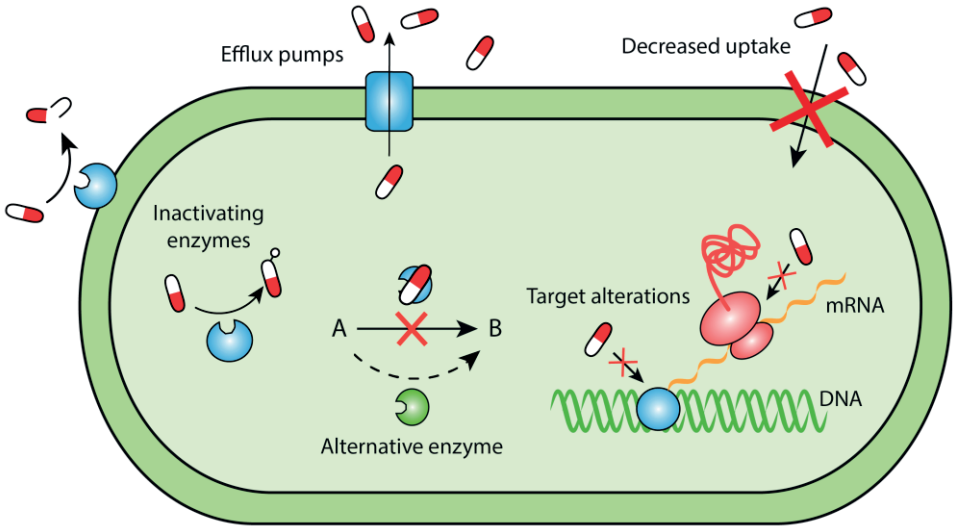

Figure 3. Mechanisms for antimicrobial resistance (Gullberg, 2014)

Intrinsic resistance occurs when bacteria are naturally resistant to certain antimicrobials and is related to the general physiology of the bacteria. For instance, Gram-negative bacteria are resistant to glycopeptides due to their outer membrane that prevents the glycopeptides from entering their cell wall. When discussing the problem of increasing AMR, intrinsic resistance plays a minor part. Acquired resistance, on the other hand, has a significant role in disseminating AMR (Munita and Arias, 2016). Bacteria can acquire resistance either by spontaneous mutations in chromosomally located genes or by horizontally acquired resistance genes. The short replication cycle of bacteria facilitates rapid adaptation to their environment. Spontaneous mutations occur during a replication cycle, potentially resulting in altered gene products, sometimes advantageous to the bacteria.

5.1.3 Horizontal gene transfer

Horizontal gene transfer (HGT) plays a central part in bacterial evolution, including the dissemination of antimicrobial resistance genes (ARGs). HGT involves sharing of DNA between cells that are not in a parent-offspring relationship, thus compensating for the disadvantages asexual reproduction entails (Soucy *et al.*, 2015). The three main mechanisms for horizontal gene transfer include transformation, transduction, and conjugation (Figure 4). Transformation comprises the uptake of foreign DNA, which subsequently is integrated by homologous recombination in the bacterial chromosome. The transformation process requires no cell-to-cell contact. Instead, it depends on genetic competence, meaning that the bacteria possess competence proteins enabling binding, uptake, and processing of exogenous DNA. Around 80 naturally competent bacterial species have been reported to date. However, only a few of these are clinical pathogens, such as *Streptococcus pneumoniae*, *Helicobacter pylori,* and *Staphylococcus aureus* (Johnston *et al.*, 2014). Transduction involves the transfer of DNA from a donor via bacteriophages (viruses that infect bacteria) to a recipient bacterium. The transduction process involves phages accidentally packaging host cell DNA segments into their capsid, followed by injection of the DNA into a new host cell. The injected DNA can then recombine with the new host cell's chromosome. The incorporation into the new host genome requires a high degree of similarity between the involved DNA segments. Hence, transduction is limited to members of the same bacterial species (Frost *et al.*, 2005). Conjugation is a sexual mode of transfer that requires physical contact between a donor and a recipient cell via a conjugation pilus through which double or single-stranded DNA is transferred. Of the three HGT mechanisms, conjugation is considered to have the greatest influence

on the dissemination of ARGs, as it enables the transfer of ARG-containing plasmids between unrelated bacteria (Carattoli, 2013; von Wintersdorff *et al.*, 2016).

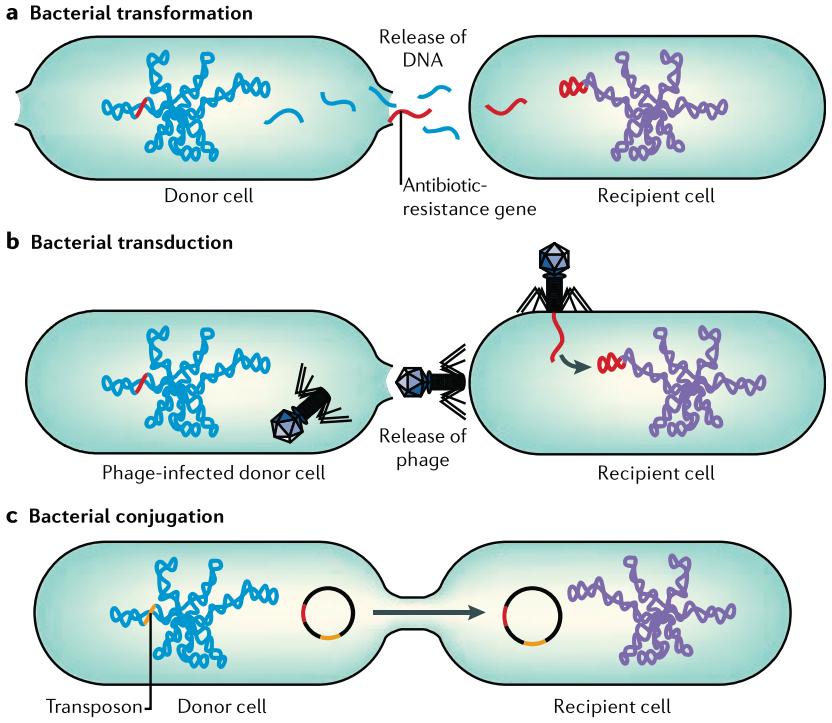

Figure 4. Horizontal gene transfer between bacteria. ***a****) Transformation occurs when another bacterium takes up naked DNA from a lysed bacterium. The antimicrobial resistance gene (ARG) can be integrated into the chromosome or plasmid of the recipient cell.* ***b****) In transduction, ARGs are transferred from a donor to a recipient cell through bacteriophages and can be incorporated into the recipient cell's chromosome.* ***c)*** *Conjugation occurs by direct contact between two bacteria. The plasmids form a mating bridge across the bacteria, and DNA is exchanged. Adapted with permission from Springer Nature, Nature Reviews Microbiology (Furuya and Lowy, 2006).*

Transduction and conjugation are facilitated by mobile genetic elements (MGEs). MGEs are protein-encoding DNA segments that promote intra- and extracellular movement of DNA (Partridge *et al.*, 2018). In addition to their core genes, MGEs typically carry several accessory genes that can favor their host cells, such as ARGs and virulence genes (Frost *et al.*, 2005). MGEs comprise a variety of elements of different sizes and properties. They can be divided into two groups: Those

facilitating intercellular transfer: Plasmids, integrative conjugative elements (ICEs), bacteriophages, and those involved in intracellular mobility: insertion sequences (ISs) and transposons. Plasmids are extrachromosomal circular, sometimes linear, DNA of varying sizes that can replicate independently from the bacterial chromosome. In addition to harboring acquired resistance genes, they can carry other MGEs and accessory genes that are dispensable to the host cell. They are intercellularly transferred by conjugation, in most cases via the type IV secretion system (Norman *et al.*, 2009). The conjugation process is mediated by the plasmids themselves (conjugative plasmids) or by ICEs that encode proteins facilitating their own transfer or transfer of other cellular DNA segments, including plasmids. Bacteriophages are the last group of MGEs that can be transmitted intercellularly. These are integrated as prophages in the bacterial genome, or in some cases, exist as plasmids within the bacteria cytoplasm (Colavecchio *et al.*, 2017). External factors such as stress may induce excision of phages from the chromosome, leading to the formation of phage particles and lysis of the host cell. The phages may subsequently infect other susceptible cells, thereby transferring DNA that is integrated into the recipient cells' chromosomes by homologous recombination (Colavecchio *et al.*, 2017).

The ISs and transposons, sometimes referred to as jumping genes, are capable of moving between different DNA molecules or within a DNA molecule. The ISs are small elements (>2.5-3 kilo base pairs) that typically carry one or two transposase genes, usually flanked by inverted repeats (Mahillon and Chandler, 1998). They can occur singly or as inverted repeats in composite transposons that can carry accessory genes, for instance, ARGs (Siguier *et al.*, 2014). Though ISs and transposons are not capable of intercellular transfer themselves, they can move horizontally as part of other MGEs.

Integrons are closely linked to the dissemination of resistance, especially in Gram-negative bacteria, due to their ability to capture and express ARGs embedded within gene cassettes (Lacotte *et al.*, 2017). Although the integrons are not mobile, they are still important vectors for spreading ARGs as they can be integrated into MGEs, including transposons and conjugative plasmids (Gillings, 2017). Among the five classes of integrons, the class 1 integrons are the most significant in the clinical context, as they can embed a broad spectrum of ARGs and genes conferring resistance to heavy metals, and their presence continues to increase in pathogenic bacteria (van Essen-Zandbergen *et al.*, 2007; Sütterlin *et al.*, 2020). The integrase,

encoded by *intI1*, mediates the insertion, excision, and shuffling of genes in class 1 integrons. The SOS response regulates the *intI1* expression, a bacterial stress response that may be induced by exposure to antimicrobials such as trimethoprim, quinolones, and beta-lactams (Guerin *et al.*, 2009). Thus, antimicrobial therapy not only enables resistant bacteria to become the predominant species in a population but also facilitates the capture and dissemination of resistance genes.

5.1.4 Methods for detecting resistance and resistance genes

Antimicrobial susceptibility testing (AST) can serve several purposes. One purpose is to detect resistance in clinical isolates or confirming susceptibility to chosen antimicrobial agents (Reller *et al.*, 2009). Bacteria are categorized as susceptible or resistant in clinical settings based on breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or by the Clinical and Laboratory Standards Institute (CLSI). Both institutions base the defined breakpoints on minimum inhibitory concentration (MIC) values, which are defined as the lowest concentration of an antimicrobial agent that inhibits visible bacterial growth (Andrews, 2001). Although CLSI and EUCAST base their breakpoints on MIC values, there are discrepancies between their breakpoints, which may impact the interpretation of clinical isolates (Cusack *et al.*, 2019).

Another purpose for AST is to monitor AMR and aims to determine whether acquired resistance is present in a population of bacteria. In this setting, epidemiological cut-off values (ECOFF) are used, which are defined as the highest MIC values of microorganisms lacking phenotypically expressed resistance. The ECOFF distinguishes between isolates in the wild type (susceptible) population and isolates with some type of acquired mechanisms that reduces the susceptibility of these isolates (Aarestrup *et al.*, 2007).

AST can be performed using several methods. The phenotypic methods are currently most used in clinical laboratories (Benkova *et al.*, 2020), but the genotypic methods are rapidly gaining increased popularity alongside the advances in sequencing technology.

5.1.4.1 Phenotypic testing

A number of phenotypic tests are available for detecting resistance, including broth dilution, disk diffusion, and test strip (Figure 5). Micro broth dilution involves a series of two-fold dilutions of antimicrobials in Mueller-Hinton broth in wells on plates. Each dilution is inoculated with standardized quantities of bacteria suspension before incubating the plate for 16- 20 hours. Bacterial growth is then

visually examined or read with automated instruments to determine the bacteria's MIC.

Agar disk diffusion is a commonly used method in routine clinical microbiology laboratories as it is suitable for testing the majority of bacterial pathogens, including fastidious bacteria, and requires no special equipment (Matuschek *et al.*, 2014). The technique involves inoculating a standardized quantity of bacterial suspension onto a Mueller-Hinton agar plate, followed by applying filter paper disks impregnated with standardized concentrations of antimicrobial agents and overnight incubation. The incubation time varies between bacteria but is usually between 16-20 hours. The antimicrobials gradually diffuse into the agar, creating a concentration gradient that decreases further from the disk. After incubation, the inhibition zones are read to the nearest millimeter and categorized as susceptible, intermediate, or resistant according to standardized clinical breakpoint tables (CLSI, 2022; EUCAST, 2022). A third method for testing antimicrobial susceptibility involves test strips and can be regarded as a hybrid between disk diffusion and micro broth dilution. In this method, plastic strips coated with decreasing concentrations of antimicrobials are applied on Mueller-Hinton agar plates pre-inoculated with bacteria (Khan *et al.*, 2019). After incubation, an ellipse appears that intersects the MIC reading scale, printed on the strip, where the concentration of the antimicrobial inhibits the bacterial growth. This concentration corresponds to the MIC value of the tested bacteria.

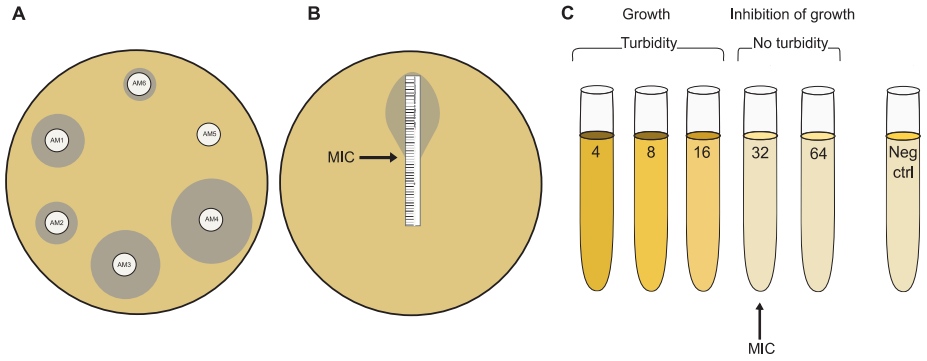

Figure 5: Illustration of agar disk diffusion (A), test strip coated with antimicrobials (B), and broth dilution (C). Methods B and C determine minimum inhibitory concentrations (MICs) for the bacteria tested.

5.1.4.2 Genotypic testing

Several molecular methods are available for detecting genes conferring AMR. The polymerase chain reaction (PCR) is a central technique for detecting specific genes or other segments of interest in a genome, including ARGs and virulence genes. It involves using short synthetic DNA fragments called primers to selectively amplify specific segments of DNA through a series of thermal cycles (Scitable, 2014). The amplified DNA can be visualized on an electrophoresis gel (Figure 6), thus determining whether the gene is present or used for further downstream analyses such as sequencing. Other techniques originating from PCR, such as multiplex PCR and quantitative PCR (qPCR), are commonly used to detect genes. Multiplex PCR enables simultaneous detection of multiple targets in a single reaction. The amplified products can subsequently be separated by size by gel electrophoresis or by probe color using qPCR. Like regular PCR, qPCR reveals the presence of genes in addition to determining the amount of the amplified product, enabling the calculation of the original copy number in the template DNA (Dymond, 2013). This is facilitated by adding fluorescent dyes or probes in the PCR mixture, which emit increasingly strong fluorescent signals alongside increasing concentrations of amplified DNA products. The qPCR cycling instrument monitors and registers the signals, providing amplification curves. By using different colored probes, multiple targets can be analyzed in the same reaction.

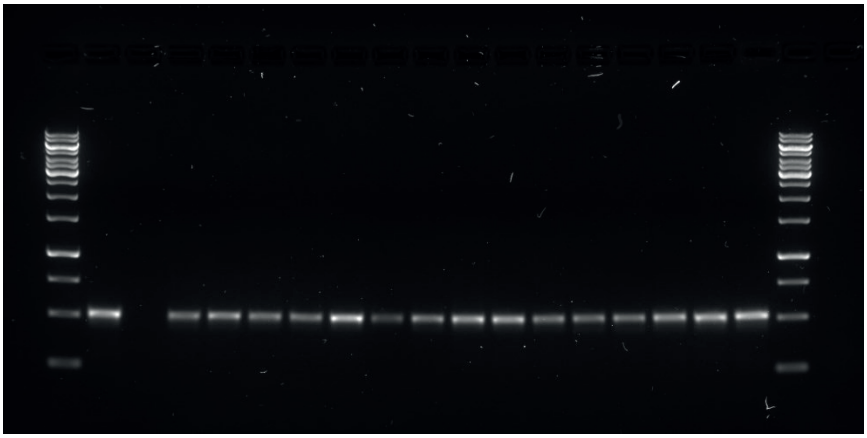

Figure 6. Amplified PCR product, in this case mecA*, visualized on an electrophoresis gel. Lane 1 and Lane 20: DNA ladder 1kB, Lane 2 and 19:* mecA *positive controls. Lane 3: Negative control. Lane 4-18:* mecA *positive samples.*

The introduction of whole-genome sequencing (WGS) into laboratory diagnostics has unlocked numerous possibilities for analyzing bacteria, including detecting ARGs. Whereas the PCR-based methods require knowledge about resistance genes and primers for detecting AMR, WGS predicts resistance by unveiling the entire collection of ARGs (resistome) present in a single isolate (Franklin *et al.*, 2021). Moreover, it provides information on allelic profiles, which can be used to track outbreaks of pathogenic bacteria. Although WGS theoretically can identify all ARGs present in an isolate, it requires accurate, up-to-date gene reference databases. Several databases are suited for the purpose, including the Comprehensive Antibiotic Resistance Database (CARD), MEGARes, and ResFinder (Lakin *et al.*, 2017; Alcock *et al.*, 2020; Florensa *et al.*, 2022). Uncovering the full resistance potential in a bacterial isolate does not necessarily mean that the phenotypic resistance profile corresponds with the predicted one, as the genes are not necessarily expressed. Nonetheless, WGS has proved to be a reliable predictor of phenotypic resistance and can, in many cases, define multidrug resistance with greater accuracy than phenotypic testing (McDermott *et al.*, 2016; Ellington *et al.*, 2017). The sequencing data can originate from various sequencing platforms, which produce outputs of different lengths. The short-read sequencing platforms such as Illumina typically provide DNA sequences ranging from 100 to 600 base pairs (bp), lengths far from covering whole genes. Consequently, the method relies on accurate assembly of overlapping sequences to reconstruct the genome of interest. A more detailed description of Illumina sequencing can be read in section 5.1.4.3 ("Illumina sequencing and workflow") below. Long-read sequencing technologies cover longer segments of the genome, usually between 5000 and 30000 bp, thus, simplifying a precise assembly of the genome. The low accuracy has been a major drawback for the long-read sequencing technologies, as the error rates could be as high as 10-15 % only a few years ago (Weirather *et al.*, 2017). Recent advances have reduced the base calling inaccuracy to between ~1 and 5 %, making these technologies more attractive for analyzing genomes (Amarasinghe *et al.*, 2020).

Metagenomic sequencing is another molecular technique of sequencing all microbial genomes within a sample. The technique is applied to composite samples and can determine the full resistome in a population of bacteria (Franklin *et al.*, 2021). One of the method's main advantages is that it requires no cultivation of bacteria prior to analysis. Therefore, fastidious or unculturable bacteria that otherwise can be challenging to study may be analyzed using this method. Shotgun metagenomic sequencing involves random fragmentation of DNA, followed by sequencing, before

contigs of DNA are assembled based on overlapping sequences. The contigs can further be aligned to a reference database for taxonomic classification or run against the databases mentioned above to identify ARGs.

5.1.4.3 Illumina sequencing and workflow

Illumina sequencing is a sequencing by synthesis (SBS) technology developed from Sanger sequencing. The method consists of three steps: Sample preparation, cluster generation, and sequencing. During sample preparation, DNA is randomly fragmented and ligated to adapter sequences. The adapter-ligated fragments are loaded into a flow cell for cluster generation, where the adapter sequences bind to complementary oligonucleotides on the flow cell. PCR clonally amplifies all bound fragments into distinct clusters through bridge amplification. The templates are then ready for sequencing, in which the sequencing machine detects and registers nucleotides from unique fluorescent signals. The sequencing process generates fastq files with millions to billions of reads with designated quality scores.
Bioinformatical pipelines, or workflows, usually begin with sequence quality checks. The Illumina output data are fastq files containing the sequences with their respective quality scores The fastqc tool can be used to assess the quality of the reads and detect sequence adapters. In the following trimming process, low-quality reads and adapter sequences are removed before downstream analysis. After trimming, the reads are assembled into longer, contiguous sequences (contigs) based on overlapping reads in a step called *de novo* assembly. SPAdes is one of the most commonly used genome assemblers for bacterial genomes and is based on the de Bruijn graph algorithm. Briefly explained, the reads are broken into smaller fragments of a specific length (k-mers) before a graph is constructed based on identified overlaps. Overlaps are then connected by an edge and assembled into contigs. Assembled genomes can be used downstream in multitudes of analyses, including identifying resistance genes by running the assemblies against antimicrobial resistance gene databases.

5.1.5 Reservoirs of resistance

To understand and mitigate the dissemination of AMR, it is crucial to map the reservoirs for AMR. In the following sections, the gut, the skin, and the environment's roles as reservoirs roles will briefly be introduced.

5.1.5.1 The gut as an AMR reservoir

The gastrointestinal tract of mammals harbors an enormous and complex population of microorganisms, termed the gut microbiota. An estimated 10^{12} to 10^{14} microorganisms form the gut microbiota, making it the most bacteria-dense surface in the body (Gill *et al.*, 2006; Thursby and Juge, 2017; Pilla and Suchodolski, 2020). The bacterial load varies along the gastrointestinal tract, with the highest numbers present in the distal intestinal segments. The microbiota is dominated by strictly anaerobic commensal bacteria whose functions span from digestion of complex carbohydrates to synthesis of essential amino acids and vitamins. Though these commensals are essential for their hosts, they may also harbor ARGs, which in turn can be transferred to pathogenic bacteria such as members of the *Enterobacteriaceae* and *Enterococcaceae* families. These bacteria are ubiquitous in the mammalian intestinal tract, though in considerably lower numbers than the anaerobic commensals (van Schaik, 2015). The gut microbiota's composition is relatively stable in healthy adult humans and animals (Jakobsson *et al.*, 2010; Thursby and Juge, 2017). However, when exposed to antimicrobials, the composition drastically shifts toward a more resistant gut flora that can persist for years (Jakobsson *et al.*, 2010). This persisting resistance is partly due to the lateral transfer of ARGs, as the conditions in the gut are ideal for HGT. Not only is the gene pool enormous, consisting of at least a thousand different ARGs at the human population level (Hu *et al.*, 2013). Also, the high cell density favors conjugation, the primary mechanism for transferring ARGs (Huddleston, 2014). Finally, clinical class 1 integrons are considered universal in the gut microbiota, facilitating the accumulation of ARGs in their bacterial hosts (Gillings, 2017).

The *Enterobacteriaceae* and *Enterococcaceae* are of particular interest when it comes to the spread of AMR, as members of these families have emerged as multidrug-resistant opportunistic pathogens in both human and veterinary medicine during the past decades (Pitout and Laupland, 2008; Wieler *et al.*, 2011).

5.1.5.2 The skin as an AMR reservoir

Bacteria form a large part of the skin microbiota, with *Corynebacterium* spp., *Streptococcus* spp., and *Staphylococcus* spp. as the dominating species in humans (Byrd *et al.*, 2018). Like in humans, staphylococci and *Corynebacterium* spp. are among the predominating species, together with *Pseudomonas* spp., in healthy dogs and cats (Older *et al.*, 2017; Chermprapai *et al.*, 2019) Of these, *Staphylococcus* spp. and *Pseudomonas* spp. hold the most extensive repertoires of AMR. The *Staphylococcus* spp. resistance potential will be described in detail in section 5.1.7 ("Resistance in staphylococci") of this thesis. *Pseudomonas* spp. comprises more than 200 species, of which some are opportunistic pathogens of humans, animals, and plants. Multidrug resistance is widespread in *Pseudomonas* spp. as they are intrinsically resistant to a broad range of antimicrobials and rapidly acquire ARGs (Lupo *et al.*, 2018). Among the *Pseudomonas* spp., *Pseudomonas aeruginosa* is the most frequently reported pathogen, often involved in otitis externa and other skin infections. The therapeutic options for treating *P. aeruginosa* are currently limited to such an extent that it takes part in the ESKAPE list of bacteria, a list of highly virulent and increasingly resistant pathogens (Mulani *et al.*, 2019).
Although the skin microbiota in healthy individuals has shown to be stable over time (Byrd *et al.*, 2018), the skin can act as a temporary residence for bacteria, otherwise not associated with skin, such as intestinal bacteria. These bacteria can, in turn, be transmitted to other individuals or the surrounding environment A prime example of this is the hands, which are in contact with countless surfaces during the day, thus functioning as mechanical vectors for microbes. Inadequate hand hygiene is an important cause of spreading AMR bacteria, especially in hospitals where resistant bacteria are frequent (WHO, 2017; Espadale *et al.*, 2018).

5.1.5.3 The environment as an AMR reservoir

AMR is ubiquitous in the environment as bacteria, through evolution, have developed protective mechanisms against antimicrobials that are naturally produced by a broad range of saprophytic organisms (Holmes *et al.*, 2016). Although many environmental microorganisms produce antimicrobial substances, little evidence supports that this contributes significantly to the selection of AMR bacteria (Martinez, 2009). Instead, human activity is responsible for a big part of AMR spread in the environment. This is especially evident in regions standing for a large part of the global production of antimicrobials (Lundborg and Tamhankar, 2017). Pharmaceutical plants release large amounts of antimicrobials into the environment. Some agents, like penicillins are quickly degraded, while substances

like fluoroquinolones, sulfonamides and tetracyclines are more persistent, causing them to accumulate in higher concentrations (Lundborg and Tamhankar, 2017). Consequently, the environmental flora is exposed to antimicrobials, forcing the selection of resistant bacteria. Moreover, many of the antimicrobial agents used in animals and humans are not completely metabolized and are excreted as parent compounds or as metabolites through urine and feces (Wasteson *et al.*, 2020). The metabolites are subsequently introduced to the wastewater system (if present), where they may interact with bacteria, selecting resistant strains, which can be spread to water and food-producing land depending on how the wastewater is treated (Paulshus, 2020).

Indoor environments may also serve as reservoirs for AMR. Hospitals and veterinary clinics are known hotspots for AMR bacteria, as these locations frequently use of antimicrobials and are often frequented by patients suffering from infections (Guardabassi and Prescott, 2015; D'Accolti *et al.*, 2019). The most common nosocomial pathogens are often associated with AMR and are also known to persist for weeks to months in dry environments (Kramer *et al.*, 2006; Boyce, 2007). Combined with a high proportion of immunocompromised patients, human and veterinary hospital outbreaks of multidrug-resistant bacteria are not uncommon (Haenni *et al.*, 2012; Grönthal *et al.*, 2014; Calbo and Garau, 2015). Patients and employees may further spread these pathogens outside the clinic environments and establish reservoirs in the community.

5.1.6 Staphylococci

5.1.6.1 Historical perspective

Staphylococci were first discovered in 1880 by the British surgeon Alexander Ogston. When examining pus from an abscess, he observed clustered micrococci. Ogston named the bacteria "staphylococci" from the Greek word "staphyle," meaning bunch of grapes, and "kokkos," meaning berry. (Licitra, 2013). In 1884, the German physician and microbiologist Friedrich Julius Rosenbach provided the first formal description of the genus *Staphylococcus* and divided the genus into two species based on the pigmentation of the colonies: *Staphylococcus aureus* (golden) and *Staphylococcus albus* (white) (Rosenbach, 1884). The number of identified species within the genus *Staphylococcus* has increased since and is continuously changing alongside the advances in genome-based classification technology. A search in the LPSN database reveals that per April 2022, 84 different staphylococcal species, of which some have been reclassified into other genera, e.g., *Staphylococcus sciuri, Staphylococcus vitulinus,* and *Staphylococcus fleuretti* which now are within the genus *Mammaliicoccus* (Parte *et al*., 2020).

5.1.6.2 Differentiation of staphylococcal species

Staphylococci are non-motile, Gram-positive cocci of approximately 0.5-1.5 µm in diameter. Upon microscopic examination, they occur singly, in pairs, tetrads, irregular clusters, or occasionally in short chains (Figure 7). Staphylococci are facultatively anaerobic, except for the strictly anaerobic species *S. aureus* ssp. *anaerobius* and *Staphylococcus saccharolyticus* (De Vos et al., 2009).
Differentiation of staphylococcal species has traditionally been based on colonial morphology, hemolysis in sheep, rabbit, or ox blood agar, coagulase test, novobiocin susceptibility, biochemical profiles, and molecular tests.

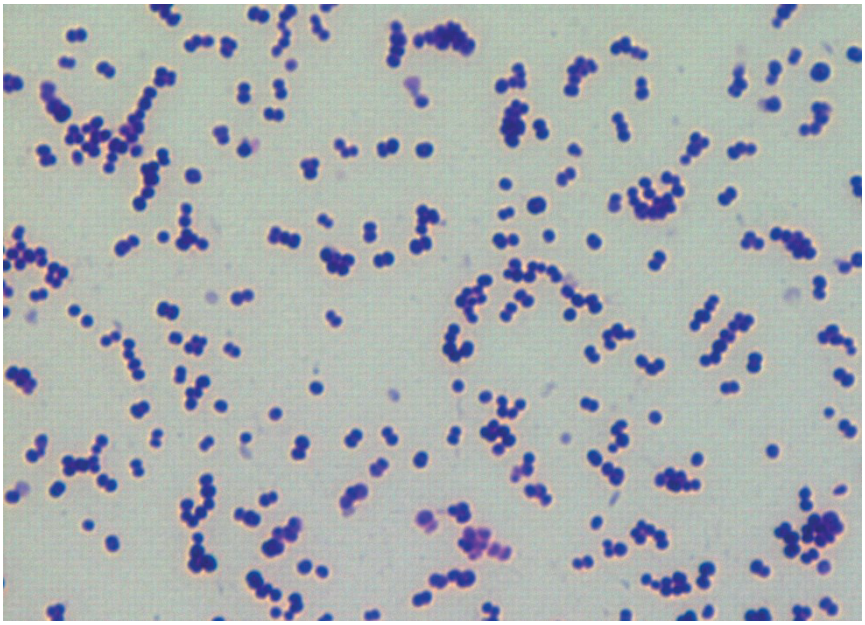

Figure 7. Microscopy picture of Staphylococcus epidermidis. *Cells appear singly, in pairs, tetrads, chains, and clusters.*

On blood agar, staphylococcal colonies usually appear as convex, opaque, pigmented white to grey, cream, or golden yellow, with a colony size varying between 1-4 mm in diameter. Staphylococcal colonies may appear beta-hemolytic, double hemolytic, or non-hemolytic, depending on which species is cultured. (Foster, 1996).
The coagulase test differentiates between coagulase-negative staphylococci and coagulase-positive (CoNS and CoPS, respectively). This subdivision is based on the staphylococci's ability to clot rabbit blood and has traditionally been a key step in differentiating staphylococcal species in routine diagnostic laboratories. Most staphylococcal species do not produce staphylocoagulase or von Willebrand factor-binding protein. Hence, they are referred to as CoNS. This group can further be differentiated by their resistance or susceptibility to novobiocin. Novobiocin-resistant CoNS are referred to as the "*Staphylococcus saprophyticus* group," while susceptible isolates belong to the "*Staphylococcus epidermidis* group."

Biochemical profiling, including sugar fermentation, β-galactosidase, and urease production, has been and is still used to differentiate between staphylococcal species. Species identification based on phenotypic tests can be laborious and time-consuming. Commercial kits such as API Staph (bioMérieux, Lyon, France) comprise

several biochemical tests, thereby reducing the workload. However, the interpretation of the test results is subjective and may vary between laboratories. In addition, phenotypic differentiation between the members of the *Staphylococcus intermedius* group (SIG) is highly complex, if not impossible (Bond and Loeffler, 2012; Murugaiyan *et al.*, 2014). Therefore, other methods may be necessary for species differentiation. Molecular methods for identifying staphylococci often include PCR with several gene targets such as *tuf, hsp60*, *sodA*, *femA*, and the *rpoB* gene (Kosecka-Strojek *et al.*, 2018). 16S rRNA sequencing is not reliable as the gene has a high degree of sequence similarity between several staphylococcal species (Kwok et al., 1999). Another method that has emerged in recent years is matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS). The MALDI-TOF analyzer generates species-unique mass spectral profiles based on the time of flight of ions released from bacterial proteins during laser desorption. Provided a database containing profiles from a large number of bacteria, MALDI-TOF enables species identification within minutes and can distinguish most staphylococcal species, including the CoNS (Croxatto et al., 2012).

5.1.6.3 Staphylococci in humans

S. epidermidis is the most frequently recovered and persistent staphylococcal species on the human skin, where they constitute the majority of the commensal microflora (Kloos and Musselwhite, 1975; Gara and Humphreys, 2001). The largest population of *S. epidermidis* is usually found in moist areas such as the anterior nares, axillae, perineal, and inguinal areas, but they may be recovered from all surfaces on the body (De Vos et al., 2009*)*. Following *S. epidermidis, Staphylococcus hominis* and *Staphylococcus haemolyticus* are the most frequently isolated CoNS species from human skin (Becker *et al.*, 2014; Heilmann *et al.*, 2019). They prefer moist areas with a high density of apocrine glands and are often recovered from the axillae, inguinal, and perineal areas (De Vos *et al.*, 2009). Likewise, *S. saprophyticus* frequently colonizes the inguinal and perineal areas. Additionally, one of the major reservoirs for *S. saprophyticus* is the human gastrointestinal tract, where it is present in as many as 40 % of all humans (Becker *et al.*, 2014).

Though CoNS were solely considered to be harmless skin and mucosal commensals for a long time, they are increasingly recognized as opportunistic pathogens. Changes in the human population, with increased numbers of elderly, chronically ill, and immunocompromised patients, have led to an acknowledgment that CoNS are responsible for a variety of nosocomial infections The infections caused by CoNS are often associated with indwelling medical devices, such as venous catheters (Becker

et al., 2014). Following their placement, the patients' commensal flora, or CoNS from already contaminated devices, airborne dust, or healthcare workers, are introduced to sites usually inaccessible to the CoNS. The bacteria can subsequently cause infections at the insertion site or disseminate from the colonization site via the cardiovascular system, causing infections ranging from endocarditis to sepsis and meningitis (Vergnano *et al.*, 2005; Vogkou *et al.*, 2016). *S. epidermidis* is by far the most frequently recovered bacteria from inserted devices, followed by *S. haemolyticus, S. hominis,* and *S. saprophyticu*s (Heilmann *et al.*, 2019). The latter species is a prime example that not all CoNS species are dependent on the aid of invasive devices to cause infections as *S. saprophyticus* is a major uropathogen, especially in young women (Lawal *et al.*, 2021).

Another common skin- and mucosa-associated commensal in humans is the coagulase-positive species, *S. aureus.* Approximately 20-40% of the adult human population are asymptomatic carriers of *S. aureus* (Sollid *et al*., 2014). The carriage rates vary by geographical location, age, and sex. Common colonization sites include the anterior nares, perineum, pharynx, and axillae. Though *S. aureus* is a commensal, it is also an opportunistic pathogen causing various soft tissue infections in humans, including impetigo, toxic epidermal necrolysis, and mastitis. *S. aureus* is also responsible for invasive infections such as sepsis, acute endocarditis, osteomyelitis, and meningitis, making it by far the most pathogenic human staphylococcal species (De Vos *et al.*, 2009).

5.1.6.4 Staphylococci in companion animals

Staphylococcus pseudintermedius is the canine equivalent to *S. aureus,* a coagulase-positive skin- and mucosal membrane commensal, causing infections ranging from pyoderma and wound infections to more invasive infections like septicemia. Previously identified as *S. intermedius*, *S. pseudintermedius* was separated into its own species in 2005 based on phenotypical characteristics and molecular techniques (Devriese *et al.*, 2005). Approximately 50-90% of dogs are colonized by *S. pseudintermedius.* Common colonization sites include the perineum, mouth, nose, anus, and groin (Bannoehr and Guardabassi, 2012). Though primarily associated with the *Canidae* family, cats can also be colonized. However, the carriage rates are reported to be lower (Bierowiec *et al.*, 2021). Additionally, *S. pseudintermedius* is increasingly recognized as an opportunistic human pathogen (Börjesson *et al.*, 2015; Somayaji *et al.*, 2016). With advances in diagnostic methods, particularly with the entry of MALDI-TOF-MS in diagnostics, members of the SIG can be correctly

identified in specimens from humans (Yarbrough Melanie *et al.*). Companion animals may also carry CoNS, although the reported carrier rates among dogs and cats are not as high as in humans (Abdel-Moein and Zaher, 2020). Common species include *Mammaliicoccus sciuri, Staphylococcus felis* (in cats), and *S. haemolyticus* (Ruzauskas *et al.*, 2015; Elnageh *et al.,* 2021). As in humans, CoNS may cause infections via medical devices in companion animals. However, as CoNS do not dominate their skin flora, Gram-negative bacteria are more frequently isolated (Marsh-Ng *et al.*, 2007).

5.1.6.5 Staphylococci in the environment

Given that the mammals' skin and mucosal membranes constitute the most extensive reservoir for staphylococci, environments closely related to humans and animals are common niches for environmental staphylococci. Staphylococci can survive and multiply in a variety of environments, including soil, food, dust, and water. This broad range of reservoirs is possible due to their hardy nature. The bacteria can grow in temperatures between 6.5 and 45°C, though most strains grow between 18-40°C. Additionally, they can survive for up to 30 minutes at temperatures reaching 60°C (Kosecka-Strojek *et al.*, 2018). Most staphylococci are halo-tolerant, able to grow in the presence of 10% NaCl, offering them a competitive advantage in environments with low water content and high salinity (De Vos *et al.*, 2009; Onyango and Alreshidi, 2018). Moreover, several staphylococcal species are capable of forming biofilm, which besides playing an essential role in staphylococcal pathogenesis, aids the bacteria's persistence outside hosts by reducing dehydration and UV exposure, as well as facilitating adhesion to non-biological surfaces (Hall-Stoodley *et al.*, 2004).

5.1.6.6 Virulence factors

Virulence factors in *S. aureus*

Staphylococci have an arsenal of virulence factors enabling them to cause disease in animals and humans. A summary of some of the major known virulence genes of *S. aureus* will be presented in this section and visualized in Figure 8. Most of the staphylococcal virulence factors do not play a significant role acting on their own, but when acting together, the pathogenic potential of the staphylococci increases (Stach *et al.*, 2018). A fine-tuned regulation of virulence genes is therefore essential for success. This role is fulfilled by the global regulators *sar* and *agr*, which control the expression of most virulence factors in *S. aureus* based on quorum sensing

(Arvidson and Tegmark, 2001; Jarraud *et al.*, 2002). In the early stages of infection, when the number of bacteria is small, *sar* promotes adhesion to host cells. At a later stage, when the staphylococci reach the exponential growth phase, *agr* counteracts *sar*, by downregulating the surface adhesins while upregulating the production of toxins and exoenzymes. Thus, *agr* expression transitions the infection from an adhesive to a more invasive state.

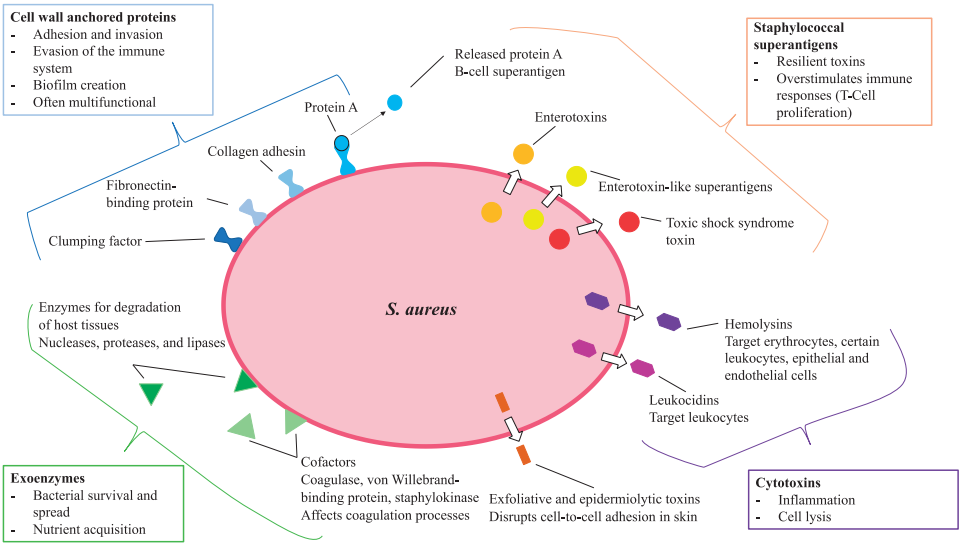

Figure 8. Simplified schematic overview of selected virulence factors in S. aureus. *Adapted with permission from Fergestad (2021)*

Cell wall-anchored proteins

The cell wall-anchored (CWA) proteins form a diverse group of virulence factors in *S. aureus*. Members of this group mediate adhesion to target cells, biofilm production, iron acquisition, invasion of host cells, inflammation, and immune evasion. The exact repertoires of expressed CWA proteins differ between strains and depend on the surrounding conditions and growth stage (Foster *et al.*, 2014). Central among the CWA proteins is the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family. This family consists, among others, of clumping factors A and B (ClfA, ClfB), fibronectin-binding proteins A and B (FnBPA, FnBPB), and collagen adhesin (Cna). The MSCRAMM proteins primarily mediate attachment to the host extracellular matrix such as fibrinogen, fibronectin, and collagen. However, some proteins have additional functions, including immune evasion and prevention of complement activation (Foster *et al.*, 2014).

Staphylococcal Protein A is a multifunctional CWA protein present in all *S. aureus* strains. Protein A has an immune evasive effect as it binds to the Fc receptor of IgG, thus preventing phagocytosis by coating the staphylococcal surface in misoriented IgG. Moreover, Protein A is a superantigen, interacting with pro-inflammatory cytokines and von Willebrand factor, leading to overstimulation of the immune system and a range of inflammatory responses (Mazigi *et al.*, 2019).

Biofilm

Many *S. aureus* strains have the ability to form biofilm, a slimy substance consisting of bacterial cells embedded in a matrix primarily made of polysaccharides (Donlan, 2002). Besides enabling the bacteria to form clusters and promote adhesion to host cells, biofilm has a protective effect against the host immune system and toxic agents such as antimicrobials, increasing the bacteria's persistence during infections (Hall-Stoodley *et al.*, 2004). Multiple mechanisms facilitate biofilm formation, including the activity of the intercellular adhesion operon, *ica,* which encodes the polysaccharide intercellular adhesin (PIA). Other staphylococcal proteins facilitating intercellular attachment include Biofilm-associated protein (Bap) and Autolysin E (AtlE) (O'Gara, 2007).

Toxins

S. aureus produces a variety of exotoxins favoring invasion of host cells. Four staphylococcal hemolysins have been identified: α, β, γ, and δ. The individual toxins differ in their effect on erythrocytes of different animal species, and staphylococcal strains and species vary in their hemolysin-producing ability. The α- and γ-hemolysins work by creating pores in cell membranes causing osmotic stress and subsequent cell lysis (Vandenesch *et al.*, 2012). The α-hemolysins' target cells include leukocytes, erythrocytes, platelets, pneumocytes, endothelial cells, and keratinocytes. Hence, the clinical manifestation varies accordingly. The γ-hemolysins primarily affect neutrophils, macrophages, and erythrocytes from various mammals (Vandenesch *et al.*, 2012). Their hemolytic effect cannot be seen on blood agar plates, as the agar inhibits their cytolytic activity. In contrast to α- and γ-hemolysins, β- hemolysins do not form membrane pores. Instead, they hydrolyze sphingomyelin, a plasma membrane lipid, damaging the cell membrane of their target cells. The δ-hemolysins form ion channels in the lipid bilayer of erythrocytes and leukocytes. Moreover, they act as surfactants disrupting membrane structures leading to cell lysis in a broad range of mammalian cells (Pontieri, 2018).
S. aureus produces other bicomponent toxins structurally similar to α- and γ-

hemolysins. These toxins specifically target leukocytes and are thus called leukotoxins or leukocidins (Otto, 2014). Like the α- and γ-hemolysins, the leukotoxins cause cell death by osmotic lysis by forming pores in the plasma membrane of their targeted cells. The Panton-Valentine leukocidin (PVL), LukDE, and LukAB are all members of this group, with PVL as the most potent toxin displaying higher leukotoxic activity, as well as proinflammatory properties (Ahmad-Mansour *et al.*, 2021). The leukotoxins are central in the early stages of infections, where their activity enables the bacteria to survive inside the host (Nawrotek *et al.*, 2018). Other exotoxins produced by *S. aureus* include the phenol-soluble modulins (PSMs). The PSMs are a multifunctional family of peptides, including, among others, the δ-hemolysins. They are central in staphylococcal pathogenesis as they are involved in the spreading of staphylococci on epithelial cell surfaces and in structuring and detachment of biofilms. Secondly, several PSMs display cytolytic activity against a broad range of cell types, namely erythrocytes, monocytes, endothelial and epithelial cells, osteoblasts, and phagocytes (Cheung *et al.*, 2014). Several PSMs facilitate survival, intracellular proliferation, and evasion from phagocytic cells by lysing the phagocytes from the inside, a property they share with the LukAB toxin.

Exoenzymes

Exoenzymes form the final group of the major virulence factors produced by *S. aureus.* This group includes a broad range of enzymes involved in immune evasion, invasion, and degradation of host tissue. The staphylococcal coagulases play a key role in staphylococcal virulence. Besides enabling the bacteria to evade the host immune system and promoting abscess formation, they are important for bacterial persistence in host tissue and enable staphylococci to cause sepsis (McAdow *et al.*, 2012). Hence, coagulase-positive staphylococci are considered more virulent than coagulase-negative. The staphylococcal proteases facilitate the migration of bacteria and provide nutrients by degrading the host tissue (Stach *et al.*, 2018). There are three types of proteases: Metalloproteases, cysteine proteases, and serine proteases. Among the serine proteases are the exfoliative toxins responsible for the skin blistering, often seen in connection with staphylococcal infections. The exfoliative toxins exert their effect by attacking desmoglein 1, responsible for the intercellular attachment in keratinocytes, causing epidermal dissociation (Ahmad-Mansour *et al.*, 2021). Staphylococcal lipases are involved in many processes in infection. Besides supplying nutrients, they are involved in biofilm production and immune evasion by interfering with granulocyte recruitment and function as well as host cell signaling

(Lepidi, 2018). Finally, *S. aureus* secretes nucleases also involved in biofilm formation and inactivation of various immune molecules, including neutrophil extracellular traps (NETs) released by dying neutrophils (Tam and Torres, 2019).

Virulence factors in *S. pseudintermedius* and CoNS

The virulence factors in *S. pseudintermedius* and CoNS are not nearly as much studied as in *S. aureus*. As *S. pseudintermedius* is coagulase-positive and cause similar types of infections as *S. aureus,* it presumably possesses many similar virulence factors, and some virulence markers are described to a certain degree. Firstly, many *S. pseudintermedius* strains are strong or moderate biofilm producers (Singh *et al.*, 2013; Meroni *et al.*, 2019). Secondly, genes encoding exotoxins, such as leukotoxins, enterotoxins, and PSMs, are present in numerous strains (Maali *et al.*, 2018; Meroni *et al.*, 2019). Moreover, certain strains, such as *S. pseudintermedius* Strain 222, produce peptides named bacteriocins that in lower concentrations exhibit bactericidal effects on related staphylococci, while at higher concentrations demonstrate cytotoxic effects towards eukaryotic cells (Wladyka *et al.*, 2015). In terms of CWA proteins, Bannoehr *et al.* (2011) revealed that the *S. pseudintermedius* genome contains a range of genes encoding proteins, some putative whose functions remain unknown and proteins whose functions are becoming increasingly clear. The CWA proteins SpsQ and SpsP are analogous to Protein A in *S. aureus* and exert the same function by binding the Fc region of IgG, while the proteins SpsD and SpsL mediate adhesion to fibrinogen and fibronectin (Pietrocola *et al.*, 2015; Balachandran *et al.*, 2018). Furthermore, SpsO mediates adherence to canine keratinocytes via unknown functions (Pietrocola *et al.*, 2015).

CoNS are less virulent than CoPS, a feature likely connected to their commensal nature. However, as many species and strains are strong biofilm producers, they can still cause various infections. To maintain their commensal lifestyle, many CoNS produce an arsenal of adhesins, such as autolysins and fibrinogen binding proteins, enabling them to attach to cell surfaces. These factors prove their importance for persistence during infections (Heilmann *et al.*, 2019). Additionally, CoNS have evolved mechanisms to sustain within a host by switching to a more intracellularly adapted lifestyle resulting in reduced host immune response. This adaptation includes forming small colony variants with slow-growing subpopulations that facilitates chronic and relapsing infections (Heilmann *et al.*, 2019).

5.1.7 Resistance in staphylococci

Historically, most staphylococcal species were naturally susceptible to narrow-spectrum antimicrobials such as penicillin G. However, shortly after penicillin's introduction in the 1940s, resistant *S. aureus* and CoNS isolates were recovered from human patients (Maranan *et al.*, 1997). Reports documented that the resistance rates among clinical CoNS and *S. aureus* isolates had increased to levels around 10 % within the next few years (Bondi and Dietz, 1945; Gallardo, 1945) and to rates higher than 90 % by the 1990s (Maranan *et al.*, 1997). Initially, penicillin resistance was mainly confined among clinical isolates. However, as time has passed, the resistance rates among community-associated strains have increased to equally high levels, resulting in the current situation where more than 90 % of staphylococcal species, regardless of origin, are penicillin-resistant (Lowy, 2003). Unfortunately, the situation is not confined to resistance solely to penicillins, as staphylococci have proven able to acquire and carry ARGs conferring resistance to a variety of antimicrobials. An overview of ARGs commonly found in staphylococci is presented in Table 2.

Table 2. Overview of frequently found antimicrobial resistance genes in staphylococci.

Antimicrobial class	Gene	Phenotypic resistance	Mechanism
Aminoglycoside	*aac(6')-aph(2')*	Amikacin, Gentamicin, Tobramycin, Kanamycin	Drug inactivation
	ant4'	Amikacin, Tobramycin, Isepamicin, Dibekacin	
	sat	Nourseothricin	
	str	Streptomycin	
Amphenicol	*cat* genes	Chloramphenicol	Drug inactivation
	fexA	Chloramphenicol, Florfenicol	Efflux
Beta-lactam	*blaZ*	Amoxicillin, Ampicillin, Penicillin, Piperacillin	Drug inactivation
	mecA	Beta-lactam antimicrobials	Target modification
Folate pathway antagonist	*dfr* genes	Trimethoprim	Target replacement
Macrolide, Lincosamide, Streptogramin B	*erm* genes	Erythromycin, Lincomycin, Clindamycin, Quinupristin, Pristinamycin IA, Virginiamycin S	Target modification
	lnuA	Lincomycin, Clindamycin	Drug inactivation
	lsa	Lincomycin, Clindamycin, Dalfopristin, Pristinamycin IIA, Virginiamycin M, Tiamulin	Target protection
	mef	Erythromycin, Azithromycin	Efflux
	msrA	Erythromycin, Spiramycin, Telithromycin	Target protection
	mphC	Erythromycin, Spiramycin, Telithromycin	Drug inactivation
	vga	Lincomycin, Clindamycin, Dalfopristin, Pristinamycin IIA, Virginiamycin M, Tiamulin	Target protection
Tetracycline	*tetM*	Doxycycline, Tetracycline, Minocycline	Target protection
	tetK	Doxycycline, Tetracycline	Efflux
	tetL	Doxycycline, Tetracycline	
Steroid antibacterial	*fus* genes	Fusidic acid	Target protection
Multidrug	*cfr*	Chloramphenicol, Florfenicol, Clindamycin, Lincomycin, Linezolid, Dalfopristin, Pristinamycin IIA, Virginiamycin M, Tiamulin	Target modification
	norA	Fluroquinolones, disinfecting agents, acridine dye	Efflux

Staphylococci have two mechanisms for resistance to beta-lactam antimicrobials. One is the production of beta-lactamases, encoded by the *blaZ* gene, that hydrolyzes the beta-lactam ring and consequently inactivates the drug (Fuda *et al.*, 2005). The second mechanism depends on the expression of the *mec* gene, encoding the penicillin-binding protein PBP2a with reduced affinity to beta-lactams. This mechanism is described more thoroughly in 5.1.7.1 ("Methicillin resistance") below.

The main mechanism behind aminoglycoside resistance in staphylococci is the activities of aminoglycoside-modifying enzymes, namely the acetyltransferases (AAC), adenylyltransferases (ANT), and phosphotransferases (APH) (Schmitz *et al.*, 1999). These enzymes alter the aminoglycosides to such an extent that they lose their ability to bind to the bacterial ribosomes and interfere with the protein synthesis. The genes are often found on MGEs but may also be chromosomally located.

Numerous resistance genes encoding macrolide, lincosamide, and streptogramin B resistance (MLS) have been identified in staphylococci, of which the most common are listed in Table 2. The different genes encode resistance through various mechanisms, thus causing resistance to different types of MLS but have in common that they are often associated with MGEs (Feßler *et al.*, 2018). The *erm* genes form the predominant group, all involved in methylation of the ribosomal target site, causing reduced binding of drug molecules (Roberts *et al.*, 1999; Roberts, 2022).

Tetracycline resistance is frequently observed among staphylococci of animal and human origin (den Heijer *et al.*, 2013; Schwarz *et al.*, 2018). Two main mechanisms in tetracycline resistance are active efflux, often encoded by *tetK* or *tetL*, and ribosomal protection encoded by *tetM.* The *tetM* gene is often chromosomally located, while TetK and TetL are usually plasmid-encoded (Schwarz *et al.*, 2018).

5.1.7.1 Methicillin resistance

Methicillin is a semi-synthetic beta-lactam introduced in the UK in 1959 to encounter the increasing penicillin resistance in *S. aureus* (Knox, 1960; Harkins et al., 2017). Although methicillin is no longer in clinical use, the term "methicillin-resistant" persists and implies resistance to virtually all beta-lactams except fifth-generation cephalosporins (Peacock and Paterson, 2015). The mechanism behind the resistance relies on the *mecA* gene or the *mecA* homologs (*mecB, mecC,* and *mecD)* that encode penicillin-binding protein 2A. This transpeptidase exhibits low

affinity to beta-lactams, enabling continued cell-wall synthesis despite the presence of beta-lactam antimicrobials (Schwendener *et al.*, 2017). The *mecA* and *mecC* genes are located on the mobile genetic element staphylococcal chromosome cassette *mec* (SCC*mec*), while *mecB* and *mecD,* present in macrococci, are usually either chromosomally encoded in SCC*mec*-like elements or found in plasmids (Schwendener *et al.*, 2017).

The SCC*mec* elements are highly diverse in their structural organization and genetic content. However, they all share essential characteristics, including a) carriage of *mecA* or *mecC* in a *mec* gene complex, b) carriage of cassette chromosome recombinase gene(s) (*ccr*) in a *ccr* gene complex, c) integration at a specific site in the staphylococcal chromosome referred to as the integration site sequence (ISS), and d) the presence of flanking direct repeat sequences containing the ISS (IWG-SCC, 2009). The *mec* gene complex comprises *mecA* or *mecC*, the regulatory genes (*mecR* and *mecI)*, and associated insertion sequences. The *ccr* gene complex consists of one or two site-specific recombinase genes responsible for the movement of the SCC*mec*. In addition, SCC*mec* elements typically contain three joining regions (J-regions) which contain genes encoding nonessential components of the cassette, including ARGs and virulence genes (IWG-SCC, 2009). The origin of SCC*mec* is still unknown, but the evidence points towards CoNS (members of the *Staphylococcus. sciuri/Mammaliicoccus.* sciuri-group) being involved in the first evolutionary stages of SCC*mec* (Rolo *et al.*, 2017).

Due to the high complexity in SCC*mec* elements, the International Working Group on the Classification of Staphylococcal Cassette Chromosome (IWG-SCC) proposed a definition and guidelines for reporting novel SCC*mec* elements in 2009 (IWG-SCC, 2009). Types and subtypes of SCC*mec* are determined by the combination of the *mec* and *ccr* gene complexes and variations within the J-regions. Based on these definitions, fourteen SCCmec types had officially been approved for *S. aureus* by the end of 2021 (Uehara, 2022). Structures of SCC*mec* elements type I (1B) to type VIII (4A) are shown in Figure 9.

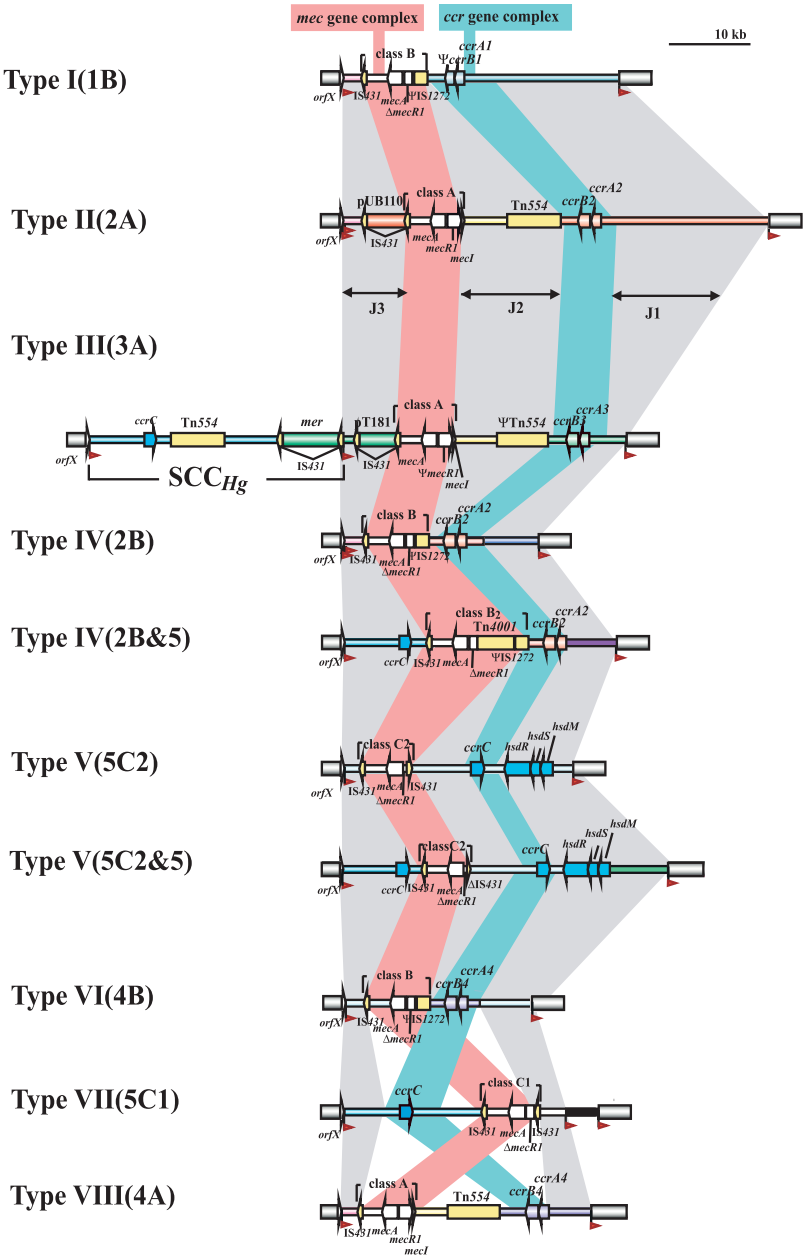

*Figure 9. Basic structures of SCC*mec *elements. Red arrowheads indicate integration site sequences. Adapted with permission from IWG-SCC, 2009, American Society for Microbiology.*

5.1.8 Epidemiology of methicillin-resistant staphylococci

Shortly after the introduction of methicillin, the first cases of methicillin-resistant *S. aureus* (MRSA) were described (Barber, 1961; Jevons, 1961). Within the following years, infections and hospital outbreaks with MRSA were reported from large parts of the world (Eriksen and Erichsen, 1964; Barrett *et al.*, 1968; Rountree and Beard, 1968). From primarily being a hospital- and health care facility-associated pathogen, community-associated MRSA strains disseminated among the general population in the 1990s (Tenover *et al.*, 2006). The high prevalence, often combined with multidrug resistance and the ability to infect nearly any body site, make MRSA one of the most successful modern pathogens (Turner *et al.*, 2019). The first reported MRSA infection in animals was in milk from mastitic cows in 1972 (Devriese *et al.*, 1972). In the following years, MRSA was reported in different animal species, and by the late 2000s it was evident that livestock-associated MRSA was established in pig farms in Europe and North America (Smith and Pearson, 2010; Verkade and Kluytmans, 2014). Companion animals may be colonized and infected by MRSA. However, the reported cases are often associated with human carriage of MRSA as the isolated strains tend to be the predominating strains in the people in the region. Moreover, evidence points towards that colonization of dogs and cats is transient. (Weese and van Duijkeren, 2010).

Methicillin-resistant *S. pseudintermedius* (MRSP) is the predominating methicillin-resistant species in companion animals. Since the mid-2000s, MRSP has spread worldwide and now represents a significant fraction of skin and soft tissue infections, especially in dogs. There are considerable variations in prevalence and clonality according to geographical locations (Ventrella *et al.*, 2017; Lee *et al.*, 2018; Adiguzel *et al.*, 2022). The clonal complexes CC71, CC258, CC45, CC68, and CC112 represent the most prevalent lineages worldwide, with CC71 and CC258 dominating in Europe, CC68 in North America, and CC45 and CC112 in Asian countries (Pires dos Santos *et al.*, 2016). Treatment of MRSP infections is often challenging as the rates of multidrug-resistant isolates are high (Cain, 2013; Pires dos Santos *et al.*, 2016). The resistance profiles are often associated with the CCs. For instance, are isolates belonging to CC258 frequently resistant to trimethoprim sulfamethoxazole and tetracyclines, whereas resistance to enrofloxacin, chloramphenicol, and gentamicin is not so commonly seen (Pires dos Santos *et al.*, 2016). Although MRSP primarily causes infections in dogs and cats (Lehner *et al.*, 2014), infections are sporadically reported in humans (Kempker *et al.*, 2009; Stegmann *et al.*, 2010; Starlander *et al.*, 2014). However, the number of human infections is likely

underestimated since *S. pseudintermedius* is easily misdiagnosed as CoNS or *S. aureus* (Guardabassi *et al.*, 2013; Börjesson *et al.*, 2015).

Methicillin resistance is widely disseminated in CoNS and mammaliicocci, especially in nosocomial strains where resistance rates up to 80 % are common (Hanssen and Ericson Sollid, 2006; Heilmann *et al.*, 2019; Becker *et al.*, 2020; Schwendener and Perreten, 2022). Resistance to methicillin, often combined with resistance to non-beta-lactam antimicrobials, offer a competitive advantage for the MRCoNS in hospital- and healthcare settings, where antimicrobials are widely used. Though primarily being associated with hospitals, non-hospital reservoirs have also been described (Stepanović *et al.*, 2008; Xu *et al.*, 2015; Seng *et al.*, 2017).

During the 1970s it became clear that methicillin resistance was more prevalent in CoNS than in *S. aureus* (John and Harvin, 2007), an observation that gave rise to the hypothesis of MRCoNS strains being the original donor of *mecA* to CoPS. This hypothesis was further supported during the 2000s when studies found *mecA* homologs from different MRCoNS/MRM species with high nucleotide sequence similarities to *mecA* in *S. aureus* (Schnellmann *et al.*, 2006; Tsubakishita *et al.,* 2010). There are still many missing links in the evolutionary history and dissemination of SCC*mec.* How *mecA* was incorporated into the SCC element is unknown, and the transfer mechanisms remain unclear (Rolo *et al.*, 2017). Transduction has been suggested as a possible mean for transfer, and this has been observed *in vitro* at low frequencies (Scharn Caitlyn *et al.*, 2013). Conjugation has also been suggested as a possible transfer mechanism based on the experiments by Ray *et al.* (2016). The successful transfer required donor manipulation by overexpressing the *ccr* genes to capture a shortened SCC*mec* into a conjugative plasmid. The findings of *mecB*-carrying plasmid with conjugative elements further supported the conjugation theory (Becker *et al.*, 2018). However, spontaneous and large element transfers between staphylococci are yet to be demonstrated. A more recent study by Maree *et al.* (2022) documented natural SCC*mec* transformation in *S. aureus* biofilm, thus adding transformation into the ranks of possible transfer mechanisms.

5.1.9 Antimicrobial resistance in companion animals

The relationship between companion animals and humans has changed drastically during the past centuries. From primarily serving instrumental purposes such as herding and hunting, companion animals have to an increasing degree, gained status as family members, living in close contact with their owners (Blouin, 2008; Amiot *et al.*, 2016). The strong bond between humans and companion animals benefits human health and well-being, as it can reduce loneliness and depression and promote physical activity while enhancing social interactions (Friedman and Krause-Parello, 2018). Alongside the changing human-companion animal relationship, veterinary small animal medicine has made advances, providing more advanced diagnostics, treatments, and intensive care, resulting in a growing population of older and immunocompromised patients at higher risk of developing infections (Wieler *et al.*, 2011).

Carriage of multidrug-resistant bacteria, such as MRS and extended-spectrum beta-lactamase (ESBL) producing *Enterobacteriaceae*, are now commonly reported in healthy dogs and cats (Davis *et al.*, 2014; Salgado-Caxito *et al.*, 2021). The prevalence of AMR bacteria in companion animals in Norway is not well characterized, but a few recent reports indicate low levels of resistance. Since 2019, some AMR bacteria, including vancomycin/linezolid-resistant enterococci, MRSP, MRSA, and fluoroquinolone/colistin-resistant/ESBL producing *Enterobacteriaceae,* became notifiable to the Norwegian Food Safety Authority (Mattilsynet, 2022). During the first six months after the implementation, ten cases of notifiable AMR bacteria were reported from dogs and cats (NORM, 2020). The NORM/NORM-VET surveillance program provides data on sales of antimicrobials and the occurrence and distribution of AMR in Norway over time. The most recent report reported low levels of AMR among humans and food-producing animals. However, the occurrence of AMR in companion animals was omitted (NORM, 2021). In the NORM-VET report from 2020, resistance data on *Escherichia coli*, *S. pseudintermedius*, and *Streptococcus canis* isolates from dogs were included, reporting of overall low levels of AMR, but a higher proportion of resistance among clinical *S. pseudintermedius* and *E. coli* isolates than in indicator bacteria from healthy dogs (NORM, 2020).

Due to the low levels of resistance, Norwegian veterinarians and pet owners are not used to handling cases where companion animals have been diagnosed with multidrug-resistant bacteria. Since pets often undergo treatment in their homes, interacting with their families and surroundings whilst recovering from the

infection, questions are raised on whether the bacteria may pose a threat to the family members. As several studies report human infections with MRSP and co-carriage of ESBL-producing *Enterobacteriaceae* among pets and owners (Stegmann *et al.*, 2010; Starlander *et al.*, 2014; Ljungquist *et al.*, 2016; Grönthal *et al.*, 2018; van den Bunt *et al.*, 2019), there is reason to question whether the close contact between companion animals and owners solely are advantageous, or if downsides can outweigh some of the benefits. The Norwegian Scientific Committee for Food and Environment (VKM, formerly known as the Norwegian Scientific Committee for Food Safety) performed a risk assessment of AMR transfer between pets and humans in Norway. The resulting report stated that AMR could be directly or indirectly between pets and humans (VKM, 2015). Further, VKM considered *S. aureus*, *S. pseudintermedius,* and members of the *Enterobacteriaceae* to be the bacteria of most concern. At the same time, VKM emphasized that there is a lack of data regarding the reservoir of AMR in the environment, pets, and humans, as well as data regarding the routes and frequencies of transmission of AMR between pets and humans. This missing knowledge forms the rationale for performing this study.

5.1.10 Aims

The overall aim of this thesis is to contribute to the knowledge gaps identified by VKM. More specifically, the thesis aims to characterize the pet's and the home environment's roles as reservoirs for AMR and investigate the transmission potential for AMR to humans staying in these surroundings.

Objectives:

1. Investigate the healthy pet's role as a reservoir for AMR by screening fecal, oral, and perineal samples for the presence of ARGs, and methicillin-resistant staphylococci (Paper I and III).

2. Investigate the transmission potential of clinical methicillin-resistant staphylococci from infected pets to their surroundings (Paper II).

3. Investigate the home environment's role as a reservoir for methicillin-resistant coagulase-negative staphylococci (Paper III).

5.2 Materials and methods

A summary of the materials and methods used in the papers is presented in this section. For further details, see the respective papers.

5.2.1 Materials

For Paper I, fecal samples collected from dogs and owners by the HUNT4 - One Health study were used. The HUNT4 – One Health study is a collaborative project between the Norwegian University of Life sciences, the Norwegian Veterinary Institute, and the Norwegian University of Science and Technology (NMBU, 2020) based on a parallel collection of fecal samples from animals and human participants in the period between 2017 and 2019. The sample collection consisted of fecal material from 836 dogs, applied on collection cards. We selected participants from this pool based on the following inclusion criteria: The owner participated in the HUNT study and had collected fecal samples from themselves, and the owner had returned the questionnaires regarding their dog's health. From the remaining pool of 111 dogs, we included family dogs whose health conditions were considered good or excellent by their owners, resulting in 35 dog-owner pairs.

The material for papers II and III was collected from households recruited through social media and small animal clinics in the Oslo area. Eight households with dogs recently diagnosed with MRS infections were included in Paper II, seven with active infections with MRSP and one with MRSE. The same eight households participated in Paper III. In addition, 14 households with healthy pets (eight with dogs, six with cats), and 11 households without pets were included in this study. From the infected dogs, swab samples were collected from the infection site, perineum, and the oral mucosa. From healthy dogs and cats, the two latter sites were sampled. Human participants collected samples from their nostrils and throat under the supervision of a veterinarian. Environmental samples were collected from the living room floor, bathroom (sink faucet and hand towel), and kitchen (kitchen counter, dish towel, cloth, and sink faucet). The samples from the bathrooms and kitchens were collected from sites out of reach for the dogs. In addition, the food bowls and the pets' sleeping places were sampled in households with pets.

5.2.2 Bacterial cultivation

The swabs from humans and infected pets were plated directly onto 5 % bovine blood agar. Additionally, the samples were selectively enriched based on the protocol made by the European Union Reference Laboratory – Antimicrobial resistance (EURL-AR, 2018). Environmental samples were only selectively enriched due to the high quantities of *Bacillus* spp. and other environmental bacteria. After incubation, 20 µl of Müller Hinton broth was plated on Oxacillin Resistance Screening Agar Base (ORSAB) plates, supplemented with 2 mg/L of oxacillin, and incubated for 24 hours. In cases of no growth, the plates were reincubated for 24 hours. Presumptive staphylococci (blue, pale blue, and white colonies, Figure 10) were subcultured on bovine blood agar before species identification and antimicrobial susceptibility testing.

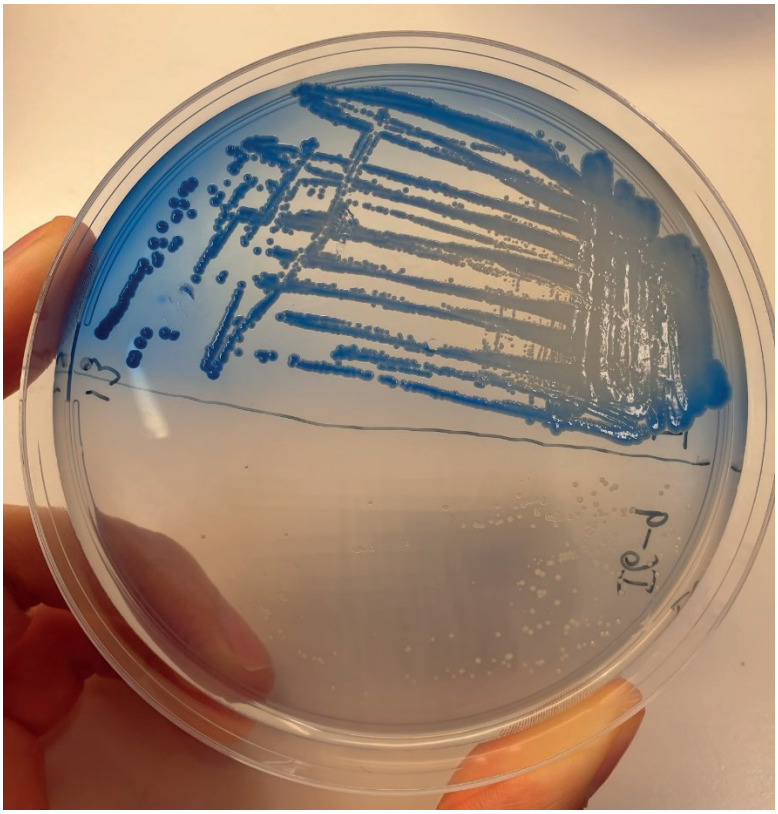

Figure 10. ORSAB plate with MRSP cultured on the upper half and MR S. hominis *on the lower half.*

5.2.3 Species identification

All bacterial isolates included in papers II and III were species identified using MALDI-TOF-MS. As MALDI-TOF-MS cannot distinguish between *S. pseudintermedius* and other species within the SIG group, we used a combination of colony morphology and biochemical tests to determine the species ID on the presumptive *S. pseudintermedius* isolates. *S. pseudintermedius* has a characteristic morphology on ruminant blood agar with white-grey colonies and surrounding double hemolysis. As *S. aureus* shares some morphologic characteristics, we used the isolates' ability to ferment ONPG and mannitol to distinguish between the two species. Furthermore, all sequenced isolates were taxonomically classified using the Microbial Genomes Atlas (MiGA) web server. The MiGA web server utilizes Average Nucleotide Identity (ANI) or Average Amino Acid Identity (AAI) values against a reference genome database to classify query genomes or assembled contigs (Rodriguez-R *et al.*, 2018). All the presumptive *S. pseudintermedius* isolates were classified as *S. pseudintermedius* by MiGA, while some of the CoNS and mammaliicoccal classifications differed between MALDI-TOF-MS and MiGA. In these cases, we used the output from MiGA when determining the ID.

5.2.4 Antimicrobial susceptibility testing

Bacterial isolates included in papers II and III were susceptibility tested using the agar disk diffusion method according to the EUCAST protocol (EUCAST, 2019). When testing resistance to oxacillin, we used Müller Hinton agar supplemented with 4 % NaCl.

5.2.5 PCR and qPCR

For papers II and III, verified staphylococcal and mammaliicoccal isolates were tested for the presence of *mecA* by PCR (Stegger *et al.*, 2012).
We used high throughput quantitative PCR (HT-PCR) to screen the fecal samples in Paper I for ARGs. The assays had been developed by NIBIO based on their clinical relevance and a list of indicators ARGs by Berendonk *et al.* (2015). The method is otherwise known as microfluidic qPCR, and uses nanoliter reaction volumes and a system of valves and microfluidic channels to automate the mixing and thermocycling of multiple assays and samples in a single chip (Crane *et al.*, 2018). To improve the method's sensitivity, we performed a preamplification step prior to the qPCR to increase the concentration of template DNA. This involved a multiplex PCR with primer sequences and a small number of PCR cycles. The assays were then run in duplicates, with controls with known concentrations of the target genes. The

standard curves of the positive controls were used to quantify the ARGs in the samples.

5.2.6 Whole-genome sequencing

To achieve sufficient concentrations and acceptable DNA quality required for whole-genome sequencing, we developed a DNA isolation protocol for the staphylococcal and mammaliicoccal samples (Papers II and III). The detailed protocol is available as an appendix in Paper II. The samples were sequenced using Illumina Miseq v3 with 300 paired-end reads. The Norwegian Sequencing Centre performed the library prep and sequencing. Two different library preps were used (Nextera DNA Flex prep and Swift Turbo 2S), as the samples were sequenced in two batches, and the Swift Turbo 2S had been phased out in the meantime.

5.2.7 Bioinformatical analyses

We used assemblies from staphylococci and mammaliicocci for taxonomic classification, multi locus sequence typing (MLST), SCC*mec* typing, resistome-mobilome, and virulence gene analysis. The workflow used in this thesis is illustrated in Figure 11. For details, see the enclosed papers.

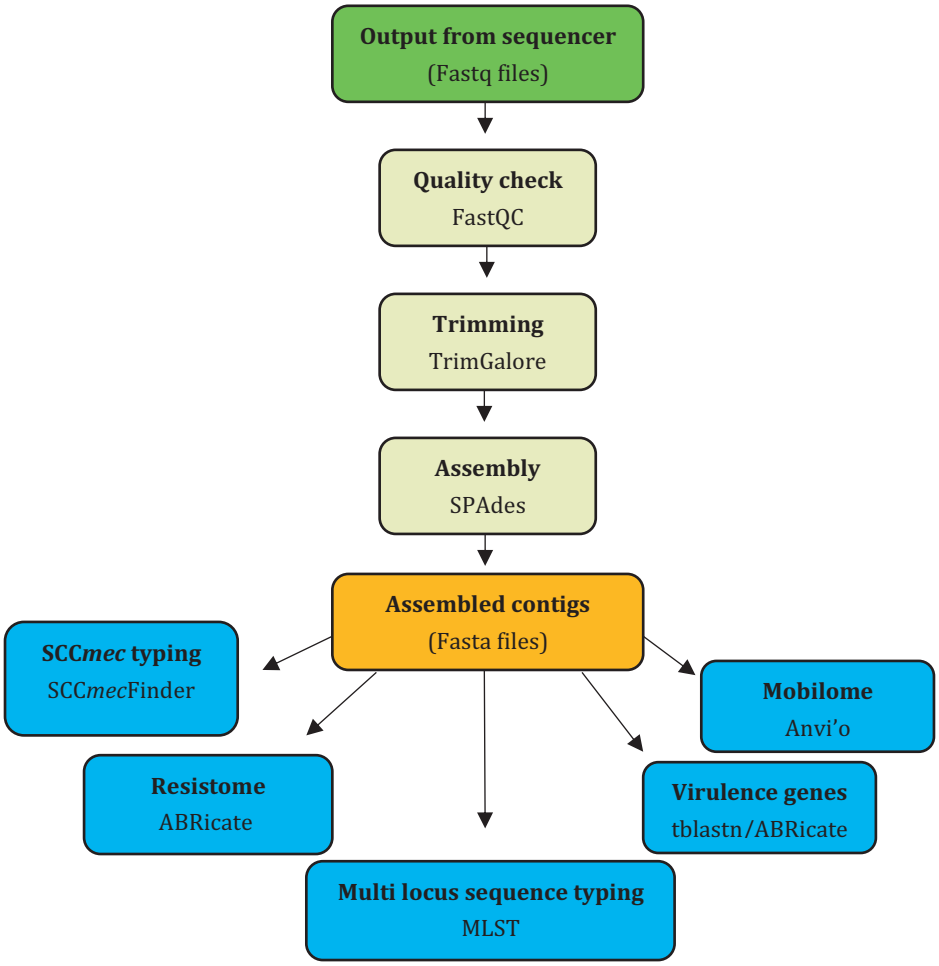

Figure 11. Workflow and analyses made in papers II and III. Light green: Input data. Gold: Steps from reads to assembly and respective tools. Orange: Input data analyses. Blue: Analyses and respective analysis tools.

5.3 Results/Summary of papers

Paper I: Antimicrobial resistance- Do we share more than companionship with our dogs?

Mari Røken, Kristin Forfang, Yngvild Wasteson, Anita Haug Haaland, Hans Geir Eiken, Snorre B. Hagen, and Ane Mohn Bjelland

Journal of Applied Microbiology, 2022, DOI: 10.1111/jam.15629

The study investigates the gut resistomes of 35 cohabiting dogs and owners, and to what degree the gut resistomes of dogs and owners contained similar antimicrobial resistance genes (ARGs). The fecal samples were screened for the presence of 34 ARGs, and the class1 integron-integrase gene *intI1* using high throughput qPCR. The results showed that tetracycline and macrolide resistance genes were the most common ARGs irrespective of host species. The examined dogs carried more aminoglycoside resistance genes than the humans. The methicillin resistance-encoding gene *mecA* was detected in 20.8% of the dogs, while 5.4% of the owners tested positive for *mecA.* At the group level, dogs and owners carried the same number of ARGs, and the slight majority of ARGs were equally represented in both groups. A mean of 9.9 unique ARGs was detected at the household level in the dogs and owners combined. Of these, dogs and owners had 3.3 ARGs in common. Older dogs had more ARGs in common with their owners than younger dogs. This relied mainly on the bigger proportion of older dogs carrying the *ermF* gene, a gene that was otherwise associated with the human samples. In conclusion, the study documents that the dogs and owners comprise reservoirs for a broad range of resistance genes. They carry many of the same resistance genes at the group level. However, the modest proportion of common ARGs at the household level is indicative of a limited level of transmission between dogs and owners.

Paper II: Transmission of methicillin-resistant *Staphylococcus* sp. from infected dogs to the home environment and owners.

Mari Røken, Stanislav Iakhno, Anita Haug Haaland, Yngvild Wasteson, and Ane Mohn Bjelland

Antibiotics, 2022, DOI: 10.3390/antibiotics11050637

Dogs with methicillin-resistant *Staphylococcus* spp. (MRS) infections often undergo treatment in their homes, interacting with their owners and surroundings. This close contact between dogs and owners may facilitate the interspecies transmission of MRS. Therefore, this study investigated the transmission of MRS from infected

dogs to their owners and home environments. Seven households with dogs diagnosed with methicillin-resistant *S. pseudintermedius* (MRSP) and one with a dog with methicillin-resistant *S. epidermidis* (MRSE) participated in the study. Dogs, owners, and the home environments were screened for the presence of clinical MRS. A selection of 36 staphylococcal isolates was whole-genome sequenced and screened for resistance genes and virulence genes. Clinical MRS were primarily identified from the dogs and their immediate surroundings but were also detected in locations out of reach for the dogs, indicating indirect transmission. Two of eight owners carried clinical MRS in their nostrils, while one owner carried methicillin-susceptible *S. pseudintermedius* (MSSP). All clinical MRS were multidrug-resistant, and several possessed resistance genes that were not expressed phenotypically. Clinical MRSP persisted in the home environment for a prolonged period, despite infection recovery, cleaning measures. and one dog being euthanized. Regardless of the stable presence of MRSP in the surroundings, the owners in these homes remained negative but tested positive for MSSP on three occasions. In conclusion, this study has documented that the home environment is a reservoir for clinical multidrug-resistant MRS shed by infected dogs, and that the home environment remains MRS positive for a prolonged period. Humans are exposed to clinical MRS directly and indirectly through interaction with their dogs and home environment, but the significance of the exposure is debatable, especially for MRSP, as human carriership tends to be temporary.

Paper III: The home environment is a reservoir for methicillin-resistant coagulase-negative staphylococci and mammaliicocci.

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This paper explores the home environment's role as a reservoir for methicillin-resistant coagulase-negative staphylococci (MRCoNS) and mammaliicocci (MRM). A total of 33 households participated in the study; 14 with healthy pets, eight with pets suffering from methicillin-resistant staphylococci infections, and 11 households without pets. The households were screened for the presence of MRCoNS and MRM by sampling the humans, eventual pets, and home environments. Selectively cultured colonies were species identified, tested for *mecA*, and antimicrobial susceptibility tested before a selection of isolates were whole-genome sequenced (WGS). Furthermore, we characterized the WGS isolates' resistance- and virulence genes, SCC*mec* elements, sequence types, and compared their mobile genetic

elements. MRCoNS and MRM were detected in 30/33 households, often with several species present in a single household. The large majority of isolates were recovered from locations in the home environment with the human-associated species *S. saprophyticus, S. haemolyticus, S. epidermidis,* and *S. hominis* being most frequently detected. Six humans (all pet owners) and three dogs (all belonging to the infection group) carried MRCoNS. The slight majority of isolates were multidrug-resistant, with resistance to macrolides and fusidic acid as the most frequent phenotypic profile. We observed a variable phenotypic expression of resistance to beta-lactams among the isolates, including resistance to cefoxitin, which is the recommended agent when screening for methicillin resistance. This suggests that methicillin resistance may be underestimated if screenings are based on phenotypic results only. In conclusion, the vast presence of MRCoNS and MRM in the home environments, without concurrent carriage in humans or pets, indicates that home environments constitute a reservoir for methicillin resistance regardless of type of household.

Supplementary material for Paper III is available at
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5.4 Discussion

5.4.1 Methodological considerations

Sample size

Based on journal records from the past years before the project started, we expected to recruit 10-20 dogs with MRS infections for paper II within the project's time frame. This was challenging to achieve, primarily due to the COVID-19 pandemic. Although additional efforts were made to increase participant recruitment, the total number of households ended at eight for paper II. Papers I and III are based on material from 35 and 33 households, respectively. Thus, the sample material in this thesis is relatively small and the observations should be interpreted with care. Consequently, no confident conclusions can be made from the papers. Instead, the work should be considered as exploratory research.

Health status

Paper I aimed to study fecal ARGs in healthy dogs and owners. In order to describe the healthy pet as a reservoir, the healthy pet must first be defined. The samples and metadata used in Paper I were collected by the HUNT project. Consequently, we did not have the opportunity to examine the dogs before including them in the study. Instead, we categorized the dogs' health based on questionnaires submitted by the owners. This was not ideal, as most owners are not trained in assessing a dog's health, and the answers were most likely based on their subjective opinion. We categorized the dogs as healthy if the owners had answered good or excellent on the question regarding health status (answering options: Poor, not so good, good, and excellent). Dogs that underwent antimicrobial treatment or suffered from gastrointestinal symptoms at the time of sampling were excluded. Although we had access to the number of times the dogs had been treated with antimicrobials during their lifetime, we did not have access to when they underwent antimicrobial therapy. Consequently, we cannot rule out that some of the dogs recently had been treated with antimicrobials, which would affect the fecal microbiota and resistome (Anthony *et al.*, 2022). To avoid the formation of subgroups among the dogs, we excluded sled dogs and hunting dogs, as many of these live outdoors, separated from their owners.

For Paper III, we categorized cats and dogs as healthy if they displayed no signs of infection and if their general health condition was compatible with vaccination.

These inclusion criteria do not necessarily exclude factors that may increase the pets' proneness to carry methicillin-resistant staphylococci, e.g., recent use of antimicrobials or visits to veterinary clinics (Lehner *et al.*, 2014). In addition, chronic skin conditions, such as atopic dermatitis, may affect the pets' susceptibility to colonization of staphylococci (Bradley *et al.*, 2016). However, as the participating owners answered questionnaires regarding their pets' health, medicine use, and eventual allergies, we got a decent overview of underlying conditions that could have affected the results in Paper III.

Due to the design of the HUNT study, we did not have access to general health data on the participating humans. Self-reported evaluation scores of the owners' health and whether they suffered from any "longstanding illness or injury of a physical or psychological nature impairing their function in their daily lives" were available through the HUNT database. However, as there was no elaborated information available on potential diagnoses, antimicrobial- or medicine use, this is a limitation of the study. The health data for the human participants in papers II and III were restricted to antimicrobial use and whether they had been hospitalized during the past 12 months before the study. In retrospect, we could have considered collecting more information on the eventual use of immunosuppressant drugs and conditions that could influence the human participants' susceptibility to carriage or infections with MRS.

Samples and sampling sites

For Paper I, the fecal samples from the humans and dogs were collected by the dog owners themselves. The owners were provided written instructions, a video on how to collect the samples, and instructions on how to dry the collection cards before sending them by mail to the HUNT Biobank. Although the sampling procedure has its benefits, facilitating a large-scale sampling over a short period, it also introduces a range of uncertainties, due to limited potential for standardization. For instance, the amount of feces varied considerably between the cards. Furthermore, the cards were supposed to be dried at room temperature for a minimum of two hours before putting them in envelopes. Thus, contamination from the surroundings, e.g., dust, as well as variations in drying time and temperature, which leads to different DNA degradation rates (Zhang *et al.*, 2019) could have affected the results. Lastly, the cards were sent by regular mail with delivery times ranging from a couple of days to a week, leaving further opportunity for DNA degradation and bacterial growth before freezing. Due to the lack of standardization in the sample collection and

treatment, we chose a qualitative approach when presenting the results from the analyses, seeing that a quantitative approach would be of limited use.

It is important to keep in mind that we have only analyzed parts of the human and canine gut resistomes. Both because of the restricted panel of ARGs included in the test panel and because we analyzed feces, and no other intestinal content. The bacterial composition varies along the gastrointestinal tract (Thursby and Juge, 2017; Pilla and Suchodolski, 2020). Even though the distal parts contain the greatest number of bacteria, it does not necessarily contain all the bacteria hosting resistance genes. Furthermore, the bacterial composition varies between the fecal/luminal and mucosal parts of the intestine (Thursby and Juge, 2017), leading to potentially biased results when only analyzing luminal content. On the other hand, by analyzing feces, one gets an overview of the ARGs that are shed to the surroundings and, thereby, ARGs with a greater potential for dissemination.

For papers II and III, the sampling sites in the pets were chosen based on the recommendations for detection of *S. pseudintermedius* by Bannoehr and Guardabassi (2012), while the human sampling sites were based on predilection sites for *S. aureus*. As emphasized in Paper III and in the general discussion of this thesis, the sampling sites in humans and pets may not have been optimal for detecting of MRCoNS, as the protocols originally were designed for detecting MRSP and MRSA. For instance, *S. hominis* and *S. haemolyticus* are often located in areas rich in apocrine glands, while *S. saprophyticus* is a frequent perineal and pubic area colonizer. Hence, we may have underestimated the MRCoNS carrier rates among pets and humans. In addition, we included only one human member in the household. Consequently, we might have missed human carriers of MRS both in papers II and III.

Culturing

For papers II and III, the samples were cultured for bacterial identification. Pets and owner samples were plated directly onto blood agar to get an overall impression of bacterial composition and if we could identify *S. pseudintermedius* directly from the human samples. Environmental samples were selectively enriched only, due to the high quantities of *Bacillus* spp. and other environmental bacteria.
We picked three colonies (if present) with similar morphology from the ORSAB plates for subculturing on blood agar. Most of the subcultured isolates from the same sampling locations, displayed similar morphology and resistance profiles.

However, there were a few examples of the opposite, exemplifying the uncertainty regarding how many colonies to pick to ensure a representative selection of bacteria.

Antimicrobial susceptibility testing

We observed phenotypic heterogenicity in resistance expression among isolates recovered from the same source, belonging to the same species in papers II and III. This was expected, as the phenomenon even occurs in homogeneous liquid cultures, where genotypic identical bacterial cells experience the same local conditions (Ackermann, 2015). Furthermore, it was noteworthy that a substantial proportion of the MRCoNS/MRM isolates did not express resistance to beta-lactams. The phenotypically cefoxitin-susceptible isolates were particularly concerning as cefoxitin is the recommended agent for most CoNS when screening for methicillin resistance (EUCAST, 2022). By using CLSI breakpoints instead of the breakpoints provided by EUCAST, more, but not all, of the isolates would have been assessed as resistant to cefoxitin. The discrepancies between the breakpoints can significantly impact susceptibility interpretation and lead to an underreported prevalence of resistance (Cusack *et al.*, 2019). Our results showed that oxacillin was more reliable than cefoxitin for predicting methicillin resistance.

HT-qPCR

The major benefit of HT-qPCR is its ability to simultaneously analyze multitudes of target genes (Waseem *et al.*, 2019). This, combined with the ability to detect and quantify low-abundance genes in multiple samples simultaneously, was the main reason for choosing HT-qPCR. In addition, the results from a pilot experiment on a similar sample set indicated that a metagenomic sequencing approach would not provide sufficient sequencing depth to classify ARGs at the gene level. Therefore, HT-qPCR appeared as the most suitable method for the sample material. Like in metagenomic sequencing, HT-qPCR detects and quantifies ARGs from the whole fecal microbiome, thereby avoiding the bias that is introduced when restricting the analysis to culturable bacteria. Provided that the gene sequences of the ARGs are known, HT-qPCR has a lower detection limit for ARGs than metagenomic sequencing (Franklin *et al.*, 2021). The method's disadvantages include that all assays are run under the same cycling conditions, which excludes the possibility of optimizing the conditions for the targeted genes. Moreover, the pre-amplification step of the method improves the detection limit but may reduce its specificity (Sandberg *et al.*, 2018). Also, the method cannot reveal the host bacteria carrying the ARG. HT-qPCR

is a highly sensitive method, making low-biomass samples prone to contamination. This was exemplified in Paper I with three negative controls testing positive for low quantities of microbial DNA. Bacterial DNA contamination within commercial DNA kits is well documented and may impact the results in microbiome studies (Salter *et al.*, 2014; Karstens *et al.*, 2019; Saladié *et al.*, 2020). In the case of Paper I, one of the negative controls tested positive for the *ant(3')* gene in addition to the 16s rRNA gene. Since only one control tested positive, we did a targeted rerun of this specific sample. The control tested negative for *ant(3'),* confirming that the contamination had occurred during the first run of qPCR. Ideally, we should have repeated the qPCR run for all samples, thereby reducing the impact of eventual contaminations in the human and canine samples as well. Due to the resources available to the project, this was not an option.

The HT-qPCR method manifested its advantages when samples from the same dogs were metagenomically sequenced in a master's thesis project by Oda Marie Bjørgum Karlsen in 2022 (Karlsen, 2022). In this project, roughly twice as much fecal material was used for DNA extraction. Still, the method detected only about one-third of the ARG number compared to the HT-qPCR method, demonstrating the lower sensitivity of the metagenomic approach. Furthermore, the ARGs detected by sequencing corresponded poorly with those detected with qPCR.

Virulence genes

Virulence factors in CoNS and mammaliicocci are far less studied than in *S. aureus*. Consequently, we used a database mainly consisting of amino acid sequences from putative and known virulence factors in *S. aureus* (Naushad *et al.*, 2019). This is not optimal for the detection of virulence genes in CoNS. However, as the method provides *Ha* scores based on percentage identity and alignment length, it appeared as a better option than VirulenceFinder (Center for Genomic Epidemiology, DTU, Denmark), considering its low detection rates in CoNS (Fergestad *et al.*, 2021). Although some studies operate with cut-off values (Li *et al.*, 2018; Nobrega *et al.*, 2018), we decided not to set a pre-defined threshold for the *Ha* scores, as we would then risk excluding sequences of importance. Instead, we included all the scores when comparing virulence genes between the MRCoNS and MRM isolates and focused on the top hits when presenting the main findings for each species.

5.4.2 General discussion

Antimicrobial resistance has been described as "the quintessential One Health issue" as it exists in all three sectors: Animals, humans, and the environment (Essack, 2018). AMR is not static, confined within one area or one species of bacteria. On the contrary, it continues spreading mainly due to the excessive use and production of antimicrobials. Therefore, the ongoing AMR crisis needs to be addressed with a holistic One Health approach as efforts in one sector alone do not necessarily reduce the extent of the problem.

There are currently no large-scale international monitoring programs for the antimicrobial consumption in companion animals, but the consumption presumably makes up a small proportion compared to the proportions used in production animals and in human medicine (Tiseo *et al.*, 2020; European Centre for Disease *et al.*, 2021; NORM, 2021; Marco-Fuertes *et al.*, 2022). Production animals have therefore received most of the attention regarding AMR (also because they share a large interface with humans through their role in the food chain). In contrast, the companion animals' role so far has been somewhat neglected. In order to understand and fight AMR, companion animals must be included as they presumably represent a reservoir for AMR, and their close contact with owners and home environment may facilitate the transmission of AMR bacteria.

5.4.2.1 The healthy pet as a reservoir for AMR

The dogs in Paper I constituted a reservoir for tetracycline and erythromycin resistance genes, of which *tetM* and *ermB* genes were particularly frequent, with nearly all dogs carrying them. Few studies have focused on the canine gut resistome. However, our findings agree with a study by Kim *et al.* (2020), at least at the class level of resistance, as Kim *et al.* did not detect any *ermB* genes. Moreover, aminoglycoside resistance genes were widespread among the dogs, often with multiple genes present in a single fecal sample. The most recent report on fecal indicator bacteria from healthy dogs in Norway displayed low aminoglycoside resistance rates in *E.coli, Enterococcus faecium,* and *Enterococcus faecalis* (NORM, 2020). Therefore, other members of the fecal microbiota likely hosted the aminoglycoside resistance genes.

The beta-lactamase gene bla_{TEM} was frequent amongst the dog samples. Variants of this gene and variants of bla_{SHV} and $bla_{\text{CTX-M}}$ encode ESBLs in *Enterobacteriaceae*

(Ramadan *et al*., 2019). The most recent report on fecal indicator bacteria in Norwegian dogs showed a prevalence of 1.3 % extended-spectrum cephalosporin producing *E. coli* and no carbapenemase-producing *Enterobacteriaceae* (NORM, 2020), indicating that the genes in paper I were not ESBL variants. However, the report was based on selectively cultured isolates, a method with lower sensitivity compared to qPCR and solely able to detect bacteria actively expressing genes, resulting in lower detection rates of ESBL genes (Singh *et al.*, 2012).

Over 20 % of the dogs tested positive for *mecA* in their fecal samples. This was a surprisingly high level considering the reported prevalence of 2.6 % MRSP-positive healthy dogs in Norway (Kjellman *et al.*, 2015) and our findings in Paper III with none of the healthy dogs carrying MRSP or MRCoNS. The *mecA*-positive rate was also high compared to the reported occurrence of 0 % in fecal samples from Portuguese dogs and cats (Belas *et al.*, 2020), a country with a relatively high prevalence of *mecA*-positive staphylococci (Couto *et al.*, 2016)
The different methodologies could explain some of the discrepancies between these studies and Paper I: Paper III and the study by Kjellman *et al.* were based on cultured perineal and oral samples, while the Portuguese study used selective culturing in addition to PCR on DNA extracted directly from fecal samples. The high sensitivity of HT-qPCR likely enabled us to detect low abundance *mecA* genes from the fecal samples that otherwise not necessarily would have been detected using PCR or culture-based methods.

Given the low reported rates of MRSP in healthy Norwegian dogs, and the high occurrence of MRCoNS/MRM from the home environments in Paper III, it seems reasonable to suspect that the *mecA* genes in the fecal samples originated from environmental MRCoNS/MRM. Dogs tend to interact closely with their surroundings, for instance, by eating off or licking the floor, and are thus exposed to MRS/MRM through their behavior. Whether the *mecA* genes originated from naked DNA, bacteria transiently passing the gastrointestinal tract, or from more permanently residing bacteria are not clear. However, *S. saprophyticus*, the most frequently detected methicillin-resistant species from the environmental samples in Paper III, is a common member of the human intestinal microbiota (Nishimura *et al.*, 2020) and has also been detected in cattle and pigs (Raz *et al.*, 2005). As the canine gastrointestinal tract has yet to be described as a reservoir for *S. saprophyticus*, we can only speculate about the source of the *mecA* genes in Paper I.

Considering the high rates of *mecA*-positive dogs in Paper I, it was peculiar that we did not detect any MRS or MRM from the perineal or oral samples from the healthy pets in Paper III. Several studies have documented that MRCoNS/MRM carriage in companion animals is far from uncommon, with carrier rates ranging from ~7-16 % (Garbacz *et al.*, 2013; Quitoco *et al.*, 2013; Gómez-Sanz *et al.*, 2019).
The discrepancies regarding MRCoNS/MRM carriage between the studies may rely on different inclusion criteria, sample size, and sampling sites, as a large proportion of isolates in the mentioned studies were recovered from the pets' nasal mucosae. Furthermore, geographical variations in the prevalence of MRS will presumably affect the probability of companion animal carriage. However, considering the vast presence of MRCoNS/MRM in the home environments in Paper III, protection from exposure does not explain the absence of carriers in the study.

5.4.2.2 The infected pet as a reservoir for AMR

MRS are often multidrug-resistant (Pires dos Santos *et al.*, 2016; Gómez-Sanz *et al.*, 2019), and the isolates from the infected pets in Paper II were no exception. The MRSP isolates displayed a high degree of resistance to MLS antimicrobials, tetracycline, and trimethoprim. Additionally, the genomic analysis revealed a broad range of aminoglycoside resistance genes, as well as trimethoprim and clindamycin resistance genes, whose presence would have gone undetected if we had relied solely on the phenotypic results. The dogs with MRS infections all carried the clinical MRS in either one or both carrier sites sampled, indicating that they were colonized by the bacteria. Long-term MRSP colonization is well documented in dogs, with individuals testing positive for months and over a year after infection recovery (Laarhoven *et al.*, 2011; Beck *et al.*, 2012; Windahl *et al.*, 2012). Therefore, it was noteworthy that one of the dogs participating in longer-term sampling deviated from this established presumption. With one exception, the dog remained negative for MRSP throughout the sampling period. This dog's samples were instead dominated by an MSSP strain, completely susceptible to all antimicrobials included in the test panel and negative for ARGs in the genomic analysis. The MSSP isolates carried *bacsp222*, a gene encoding the peptide BacSp222 that possesses both bacteriocin and virulence properties (Wladyka *et al.*, 2015). As BacSp222 kills gram-positive bacteria, including phylogenetically related strains at low concentrations, it is tempting to hypothesize that the MSSP strain outcompeted the MRSP residing on the dog's skin and mucosal surfaces, thus providing a protective effect against further colonization of MRSP.

Interestingly, we detected two dogs carrying multidrug-resistant MRSE in the perineal area in addition to MRSP. As we had no longer-term data on these dogs, it was impossible to determine whether they were colonized or if it was a transient carriage. Both these dogs had been treated with beta-lactam antimicrobials shortly before sampling, which may have favored the MRSE to reside on the dogs.

5.4.2.3 The home environment as a reservoir for AMR

Through papers II and III we documented that MRS and MRM could be recovered from various locations in the home environments. Moreover, most isolates expressed resistance to additionally two or more classes of antimicrobials, thereby demonstrating multidrug resistance (Magiorakos *et al.*, 2012).
The clinical MRS were primarily located in the immediate surroundings of the dogs but were also present in locations out of reach for the dogs (Paper II). Furthermore, we documented that clinical MRSP was present in the home environment over a prolonged period despite infection recovery and one dog being euthanized. The environmental finding of MRSP in the infection recovery case was hardly surprising, considering MRSP's persistence in dogs after infection recovery. Hence, bacterial shedding may persist equally long (Laarhoven *et al.*, 2011). As exemplified by our findings of MRSP in the home environment of the euthanized dog, MRSP may survive for weeks outside of a natural host. This resilience is problematic as it may enable the bacteria to re-colonize and infect the host again. Moreover, it raises the question of when it is "safe" to introduce MRSP naïve dogs into environments that have been occupied by MRSP-positive individuals, as it is easily transmitted between dogs (Laarhoven *et al.*, 2011). In the case of the household with the euthanized dog, the owner had implemented several hygienic measures in the weeks after the dog had been put down. The measures included vacuum cleaning and washing floors multiple times a week, and the use of alcohol and quaternary ammonium compound containing disinfecting spray on floors, rugs, and surfaces. Despite the effort, we were still able to recover MRSP from two locations in the living room five weeks after the dog had been put down, underlining the staphylococcal resilience.

When analyzing environmental samples collected from the households for Paper II, we identified several species of MRCoNS and MRM that we could recover from neither the pet nor the owner. These findings made us wonder whether the home environment could serve as a reservoir on its own. Our suspicion was further increased when we observed the same trend in households with healthy pets and

households without pets: MRCoNS and MRM were almost ubiquitous regardless of the type of household and carrier status of owners and pets. Thus, our findings add to the ranks of non-hospital locations from which MRCoNS/MRM have been detected (Stepanović *et al.*, 2008; Xu *et al.*, 2015; Seng *et al.*, 2017).

As discussed in the «Samples and sampling sites» paragraph in Section 5.4.1 ("Methodological considerations"), we might have underestimated the MRCoNS carriage rates, at least in the human participants. Still, the vast presence of MRCoNS/MRM in the environmental samples indicates that the home environment serves as a reservoir for these bacteria. A reservoir is defined as «the habitat in which the agent normally lives, grows, and multiplies» (CDC, 2012). Although, CoNS are widespread in natural environments (Silva *et al.*, 2020), the most frequently detected species in Paper III are associated with humans. Therefore, it is natural to suspect that the source for these MRCoNS is, in fact, humans and that the home environments become reservoirs because of the human presence.

Although we detected MRCoNS and MRM in all types of households, the households with infected pets tended to have a broader range of MR species in the home environment than in households with healthy pets and without pets. The significant difference between the infected pet households and those without pets may be due to fewer sampling locations in the latter. However, the species diversity may also reflect an antimicrobial-induced selection of MRCoNS/MRM. Given that more than half of the dogs in the infection group had been treated with beta-lactams shortly before sampling, MRCoNS/MRM potentially residing on these dogs or in the surroundings could gain a competitive advantage compared to susceptible surrounding flora. However, further investigation is needed to determine if this is in fact the case.

5.4.2.4 Transmission of AMR

To assess the risk of AMR transmission between pets, humans, and the environment, it is essential to investigate the transmission frequency of AMR between these three. This, however, is challenging to quantify, especially in regions where AMR is highly prevalent. Also, the direction of transmission may be difficult to determine, considering that many bacteria have a broad range of hosts and habitats. Given the favorable situation in Norway, with low levels of AMR, we are in a good position to study resistance development and horizontal gene transfer at an early stage (Hanssen and Ericson Sollid, 2006; NORM, 2021).

In Paper I, we compared the ARGs in feces from dogs and owners. This study design was not suited to determine whether there had occurred interspecies transmission of ARGs. Still, the modest proportion of shared ARGs between cohabiting dogs and owners indicates that ARG transmission between the dog and human gut is of minor concern. The number of owners and dogs carrying the *intI1* gene was notably low, considering that class 1 integrons are regarded as universal in the microbiota of humans and domesticated animals (Gillings, 2017). This low number may be a contributing factor to the low proportion of shared ARGs, as class 1 integrons are closely linked to the dissemination of ARGs.

Interestingly, we observed that the older dogs had more ARGs in common with their owners than younger dogs. This relied mainly on a larger proportion of the older dogs carrying the *ermF* gene, a gene that was otherwise more frequent among the owners. Whether this was caused by interspecies transmission of ARGs, a shift in the dogs microbiomes with age, or if it was purely coincidental, remains unknown.

Although healthy individuals may carry and transmit AMR bacteria to their surroundings, there is reason to believe that infected individuals play a more prominent role in transmitting bacteria, as bacteria rapidly multiply during infection, and clinical signs often are correlated with bacterial shedding (Keefe, 2012; O'Brien *et al.*, 2013). Another aspect that may influence the risk of transmission is that pets often undergo treatment in their homes, interacting with their owners and home environment whilst recovering. This interaction may facilitate the transmission of bacteria, especially if the infection is located on sites such as skin and mucosa that are directly in contact with the surroundings. As documented in Paper II, the infected pets primarily shed clinical MRS to their immediate surroundings, meaning their food bowls, sleeping places, and the floor. There was, however, one exception to this trend. In one of the households, we could only detect the clinical MRS in the dog's food bowl and not from any other locations in the home environment. Unlike the other participating dogs, this dog's movements were confined to an enclosure in the living room, and the infection site was covered in bandages, presumably providing a protective barrier against bacterial shedding to the surroundings. Despite the hygienic precautions, the contact dog in the household tested positive for MRSP, thus demonstrating how easily MRSP transmits between dogs (Windahl *et al.*, 2016). In half of the households, we detected MRSP in locations out of reach for the dogs, indicative of indirect transmission routes, for

instance, by dust particles or mechanical vectors such as hands or cleaning cloths (Laarhoven *et al.*, 2011; van Duijkeren, E. *et al.*, 2011). We detected human carriage of clinical MRS in two cases, one case of MRSP and one case of MRSE. In the MRSP case, we can be reasonably certain of the transmission direction, considering that dogs are the natural host for *S. pseudintermedius*. In the MRSE case, however, the direction is less obvious. Although *S. epidermidis* is mainly associated with humans, dogs may also be carriers (Kern and Perreten, 2013; Gómez-Sanz *et al.*, 2019). This particular sequence type has previously been identified in humans and dairy cows (Kim *et al.*, 2019; Asante *et al.*, 2021), and not in dogs, which may indicate that the owner was the "original" source for the bacteria. Dogs and owners concurrently carried MRSE in two more cases, but there were no indications that transmission had occurred since the sequence types and SCC*mec* elements differed between the human and canine isolates. Three more owners carried MRCoNS while their pets tested negative. In most cases of human carriage, isolates with identical sequence types were present in the home environment, indicative of a more significant interplay between the environment and the humans than between the pets and owners regarding MRCoNS transmission. The high presence of MRCoNS in the home environment compared to relatively low human carriage rates still points in the direction of low transmission frequencies. However, it is important to remember that this impression is based on a cross-sectional study in a small number of households.

Through papers II and III, we documented that humans and pets are exposed to MRS and MRM by interacting with each other and their home environments, but the significance of the exposure is debatable. Similar to *S. aureus*, colonization is likely a contributing factor to infection development for *S. pseudintermedius* (Bhooshan *et al.*, 2020). Several studies have documented human carriage of *S. pseudintermedius*. Even though longer-term carriage has been documented (Kronbichler *et al.*, 2019), the majority of studies, as well as the findings in Paper II, indicate that human carriage is rare and temporary (Hanselman *et al.*, 2009; Laarhoven *et al.*, 2011), The vast number of companion animals relative to the reported human cases of *S. pseudintermedius* infections implies that *S. pseudintermedius*' reputation as an "emerging human pathogen" (Somayaji *et al.*, 2016; Bhooshan *et al.*, 2020) is somewhat overstated. That said, human infections occur, and the number of cases is likely underreported (Guardabassi *et al.*, 2013; Börjesson *et al.*, 2015). Demonstrated by the clinical infections, we know that the MRSP strains in this study were able to cause disease in dogs. Furthermore, the isolates possessed known and

putative virulence genes enabling them to do so. As many of these are orthologues to important virulence factors in *S. aureus* (van Duijkeren, Engeline *et al.*, 2011), the question is raised if any of these factors may contribute to crossing the species barrier from dogs to humans. It is clearly sufficient in some cases but which factors that are of significance are yet unidentified. Given that many reported human *S. pseudintermedius* infections have been observed in elderly or immunocompromised patients (Somayaji *et al.*, 2016), host factors, such as age and health status, seem to be of importance.

The significance of MRCoNS and MRM exposure is likely limited since CoNS are primarily associated with nosocomial infections and invasive procedures. Nonetheless, with the vast presence in the home environment, humans and pets are continuously exposed, which offers the MRCoNS ample opportunity to colonize potential hosts. The observed trend of human- and pet-carriage being associated with recent antimicrobial use suggests that the MRCoNS had seized this opportunity. However, this is again speculative, as there might have been other factors affecting the results or cases of transient carriage. A different study design would therefore be necessary to support such a hypothesis.

Another aspect of the high presence of MRCoNS in the households is their reservoir of ARGs and virulence genes which potentially may be transferred to other more virulent bacteria such as *S. aureus* or *S. pseudintermedius* (Otto, 2013). As the transfer mechanisms and frequencies of SCC*mec* have not yet been clarified, it is impossible to assess the risk for SCC*mec* transfer in a household setting. However, seeing that natural transformation now has been demonstrated in biofilm (Maree *et al.*, 2022), it was somewhat reassuring to observe a low frequency of genes encoding biofilm formation in the isolates in Paper III. Furthermore, the mobilome analysis performed on these isolates displayed a species-specific profile more than a household-related pattern, suggesting a low level of genetic exchange at the household level.

5.5 Conclusions

- Healthy pets constitute a reservoir for certain antimicrobial resistance genes, emphasizing that the potential for selection and propagation of resistant bacteria is highly present.

- The high rates of *mecA* in the canine fecal samples may have originated from environmental MRCoNS/MRM, given their vast presence in the home environment.

- Dogs with MRS infections primarily shed clinical MRS to locations in direct contact with the dogs, however, indirect transmission within the household is also possible.

- The home environment serves as a reservoir for clinical MRS in households with infected pets, and for MRCoNS and MRM regardless of type of household.

- Humans are exposed to AMR through interaction with infected pets and home environments.

- The moderate proportion of shared ARGs and concurrent carriage of MRS/MRM indicate a low level of transmission between pets and owners, home environment and humans, and between healthy pets and the home environment.

- The environmental MRCoNS and MRM isolates expressed variable phenotypic resistance to beta-lactam antimicrobials.

- Oxacillin was more reliable for detecting methicillin-resistant isolates than cefoxitin.

5.6 Future perspectives

To mitigate the dissemination AMR, there is no doubt that the AMR crisis needs to be addressed with a One Health approach. In this regard, it is necessary to learn more about the AMR reservoirs and mechanisms involved in ARG transmission between the different sectors of One Health. Thus, studies investigating the resistomes of different reservoirs are required. Despite the close contact companion animals have with humans in daily life, surprisingly little is known about their role as reservoirs, as few resistome-level studies are available. Furthermore, the ARG flow between the different sectors of One Health should be investigated by monitoring the interfaces among these sectors. Regarding the ARG flow and bacterial transmission between companion animals and humans, larger-scale longitudinal studies, including control groups of humans with no contact with companion animals, should be performed. In addition, studies on factors and mechanisms influencing horizontal gene transfer between different bacterial species are required to understand and mitigate dissemination of ARGs.
As for the staphylococci, many questions remain to be answered. From a One Health perspective, the horizontal transfer of SCC*mec* in nature is of particular interest and is not yet understood. Moreover, research on the staphylococci's host specificity and virulence factors (also including other species than *S. aureus*) is necessary to understand their pathogenic potential, as well as the risk for zoonotic transmission.

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7 Scientific papers I-III

Paper I

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ORIGINAL ARTICLE

Antimicrobial resistance—Do we share more than companionship with our dogs?

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Abstract

Aims: To investigate and compare antimicrobial resistance genes (ARGs) in faeces from cohabiting dogs and owners.

Methods and Results: DNA from faecal samples from 35 dogs and 35 owners was screened for the presence of 34 clinically relevant ARGs using high throughput qPCR. In total, 24 and 25 different ARGs were present in the dog and owner groups, respectively. The households had a mean of 9.9 ARGs present, with dogs and owners sharing on average 3.3 ARGs. ARGs were shared significantly more in households with dogs over 6 years old (3.5, interquartile range 2.75–5.0) than in households with younger dogs (2.5, interquartile range 2.0–3.0) ($p = 0.02$). Dogs possessed significantly more *mecA* and aminoglycoside resistance genes than owners.

Conclusions: Dogs and owners can act as reservoirs for a broad range of ARGs belonging to several antimicrobial resistance classes. A modest proportion of the same resistance genes were present in both dogs and owners simultaneously, indicating that ARG transmission between the dog and human gut is of minor concern in the absence of antimicrobial selection.

Significance and Impact of the Study: This study provides insight into the common dog and human gut resistomes, contributing to an improved knowledge base in risk assessments regarding ARG transmission between dogs and humans.

KEYWORDS

antimicrobial resistance genes, dog, faecal resistome, high throughput qPCR, human, one health

INTRODUCTION

Antimicrobials are amongst the most prescribed medicines globally, and consumption continues to increase (Klein et al., 2018; Sriram et al., 2021). Bacteria have proven to be highly adaptive to antimicrobials, as they have managed to develop resistance mechanisms to nearly all antimicrobials shortly after they were introduced (Ventola, 2015). The use and misuse of antimicrobials in human and veterinary medicine have contributed to the global spread of drug-resistant bacteria by driving the selection of bacteria in possession of antimicrobial resistance genes (ARGs) (Holmes et al., 2016). This complicates the treatment of infections in both human and veterinary medicine to such a degree that WHO has declared antimicrobial resistance one of the top 10 global public health threats to humanity (WHO, 2020a).

Humans live in an environment interacting with animals that may carry pathogens, which occasionally cross-species barriers (WHO, 2020b). Animals are essential to humans both as a source of food and as companionship, but this relationship does not come without risks. The animal kingdom is a reservoir for micro-organisms causing 60%–70% of infectious diseases in humans (Woolhouse & Gowtage-Sequeria, 2005). Furthermore, most pathogens involved in emerging infectious disease events are caused by drug-resistant strains (Jones et al., 2008). Companion animals are often in close direct contact with humans. For instance, dogs may share housing, food, sofas, and perhaps even beds with their owners. Hygienic measures like hand wash are not necessarily performed after direct or indirect contact with these animals. Hence, the potential for transmission of antimicrobial-resistant (AMR) bacteria between companion animals and owners is present. As emphasized in an assessment report by the Norwegian Committee for Food and Environment, there is a lack of data regarding AMR reservoirs in pets and humans (VKM, 2015). This identified knowledge gap hampers the development of proper risk assessments.

Culturable AMR bacteria such as methicillin-resistant *Staphylococcus* spp. (Ferreira et al., 2011) and extended-spectrum β-lactamase producing members of the Enterobacteriaceae family (Grönthal et al., 2018; Ljungquist et al., 2016) have received most of the attention as these are opportunistic pathogens and have been simultaneously isolated from cohabiting dogs and owners. However, non-pathogenic gut commensals may also host ARGs (Bag et al., 2019). These bacteria may be overlooked since culture conditions for a significant part of the gut commensals are unknown (Juricova et al., 2021). To better understand the occurrence of ARGs, the possible interplay and exchange of ARGs between companion animals and their owners, and their respective gut resistomes must be explored more comprehensively and independently of isolation of specific bacterial species.

This study aimed to investigate and compare the presence of ARGs in faeces from cohabiting dogs and owners. Using high throughput qPCR, we screened faecal samples from 35 dogs and owners for the presence of 34 clinically relevant ARGs.

MATERIALS AND METHODS

Recruitment and enrolment criteria

This project was approved by the Regional Committee for Medical and Health Research Ethics Southeast, approval number: 62346. Participants were recruited and samples were collected through the HUNT4-One Health survey (NTNU, 2019; NMBU, 2020). All participants signed consent forms before enrolment. A total of 836 dogs participated in the survey. Questionnaires about the dogs' breed, health condition, diet, activities and primary use were sent to the owners after sample collection. One hundred and eleven completed questionnaires were returned. Dog and owner pairs ($n = 35$) were selected from the pool of 111 dogs based on the following criteria: The dog's primary use was being a family dog, and the owner considered the dog's health condition to be good or excellent at the time of sampling. To avoid the formation of subgroups amongst the dogs, sledge dogs, hunting dogs and dogs who underwent antimicrobial treatment or had gastrointestinal symptoms at the time of sampling were excluded. No information on antimicrobial use was available through the HUNT study for the owners. However, all the owners participating in this study had submitted self-evaluation scores of their health with answering options poor, not so good, good and excellent. In addition, participants had reported whether they suffered from any long-standing illness or injury of a physical or psychological nature impairing their function in their daily lives with answering options yes or no.

Sampling

All participants received written instructions and a video link on how to collect faecal samples. Participants collected about a teaspoon of fresh faeces using EasySampler for stool collection (GP Medical Devices), gloves, and a wooden spatula to apply faeces on a collection card (LipiDx). The same participants collected faecal samples from their respective dogs and applied them to collection cards. The collection cards were left to dry for approximately 2 h and then put into separate sterile envelopes. Samples were sent to the HUNT Biobank by mail for storage at −20°C until further handling and genomic DNA extraction.

DNA extraction

Depending on the visible amount of faecal material on the collection card, one to two 8 mm biopsy punches from the dog samples ($n = 35$) were used for DNA extraction. One 6 mm biopsy punch from the human collection cards was used for the analysis. One 8 mm punch from empty collection cards was included as a negative control for each extraction batch ($n = 4$). The DNA extractions were performed using the QIAamp PowerFaecal Pro DNA kit (Qiagen GmbH) according to the manufacturer's protocol. For the bead beating step, we used the TissueLyser II system at 30 Hz for 10 min.

We used the supplied C6 solution as elution buffer with a final volume of 50 µl. Quantification of eluted DNA was performed by Qubit 3.0 fluorometer using dsDNA Broad Range Assay Kit (Invitrogen). The DNA quality was measured using a NanoDrop™ ND-1000 spectrophotometer (Thermo Scientific). The eluates were stored at 4°C for no more than 4 days before 20 µl were sent overnight on ice for HT-qPCR analysis.

HT-qPCR analysis

The qPCR analysis was performed at the Norwegian Institute of Bioeconomy Research (NIBIO) using a high-throughput setup with the Biomark HD system for real-time PCR (Fluidigm). Pre-amplification was performed with 1.25 µl of DNA and a final primer concentration of 0.05 µmol l^{-1} in a 14-cycled specific target amplification. The primers used are listed in Table 1. The pre-amplification conditions were as follows: Initial denaturation at 95°C for 15 min, 14 cycles at 95°C for 15 s and 60°C for 4 min. The presence of ARGs in the faecal samples was determined using a qPCR chip with 46 assays developed to detect 34 ARGs. We selected these ARGs based on the list of indicators by Berendonk et al. (2015) and expanded with other clinically relevant ARGs. The ARGs are responsible for genotypic resistance to 10 antimicrobial classes, including beta-lactams, tetracyclines, aminoglycosides, amphenicols, fluoroquinolones, sulphonamides, dihydrofolate reductase (DHFR) inhibitors, glycopeptides, colistin, and macrolide-lincosamide-streptogramin B (MLS). In addition, the chip contained two assays for the detection of microbial DNA (16S rRNA) and the class 1 integron-integrase gene (*intl1)*. Eleven positive controls with confirmed presence of specific ARGs and four negative controls were included in each run. The chip was primed and loaded with pre-amplified DNA (2.25 µl) and EvaGreen assays (Invitrogen) in two replicates according to the manufacturer's protocol. Initially, the samples were thermal mixed at 70°C for 40 minutes, followed by 60°C for 30 s. Then, the thermal profile was: Initial hot start at 98°C for 2 min, 40 cycles at 98°C for 5 s, and 60°C for 20 s, ending with a melting curve analysis at 60°C for 3 s followed by a 1°C/3 s increase to 95°C. All 46 assays were tested against standard curves of the 11 positive controls to determine the slopes and intercept for quantification of each assay. Data collection was performed using Biomark HD Data Collection software (Fluidigm, USA). The positive controls were used to correct the cycle threshold (CT) value before quantification. Quantification of ARGs present was conducted in Fluidigm Real-time PCR analysis software (version 4.5.2) using Equation 1, in which the CT value represented the mean of the duplicates.

$$\mathrm{ARG}\left(\frac{\mathrm{ng}}{\mu\mathrm{l}}\right) = 10\left(\frac{\mathrm{CT} + \mathrm{CT}_{\mathrm{corr}} - \mathrm{intercept}}{\mathrm{slope}}\right) \quad (1)$$

Statistical analysis

Statistical analysis was performed using JMP® Pro Software (Version 15.2.1, SAS Institute Inc.). The quantitative output of ARGs was transformed to binominal values and treated in general as categorical variables in the statistical analysis. Fisher's exact test was applied when comparing the presence/absence of genes between dogs and owners at the group level. When comparing the number of ARGs and antimicrobial resistance classes between the groups, the data were treated as continuous variables, and a two-tailed Mann–Whitney test was applied. The significance level was set at 5%. The mean values are reported with their corresponding interquartile ranges (IQR). Pearson's correlation coefficient was used to assess the correlation between the number of ARGs in dogs and owners at the household level.

RESULTS

Enrolment and participant data

Of the 35 dogs enrolled, 24 were purebreds from 19 different breeds, and 11 dogs were of mixed or unknown breeds. The group consisted of 17 males and 18 females, four of whom were neutered. Their median age was 6 years. Twelve dogs had never received antimicrobial treatment; eight dogs had received antimicrobial treatment between one and three times. Three dogs had received antibiotics more than three times. Four owners did not recall whether their dogs had been treated with antibiotics during their lifetime.

The owner group consisted of 18 women and 17 men with a median age of 55 years. Of these, 29 considered their health to be good or excellent. None of the participants reported their health to be poor, whilst five considered their health not good. Twenty-three owners reported not to be suffering from any longstanding illness or injury of a physical or psychological nature impairing their functioning in their daily lives, whilst 12 reported suffering from this.

Analysis

Of the dog and owner samples, 69/70 tested positive for the presence of microbial DNA (16S rRNA). The negative sample was of canine origin and was excluded from further analysis. The owner of this dog was included in the analysis of human samples but excluded from the household

TABLE 1 List of primers included in the qPCR chip for detection of ARGs

Assay	Forward primer	Reverse primer	References
16S_1	CCCAGATGGGATTAGCTTGT	TCTGGACCGTGTCTCAGTTC	Kim and Lee (2014)
aac6_1	CTGTTCAATGATCCCGAGGT	TGGCGTGTTTGAACCATGTA	Hu et al. (2013a, 2013b)
aac3_2	GCGCACCCCGATGCMTCSATGG	GGCAACGGCCTCGGCGTARTGSA	Heuer et al. (2002)
ant3_1	CAGCGCAATGACATTCTTGC	GTCGGCAGCGACAYCCTTCG	Walsh et al. (2011)
ant3_2	ATCTTGCGATTTTGCTGACC	TGTACCAAATGCGAGCAAGA	Szczepanowski et al. (2009)
aph3_2	ATTCAACGGGAAACGTCTTG	ACGCTACCTTTGCCATGTTT	Szczepanowski et al. (2009)
blaACT_3	GTRCCGGATGAGGTCRMGGAT	TGGYRTTRGCGTAAAGACG	Chavda et al. (2016)
blaCTX_2	GCGATAACGTGGCGATGAAT	GTCGAGACGGAACGTTTCGT	Zhu et al. (2013)
blaCTX_3	CGTCACGCTGTTGTTAGGAA	CGCTCATCAGCACGATAAAG	Szczepanowski et al. (2009)
blaDHA_1	AACTTTCACAGGTGTGCTGGGT	GCTGCCACTGCTGATAGAA	Pérez-Pérez and Hanson Nancy (2002)
blaKPC_1	GGCAGTCGGAGACAAAACC	CCCTCGAGCGCGAGTCTA	Chen et al. (2012)
blaNDM_1	TTGGCGATCTGGTTTTCC	GGTTGATCTCCTGCTTGA	Zheng et al. (2013)
blaNDM_2	CGCAACACAGCCTGACTTT	TCGATCCCAACGGTGATATT	Ong et al. (2011)
blaSHV_1	TCCCATGATGAGCACCTTTAAA	TCCTGCTGGCGATAGTGGAT	Roschanski et al. (2014)
blaTEM_1	GCATCTTACGGATGGCATGA	GTCCTCCGATCGTTGTCAGAA	Roschanski et al. (2014)
blaVIM_1	GGTCTCATTGTCCGTGATGGTGATGAG	CTCGATGAGAGTCCTTCTAGAG	Kaczmarek et al. (2006)
blaVIM_2	TGGCAACGTACGCATCACC	CGCAGCACCGGGATAGAA	Weiß et al. (2017)
blaVIM_3	GCACTTCTCGCGGAGATTG	CGACGGTGATGCGTACGTT	Zhu et al. (2013)
catA_2	GGGTGAGTTTCACCAGTTTTGATT	CACCTTGTCGCCTTGCGTATA	Zhu et al. (2013)
cmlA_3	TAGTTGGCGGTACTCCCTTG	GAATTGTGCTCGCTGTCGTA	Szczepanowski et al. (2009)
dfrA_2	GAGCTGAGATATACACTCTGGCACT	GTACGGAATTACAGCTTGAATGGT	Grape et al. (2007)
ermB_1	GGTTGCTCTTGCACACTCAAG	CAGTTGACGATATTCTCGATTG	Koike et al. (2010)
ermB_2	GGATTCTACAAGCGTACCTTGGA	TGGCAGCTTAAGCAATTGCT	Schmidt et al. (2015)
ermB_3	GGATTCTACAAGCGTACCTTGGA	AATCGAGACTTGAGTGTGCAAGAG	Belén Flórez et al. (2014)
ermF_1	TCGTTTTACGGGTCAGCACTT	CAACCAAAGCTGTGTCGTTT	Schmidt et al. (2015)
ermF_2	TGATGCCCGAAATGTTCAAGT	AAAGGAAATTTCGGAACTGCAA	Belén Flórez et al. (2014)
floR_2	ATTGTCTTCACGGTGTCCGTTA	CCGCGATGTCGTCGAACT	Zhu et al. (2013)
intl1_1	CCTCCCGCACGATGATC	TCCACGCATCGTCAGGC	Bass et al. (1999)
mcr1_2	ACACTTATGGCACGGTCTATG	GCACACCCAAACCAATGATAC	Bontron et al. (2016)
mecA_1	CATTGATCGCAACGTTCAATTT	TGGTCTTTCTGCATTCCTGGA	Francois et al. (2003)
oqxA_3	GCGATGATGCTCTCCTTTCT	GATCGACTTCACCAGCACCT	Pitt et al. (2020)
oqxB_1	TCCTGATCTCCATTAACGCCCA	ACCGGAACCCATCTCGATGC	Kim Hong et al. (2009)
qnrA1_1	ATTTCTCACGCCAGGATTTG	CAGATCGGCATAGCTGAAG	Marti and Balcázar (2013)
qnrB1_2	GGMATHGAAATTCGCCACTG	TTYGCBGYYCGCCAGTCG	Cattoir et al. (2007)
qnrS_1	GACGTGCTAACTTGCGTGAT	TGGCATTGTTGGAAACTTG	Marti and Balcázar (2013)
strA_3	CCAGTTCTCTTCGGCGTTAG	ACTCTTCAATGCACGGGTCT	Faldynova et al. (2013)
strB_2	CGGTCGTGAGAACAATCTGA	ATGATGCAGATCGCCATGTA	Pyatov et al. (2017)
sul1_3	ACGAGATTGTGCGGTTCTTC	CCGACTTCAGCTTTTGAAGG	Li et al. (2007)
sul2_2	CTCCGATGGAGGCCGGTAT	GGGAATGCCATCTGCCTTGA	Luo et al. (2010)
sul3_3	TTCGTTCAGCGAATTGGTGCAG	TTCGTTCACGCTTTACACCAGC	Muziasari et al. (2014)
tetA_3	CTCACCAGCCTGACCTCGAT	CACGTTGTTATAGAAGCCGCATAG	Zhu et al. (2013)
tetB_2	GCCCAGTGCTGTTGTTGTCAT	TGAAAGCAAACGGCCTAAATACA	Zhu et al. (2013)
tetM_2	TAATATTGGAGTTTTAGCTCATGTTGATG	CCTCTCTGACGTTCTAAAAGCGTATTAT	Zhu et al. (2013)

TABLE 1 (Continued)

Assay	Forward primer	Reverse primer	References
vanA_1	CTGTGAGGTCGGTTGTGCG	TTTGGTCCACCTCGCCA	Volkmann et al. (2004)
vanA_2	AGCTGTACTCTCGCCGGATA	CGCAGCCTACAAAAGGGATA	Cantarelli et al. (2011)
vanA_3	GCCGGAAAAAGGCTCTGAA	TTTTTTGCCGTTTCCTGTATCC	He et al. (2020)

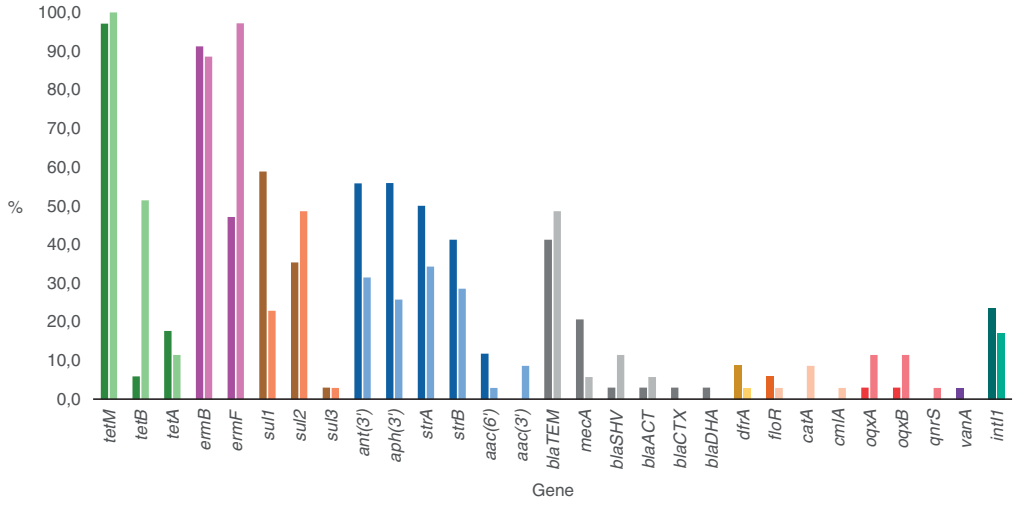

FIGURE 1 Detected antimicrobial resistance genes and the class 1 integron-integrase gene intI1 in dogs, represented by the darker shades, and owners, represented by lighter shades. Different colours represent the different antimicrobial classes. From left to right: Tetracyclines, macrolides-lincosamides-streptogramins (MLS), sulphonamides, aminoglycosides, beta-lactams, dihydrofolate reductase (DHFR) inhibitors, amphenicols, fluoroquinolones, glycopeptides, and the class 1 integron-integrase gene intI1.

level analysis, making the number of participating households 34. Three negative batch controls tested positive for low amounts of 16S rRNA, including one testing positive for the *ant(3′)* gene. Due to suspicion that the *ant(3′)* positive control had been contaminated during the first qPCR run, it was rerun under the same conditions. The control was then negative for *ant(3′)*; however, positive for low concentrations of the 16S rRNA gene.

Antimicrobial resistance genes

Our results show that 68/69 dog and owner samples tested positive for two or more ARGs. The remaining sample was of canine origin and lacked all the targeted ARGs. The detected ARGs in dogs and owners are listed in Figure 1 and Table 2. Overall, 28 different ARGs were detected in the human and canine samples combined, 24 ARGs in dogs and 25 ARGs in humans. The mean number of ARGs was 6.7 (IQR: 4.0–9.25) amongst the dogs and 6.7 (IQR: 4.-10.0) amongst the owners. The most frequently occurring ARGs in the dog group were *tetM* (97.1%, 33/34), *ermB* (91.2%, 31/34), *sul1* (58.8% 20/34), and *ant(3′)* (58.8%, 20/34). Likewise, *tetM* was the most frequent ARG amongst the owners, detected in all (100%, 35/35) samples, followed by *ermF* (97.1%, 34/35) and *ermB* (88.6%, 31/35). Seven dogs (20.6%) and two owners (5.4%) tested positive for the *mecA* gene. None of the dog nor owner samples tested positive for *qnrA1*, *qnrB1*, *mcr1*, bla_{KPC}, bla_{NDM} or bla_{VIM}.

Of the ARGs analysed, 61.8% (21/34) were equally represented in the two groups. The remaining 38.2% (13/34) ARGs were unique to one, or their presence differed significantly between the groups. Four of the ARGs, *floR*, bla_{CTX}, bla_{DHA} and *vanA*, were unique to the dog group. The *aac(3′)*, *catA*, *cmlA* and *qnr*S genes were found exclusively amongst the owners. Five ARGs, *ermF*, *tetB*, *ant(3′)*, *aph(3′)* and *sul1*, occurred in both groups but with significantly different frequencies (Table 2). The *ermF* gene was detected in 97.1% (34/35) of the owner samples and 47.1% (16/34) of the dog samples. Worth noticing is that 81.2% (13/16) of the *ermF*-positive dogs were at the median age of six or older, making it the only gene associated

TABLE 2 Results of the detection of antimicrobial resistance genes and their corresponding antimicrobial classes in dogs and owners. Numbers represent the percentage of individuals testing positive and the percentage of households in which both dog and owner tested positive for the same ARG. Listed *p*-values refer to differences in gene occurrence between dogs and owners. The class 1 integron-integrase gene intI1 is included at the bottom of the table. DHFR = dihydrofolate reductase. MLS = macrolide- lincosamide-streptogramin B.

Antimicrobial resistance class	Antimicrobial resistance gene	Dogs %	Owners %	*p*-value	Households with shared gene %
Aminoglycosides	*aac(6′)*	11.8	2.9	0.1981	0.0
	aac(3′)	0.0	8.6	0.2391	0.0
	ant(3′)	58.8	31.4	**0.0301**	14.7
	aph(3′)	55.9	25.7	**0.0146**	17.6
	strA	50.0	34.3	0.2270	11.8
	strB	41.2	28.6	0.3185	5.9
Amphenicols	*catA*	0.0	8.6	0.2391	0.0
	cmlA	0.0	2.9	1.0000	0.0
	floR	5.9	2.9	0.6139	0.0
Beta-lactams	bla_{ACT}	2.9	5.7	1.0000	0.0
	bla_{CTX}	2.9	0.0	0.4928	0.0
	bla_{DHA}	2.9	0.0	0.4928	0.0
	bla_{KPC}	0.0	0.0	—	0.0
	bla_{NDM}	0.0	0.0	—	0.0
	bla_{SHV}	2.9	11.4	0.3565	0.0
	bla_{TEM}	41.2	48.6	0.6307	23.5
	bla_{VIM}	0.0	0.0	—	0.0
	mecA	20.6	5.7	0.0840	2.9
Colistin	*mcr1*	0	0	—	0
DHFR inhibitors	*dfrA*	8.8	2.9	0.3565	0
Glycopeptides	*vanA*	2.9	0	0.4928	0
MLS	*ermB*	91.2	88.6	1.0000	79.4
	ermF	47.1	97.1	**<0.0001**	47.1
Quinolones	*oqxA*	2.9	11,4	0.3565	0.0
	oqxB	2.9	11,4	0.3565	0.0
	qnrA1	0.0	0.0	—	0.0
	qnrB1	0.0	0.0	—	0.0
	qnrS	0.0	2.9	1.0000	0.0
Sulphonamides	*sul1*	58.8	22.9	**0.0033**	5.9
	sul2	35.3	48.6	0.3319	23.5
	sul3	2.9	2.9	1.0000	0.0
Tetracyclines	*tetA*	17.6	11.4	0.5130	0.0
	tetB	5.9	51.4	**<0.0001**	2.9
	tetM	97.1	100	0.4928	97.1
Class 1 integron-integrase	*intI1*	23.5	17.1	0.5613	8.8

Significant *p*-values are emphasized in bold.

with age ($p = 0.0342$). In general, dogs possessed a wider range of aminoglycoside resistance genes than the owners (Table S1); 64.7% (22/34) of the dogs tested positive for two or more aminoglycoside resistance genes, compared to 37.1% (13/35) of the owners ($p = 0.0306$). Concurrent carriage of *ant(3′)* and *aph(3′)* occurred in 38.2% (13/34) of the dogs, compared to 11.4% (4/35) of the owners ($p = 0.0125$). In addition, 10 of these dogs tested positive for *strA* and *strB*, thus contributing to the high number of aminoglycoside resistance genes.

Class 1 integron-integrase gene (int*I*1)

Eight dogs (23.5%) and six owners (17.1%) tested positive for the *intI1* gene. The mean number of ARGs detected in the *intI1*-positive dogs was 9.4 (IQR: 7.25–12.75), significantly higher than the *intI1* negative dogs' mean of 5.9 (IQR: 4.0–7.5, $p = 0.0257$). The difference relied on more *intI1* positive dogs possessing *ant(3′)* ($p = 0.0109$), *strA* ($p = 0.0391$), bla_{TEM} ($p = 0.0039$) and *tetA* ($p = 0.018$) compared to the *intI1* negative dogs (Figure 2). We observed the same association amongst the *intI1* positive owners with a mean of 10.5 ARGs (IQR: 9.25–11.5) compared to *intI1* negative owners with a mean of 5.9 ARGs (IQR: 4.0–7.5, $p = 0.0009$). The *intI1*-positive owner samples contained more *ant(3′)* ($p = 0.0047$), bla_{TEM} ($p = 0.006$), *strA* ($p = 0.0082$), *strB* ($p = 0.0477$) and *sul1* ($p = 0.0096$) compared to the samples of the *intI1* negatives.

Household-level

On average, we detected 9.9 (IQR: 7.0–12.25) different ARGs in each of the 34 households included in the study. In total, 35.3% (12/34) of the different ARGs were identified simultaneously in both dogs and owners. These genes confer genotypic resistance to aminoglycosides, beta-lactams, MLS, sulphonamides and tetracyclines (Figure 3). We observed close to no correlation between the number of ARGs detected in cohabiting dogs and owners ($r\,[32] = -0.11$ $p = 0.52$). On average, dogs and owners had 3.3 (IQR: 2.0–4.25) ARGs in common. All except one household had a minimum of two shared ARGs, the exception being the household in which the dog tested negative for all ARGs (Figure 4). Households with dogs aged 6 years and older shared significantly more ARGs (3.5, IQR: 2.75–5.0) than households with younger dogs (2.5, IQR: 2.0–3.0) ($p = 0.0204$). The difference relied mainly on *ermF* being shared in 59% (13/22) of the older-dog households versus 18.2% (2/11) of the younger-dog households ($p = 0.0342$). Furthermore, in seven older dog households, both dog and owner had positive matches on *sul2*, whilst none in the younger dog households shared this gene. However, this difference was not significant ($p = 0.0674$). For one household, the dog's age was not listed and was excluded from the analysis. The *intI1* gene was simultaneously present in the dog and owner in three cases (Table S1). These dog-owner pairs had two, four and seven ARGs in common, respectively.

DISCUSSION

Literature on the occurrence of common ARGs amongst cohabiting dogs and humans is scarce, and few studies, e.g. Kim et al. (2020) and Liu et al. (2021), have focused on the canine gut resistome. Therefore, we aimed to describe the canine resistome and investigate to what degree cohabiting dogs and owners share ARGs in the gut by screening the samples for a panel of 34 ARGs and the class 1 integron-integrase gene *intI1*. Although most of the investigated ARGs were equally represented in both groups, the dogs and owners had few ARGs in common (3.3 ARGs on average) at the household level.

Our results show that tetracycline and MLS resistance genes were the most abundant ARGs irrespective of host species. These results correspond well with previous research on human faecal samples (Feng et al., 2018; Hu et al., 2013a, 2013b; Seville et al., 2009) and seem to comply with the dog samples as well. In striking contrast to our results that show a high representation of *ermB* in the dogs, Kim et al. (2020) did not detect any *ermB* genes amongst the canine faecal samples they investigated. Similar to us, Kim et al. (2020) found the tetracycline- and MLS resistance genes to be the most occurrent ARGs.

The slight majority of ARGs were equally present in both groups. However, 38.6% of the ARGs were unique to one group, or their presence differed significantly. The limited sample size may have contributed to the ARGs being unique to one group or absent in all samples. Nevertheless, the differences in the prevalence of

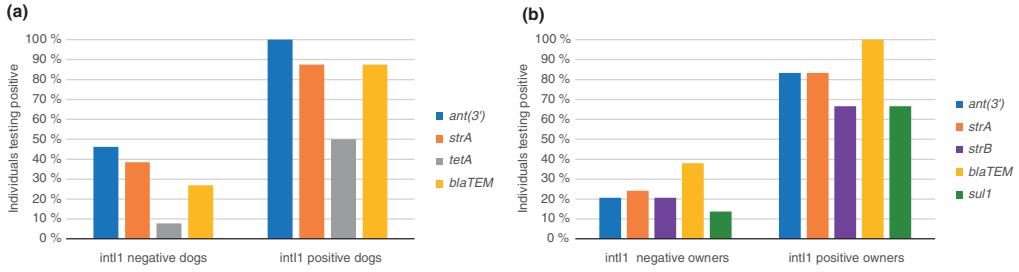

FIGURE 2 Distribution of selected ARGs in intI1 positive and negative dogs (a) and owners (b).

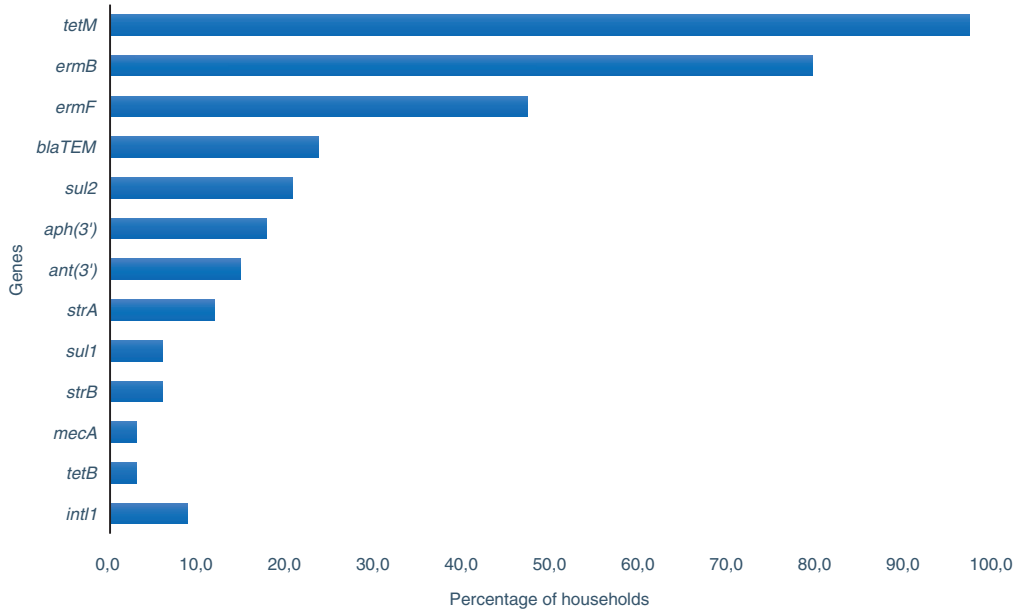

FIGURE 3 Percentage of households in which dogs and owners possessed the same ARGs. tetM and ermB were the dominating shared ARGs in the 34 households tested.

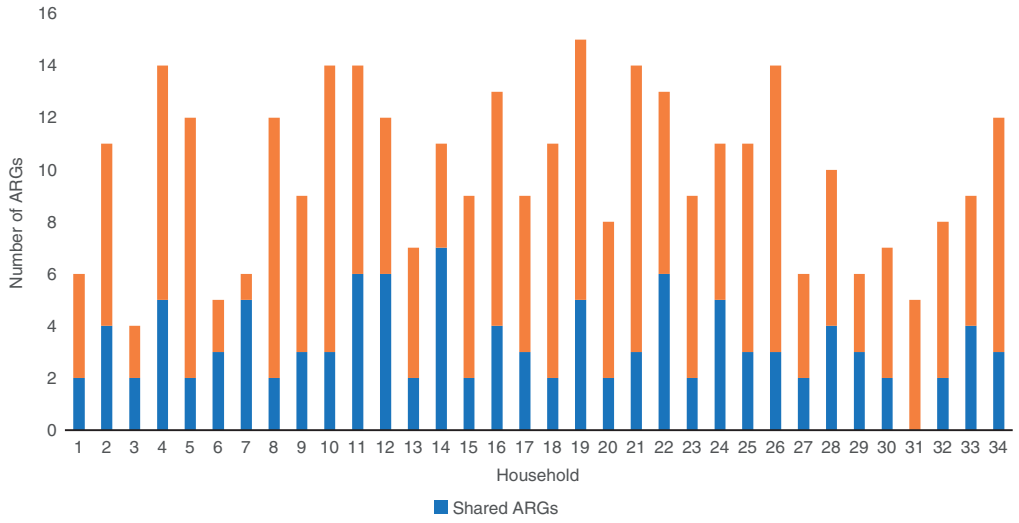

FIGURE 4 Total number of unique ARGs detected in the different households presented as columns. The blue proportions of the columns present the number of shared ARGs in the households. The total number of unique detected ARGs ranged between 4 and 15 in the households, whilst the shared ARGs ranged between 0 and 7.

sul1 and *tetB* between dogs and owners may point to species-specific compositional differences between the canine and the human gut microbiome. The *ant(3′)* gene was significantly more occurrent in the dog samples. Concurrent carriage of *aph(3′)* and, in many cases, also *strA* and *strB* contributed to a higher total number of aminoglycoside resistance genes amongst the dogs. According to the NORM-VET surveillance programme,

the usage of aminoglycosides is low in Norway (NORM, 2020). In faecal samples from healthy dogs, the surveillance programme reports a low aminoglycoside resistance level in *Escherichia coli*, *Enterococcus faecium* and *Enterococcus faecalis*. Hence, bacteria hosting the aminoglycoside resistance genes detected in the dog samples were most likely other bacteria. Our findings emphasize the importance of maintaining the low usage of aminoglycosides in small animal clinical practice to avoid the selection and dissemination of aminoglycoside-resistant bacteria.

Surprisingly many of the dog samples tested positive for *mecA*, *the gene mediating* methicillin resistance in staphylococci. The *mecA* gene is often associated with *Staphylococcus pseudintermedius* in dogs. However, it may also be present in coagulase-negative staphylococci (MRCoNS) and *Staphylococcus aureus* (MRSA), the latter being more often associated with humans (Gómez-Sanz et al., 2019; Turner et al., 2019; Weese & van Duijkeren, 2010). A prevalence screening of methicillin-resistant *S. pseudintermedius* (MRSP) in healthy dogs in Norway showed carriage rates of 2.6% (5/189) (Kjellman et al., 2015). Additionally, the 2019 surveillance report on antimicrobial resistance in Norway stated that none out of 230 healthy dogs carried methicillin-resistant staphylococci, whilst 4.5% (7/157) of the *S. pseudintermedius* clinical isolates were identified as MRSP (NORM, 2020). Staphylococci are primarily associated with skin and mucosal membranes (Bannoehr & Guardabassi, 2012; Foster, 2002). Our results may partly reflect the self-contamination of the faeces from these sites and not the state in the gut. Still, the level of *mecA* positive samples was notably high considering the low reported prevalence of methicillin-resistant staphylococci in Norwegian dogs. The HT-qPCR method used in this study may have contributed to the high number of *mecA*-positive individuals, as it can detect low-abundance genes (Franklin et al., 2021; Waseem et al., 2019) and does not discriminate between different staphylococcal species. Hence, the *mecA* may originate from other sources such as coagulase-negative staphylococci that frequently carry *mecA (*Garza-González et al., 2010*)*.

In this study, individuals carrying *intI1*-positive bacteria had more ARGs in the gut than individuals who were negative for *intI1*. We expected this as the *intI1* gene encodes the integrase in class 1 integrons, enabling the integrons to capture and express a wide range of resistance genes (Lacotte et al., 2017). Class 1 integrons can be carried by conjugative plasmids and are thus believed to be a significant contributor to the acquisition and dissemination of ARGs (Gillings et al., 2017). However, a study by Zhang et al. (2018) suggested that the contribution of class 1 integrons to the dissemination of ARGs might be limited as they are mainly within *Gammaproteobacteria*. Furthermore, Zhang et al. showed that more than half of the class 1 integrons were chromosomally embedded with less potential for horizontal gene transfer. In this study, eight dogs and six owners tested positive for *intI1*, of which three dog-owner pairs simultaneously carried the gene. Seeing that class 1 integrons are considered almost universal in the microbiota of humans and domesticated animals (Gillings, 2017), the number of *intI1* carrying individuals in this study was notably low. Moreover, the low number indicates a limited transmission rate of *intI1*-carrying bacteria between dogs and owners.

Considering the close contact humans and their pets often have, it is surprising that dogs and owners from the same household had such a small proportion of the same ARGs in common. Undoubtedly, factors such as species barriers, the extent of contact in the individual homes, and the limited sample size may have affected the results. The observed association between shared ARGs and age may imply that the dogs' age and perhaps even cohabiting time are factors that affect the degree of common ARGs. Whether this is caused by the inter-species transmission of bacteria, a shift in the dogs' microbiomes with age, or is purely coincidental, remains unanswered. Resistance determinants persist for at least a year in the human gut (Forslund et al., 2013). With that in mind, our results suggest that the exchange of ARGs between dogs and owners and subsequent carriage of ARGs are of limited concern. However, the situation might have looked differently if the dog or owner had undergone antimicrobial treatment. In which case, the selection pressure would increase the population of resistant bacteria and potentially increase the risk of exposure to either the dog or owner (Francino, 2016).

The HT-qPCR approach used in this study proved to be a quick and efficient method to screen for multiple ARGs in many samples simultaneously. The technique is often used to detect ARGs in environmental samples as it requires a limited amount of DNA per sample and can detect low abundance genes (Franklin et al., 2021; Waseem et al., 2019). Nevertheless, some studies have successfully applied the method to detect ARGs in faecal samples from animals and humans (Zhao et al., 2018; Zhou et al., 2018). A downside of the method is that it fails to connect the ARGs to the host bacteria. However, the method's strength is that it enabled us to identify ARGs from the whole faecal microbiome, not only ARGs in culturable faecal bacteria. As exemplified in this study, low-biomass samples, like negative controls are prone to contamination as DNA is ubiquitous and can even be found in DNA extraction kits (Karstens et al., 2019; Saladié et al., 2020; Salter et al., 2014). Therefore, we accepted that some of the controls contained low amounts of the 16S rRNA gene. *We*

suspected that the ant(3′)-positive negative control had been contaminated by a neighbouring well due to the close positioning of the wells. A targeted rerun of this specific sample confirmed this assumption. The pre-amplification step of the method improves the detection limit but may also reduce the specificity of the analysis leading to false positives (Sandberg et al., 2018). A metagenomic sequencing analysis may be another option, as it provides data on the taxonomic composition of the gut microbiome as well as detecting ARGs. However, detecting low-abundance genes requires high-depth sequencing, which may be challenging and costly to achieve (Waseem et al., 2019).

In conclusion, despite a reported low level of antimicrobial resistance in Norway (NORM, 2018, 2019, 2020), a wide range of ARGs belonging to several AMR classes was present in faecal samples from both dogs and owners. Thus, both groups may act as reservoirs for bacteria carrying these ARGs. A modest proportion of the same resistance genes was present in both dogs and owners simultaneously. This indicates that the transmission of resistance genes between dogs and owners is of limited concern, provided a low antimicrobial selection pressure. Furthermore, this study has provided valuable insight into the common dog and human resistome and improved the knowledge base for risk assessments regarding the zoonotic potential of antimicrobial resistance.

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CONFLICT OF INTEREST

No conflict of interest was declared.

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Paper II

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Transmission of Methicillin-Resistant *Staphylococcus* spp. from Infected Dogs to the Home Environment and Owners

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Abstract: Dogs with methicillin-resistant *Staphylococcus* spp. (MRS) infections often undergo treatment in their homes, interacting with their owners and surroundings. This close contact between dogs and owners may facilitate the interspecies transmission of MRS. Therefore, this study aimed to investigate the transmission of MRS from infected dogs to their owners and home environments. Seven households with dogs that had been diagnosed with methicillin-resistant *S. pseudintermedius* (MRSP) and one household with a dog with methicillin-resistant *S. epidermidis* (MRSE) participated in the study. Dogs, owners, and the home environments were screened for the presence of clinical MRS. A selection of 36 staphylococcal isolates were whole-genome sequenced and screened for resistance genes and virulence genes. Clinical MRS were primarily identified from the dogs and their immediate surroundings, but these were also detected in locations that were out of reach for the dogs, indicating indirect transmission. Two of eight owners carried clinical MRS in their nostrils, while one owner carried methicillin-susceptible *S. pseudintermedius* (MSSP). All clinical MRS were multi-resistant, and several possessed resistance genes that were not expressed phenotypically. Clinical MRSP persisted in the home environment for a prolonged period, despite infection recovery and one dog being euthanized. Regardless of the stable presence of MRSP in the surroundings, the owners in these homes remained negative, but tested positive for MSSP on three occasions.

Keywords: antimicrobial resistance; methicillin-resistance; one health; *Staphylococcus pseudintermedius*; *Staphylococcus epidermidis*

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1. Introduction

Methicillin-resistant *Staphylococcus* spp. (MRS) cause a substantial number of infections in humans worldwide. Methicillin-resistant *Staphylococcus aureus* (MRSA) has been estimated to cause almost 150,000 infections annually, and over 7000 attributable deaths in the European Union and the European Economic area [1]. Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) is among the most common MRS carried by and causing infections in dogs [2], and it is the canine equivalent to *S. aureus*. Despite initially being described as an animal pathogen, an increasing number of studies now recognize *S. pseudintermedius* as an opportunistic human pathogen [3–6]. In human medicine, methicillin-resistant coagulase negative *Staphylococcus* spp. (MRCoNS), and methicillin-resistant *S. epidermidis* (MRSE) are major contributors to nosocomial infections [7–9]. Similarly, in veterinary medicine, MRCoNS are recognized to colonize and cause infections in dogs, with MRSE being one of the most commonly occurring MRCoNS species [10–12]. In addition to *mecA*-encoded resistance to all beta-lactam antibiotics, clinical strains of MRS often have multidrug-resistant properties that complicate the treatment of these infections [13,14].

The close relationship between dogs and humans may facilitate the bidirectional transmission of bacteria. Transmission may occur through direct contact and/or indirectly

through contact with bacteria in the surrounding home environment. The staphylococci's ability to survive without a host from weeks to months in dry environments increases the probability of MRS exposure and allows for the recolonization of hosts after successful antimicrobial treatment of the primary infection [15,16].

As clinical microbiologists and veterinarians, we are often contacted by dog owners who worry about the risk of becoming infected by their dogs. Therefore, this study aimed to investigate the transmission of clinical MRS from dogs to their immediate surroundings. By screening the dogs, their owners, and home environments for clinical MRS, we assessed the transmission potential of MRSP and MRSE. Furthermore, we aimed to describe the MRS' resistome and virulence genes to evaluate the severity of zoonotic transmission.

2. Results

2.1. Identification of MRS Isolates

An extended summary of all MRSP, MSSP, and MRSE isolates included in the study is presented in Table S1. A total of 103 isolates were included, 62 from Sampling 1, and 41 from the follow-up samplings (Sampling 2 and 3). Tables 1–4 present summaries of the data from Table S1.

Table 1. Location of clinical methicillin-resistant *Staphylococcus* spp. (MRS) in households in Sampling 1. Contact dogs in the same household were tested if present. The contact dog of Dog C tested negative for methicillin-resistant *S. pseudintermedius* (MRSP) at the time of sampling but had tested positive for MRSP in a screening approximately one month earlier.

		Dog			Owner			Environment				Contact Dog
Household	**Isolate**	**Infection Site**	**Perineum**	**Mouth**	**Nose**	**Throat**	**Food Bowl**	**Sleeping Place**	**Floor**	**Bathroom**	**Kitchen**	**Perineum/Mouth**
A	MRSP	+	+	+	+	-	+	+	+	-	+	n/a
B	MRSP	+	-	-	-	-	-	+	+	-	-	n/a
C	MRSP	+	+	+	-	-	+	+	+	-	-	-
D	MRSP	+	+	+	-	-	+	+	+	+	+	n/a
E	MRSP	+	+	-	-	-	+	+	+	-	-	+
F	MRSP	+	+	-	-	-	+	-	-	-	-	+
G	MRSP	+	+	+	-	-	+	+	+	+	+	n/a
H	MRSE	+	+	-	+	-	+	+	+	-	+	n/a

Table 2. Phenotypic resistance in MRSP, MSSP, and MRSE in the eight households. The table presents a summary of all isolates from Sampling 1. T/S = Trimethoprim/Sulfamethoxazole, Tet = Tetracycline, Fus = Fusidic acid, Enr = Enrofloxacin, Gen = Gentamicin, Cli = Clindamycin, Oxa = Oxacillin, Cef = Cefoxitin, Chl = Chloramphenicol, Ery = Erythromycin.

Household	**Isolate(s)**	**T/S**	**Tet**	**Fus**	**Enr**	**Gen**	**Cli**	**Oxa**	**Cef**	**Chl**	**Ery**
A	MRSP		R				S/R	R	n/a		R
B	MRSP	R	R		R	R	R	R	n/a		R
C	MRSP	R	R		R	R	R	R	n/a		R
D	MRSP	R			R	R	R	R	n/a		R
E	MRSP	R	R					R	n/a		
F	MRSP	R	R			R	R	R	n/a		R
G	MRSP	R	R				R	R	n/a		R
G	MSSP	R	R				R		n/a		R
H	MRSE			R			S/I	R	R		R

Table 3. Summary of sequence types (ST), staphylococcal cassette chromosome *mec* (SCC*mec*) elements, and resistance genes of the MRSP and MRSE isolates isolated from Sampling 1 in all households. The ST of the MRSP isolate from household C could not be determined by multilocus sequence typing (MLST).

	Household	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**
	Isolate	MRSP	MRSP	MRSP	MRSP	MRSP	MRSP	MRSP	MRSE
	ST	258	551	-	680	258	386	258	640
AB class	SCC*mec*	IVg (2B)	Vc (5C2&5)	V (5C2&5)	III (3A)	IVg (2B)	IVg (2B)	IVg (2B)	IVd (2B)
Aminoglycoside	*ant(6′)-Ia*		+	+	+	+	+	+	
	aph(3′)-IIIa		+	+	+	+	+	+	
	aac(6′)-Ie		+	+	+		+		
	aph(2″)-Ia		+	+	+		+		
	ant(4′)-Ib								+
	ant(9)-Ia				+				
	sat4		+	+	+	+		+	
Beta-lactam	*blaZ*	+	+	+	+	+	+	+	+
	mecA	+	+	+	+	+	+	+	+
Folate pathway antagonist	*dfrG*	+	+	+	+	+	+	+	
	dfrC								+
Macrolide, Lincosamide, Streptogramin B	*ermB*	+	+	+	+		+	+	
	lsaE						+		
	mefE			+			+		
	msrA								+
Tetracycline	*tetM*	+	+	+		+	+	+	
	tetK		+						
Steroid antibacterial	*fusB*								+
Multidrug	*mgrA*								+
	norA								+

Table 4. Persistence of MRSP over time in Households A and B. Sampling Period 2 started two weeks after Sampling 1. Sampling Period 3 started four weeks after Sampling Period 2. The owners tested negative for MRSP in the follow-up sampling periods, but tested positive for MSSP (*) on two occasions each. The home environments remained positive for MRSP throughout the sampling periods.

	Household A	**Sampling 1**	**Sampling Period 2**					**Sampling Period 3**				
		Day 1	**D1**	**D2**	**D3**	**D4**	**D5**	**D1**	**D2**	**D3**	**D4**	**D5**
Dog	Infection site	+	+		+	+	+	+	+	+	+	+
	Perineum, mouth	+	+	+					+	+		+
Owner	Nose/Throat	+	*									
Environment	Floor	+	+	+		+			+	+		
	Bathroom	+	+						+			
	Kitchen	+										
	Household B	**Sampling 1**	**Sampling Period 2**					**Sampling Period 3**				
		Day 1	**D1**	**D2**	**D3**	**D4**	**D5**	**D1**	**D2**	**D3**	**D4**	**D5**
Dog	Infection site	+	+									
	Perineum, mouth								+			
Owner	Nose/Throat					*					*	
Environment	Floor	+	+	+	+	+		+	+	+	+	+
	Bathroom			+								
	Kitchen			+		+		+				

2.2. Location of Clinical Methicillin-Resistant Staphylococci

Overall, the results from Sampling 1 showed that clinical MRS were frequently present on the dogs' carrier sites (perineum/mouth) and in the home environment (Table 1). Two of eight owners carried the same MRS that their dogs were infected with, one case of MRSP (Household A), and one case of MRSE (Household H). In both cases, the isolates were recovered from the owners' nostrils. In addition, the owner of household G carried MSSP in the nose. Except for Dog B, all of the dogs tested positive for clinical MRS at either one or two carrier sites. We detected clinical MRS in all home environments, but in varying locations and frequencies. In all households, the MRS were identified at a minimum of one of the dog-associated locations; the food bowl, the sleeping place, or the floor, while we could identify the MRS in four of the kitchens and two of the bathrooms.

2.3. Contact Dogs

Contact dogs were present in three households. Despite having tested positive for MRSP in a screening a month before, the contact dog in household C tested negative for MRSP at the sampling for this study. The contact dog in household F tested positive for MRSP from the perineum and from pyotraumatic dermatitis on the cheek. Dog E had 10 four-week-old puppies that all tested positive for MRSP.

2.4. Phenotypic Resistance

All MRS isolates were multidrug-resistant by the definition proposed by Magiorakos et al. [17]. The number of resistance classes ranged from three to seven, with the MRSP isolates in households B and C expressing phenotypic resistance to most classes of antibiotics (Table 2). Resistance to erythromycin, trimethoprim/sulfamethoxazole, clindamycin, and tetracycline were the most frequent. None of the MRS were resistant to chloramphenicol, while the MRSE isolate was the only isolate expressing resistance to fusidic acid. Of the eight MRSP isolates in household A, three were susceptible, while the remaining were resistant to clindamycin. The MSSP isolated from the owner in household G was susceptible to oxacillin, but had an otherwise identical resistance pattern to the MRSP isolated from the dog and the home environment.

2.5. Genomic Data Analysis

Table 4 presents the staphylococcal cassette chromosome *mec* (SCC*mec*) elements, STs, and antimicrobial resistance genes of clinical MRS from Sampling 1. SCC*mec* IVg (2B) was the most frequent SCC*mec* element in the MRSP, being detected in four of seven isolates. Three of seven MRSP were typed to ST258. This ST was shared by the MRSP and MSSP isolates from household G (Table S1). Furthermore, the MRSP and MSSP isolates carried the same resistance genes, except for the *mecA* gene.

Overall, the genotypic resistance corresponded well with the phenotypic resistance, with some exceptions: A broad spectrum of aminoglycoside resistance genes were present in all MRSP isolates, except for Household A. In Household A, none of the MRSP isolates expressed phenotypic resistance to trimethoprim/sulfamethoxazole, while the resistome analysis uncovered the *dfrG* gene in all of the sequenced isolates. Furthermore, despite their phenotypic heterogenic resistance to clindamycin, all of the MRSP isolates from Household A possessed the *ermB* gene. A Blast analysis revealed a C251T mutation (Ser84Leu) in the *gyrA* genes of the fluoroquinolone-resistant MRSP isolates in Households B–D. The MRSE isolates were susceptible to enrofloxacin despite possessing *norA*, a gene encoding a multidrug efflux pump conferring resistance to fluoroquinolones. As with *norA*, the trimethoprim resistance gene *dfrC* was present in the MRSE genomes but this was not expressed phenotypically.

2.6. Persistence over Time

Households A and B were sampled for two periods of five days. During both sampling periods, Dog A displayed infection symptoms and tested positive for MRSP until the

sampling was terminated, while the home environment was intermittently positive (Table 4). The owner tested negative for MRSP during both sampling periods, but tested positive for MSSP on one occasion. The MSSP isolates were phenotypically susceptible to all antibiotics included in the panel, and the resistome analysis confirmed the absence of resistance genes. The MSSP sequence type could not be established using multilocus sequence typing (MLST).

The situation in Household B differed from Household A. The dog displayed no symptoms of infection on the first day of the follow-up sampling. It tested positive for MRSP from the primary infection site on the first day, but remained negative on the following test days. With one exception, in Sampling period 3, the perineal and mouth samples were negative for MRSP. Dog B's samples were dominated by an MSSP strain that we also isolated from the owner on two occasions, one in Sampling Period 2 and one in Sampling Period 3. On both occasions, the owner tested negative for MSSP the following day. MLST could not establish the MSSP sequence types. The isolates expressed no phenotypic resistance to the antibiotics in the test panel, and we detected no resistance genes in the search against the CARD database. Despite Dog B's recovery from the infection and the negative carrier status, the home environment remained positive for MRSP throughout the testing period.

Household C was sampled 5 and 10 weeks after the dog had been euthanized. The home environment tested positive for MRSP, with two different phenotypic resistance patterns in the first follow-up sampling. One isolate expressed the same phenotypic susceptibility profile as the MRSP recovered from the dog six weeks earlier (T/S, Tet, Enr, Gen, Cli, Oxa, and Ery). In contrast, the other isolate was susceptible to trimethoprim/sulfamethoxazole, gentamicin, and erythromycin. The CARD analysis showed that the less resistant isolate lacked *ant(6′)*, *aph(3′)*, *dfrG*, *ermB*, and *sat4*. Like the isolate recovered from the dog, we could not determine the STs by MLST on either of the two environmental MRSP isolates. The SCC*mec* elements were identical (V) to the MRSP isolated from the dog from Sampling 1. In addition, the virulome analysis revealed that both isolates had identical virulence genes (Table S2). No MRSP was detected in the second follow-up sampling 10 weeks after Dog C was euthanized. The owner tested negative for MRSP on both follow-up occasions.

2.7. Virulence

All MRSP/ MSSP isolates possessed genes that are involved in adhesion and biofilm production, *ebpS* and *icaA-D*, and the toxin-encoding genes *hlB*, *lukF-I*, *lukS-I*, *se-int*, *siet*, and *speta* (Table S2). Except for MRSP from Households B and D, all isolates had the adhesin gene *spsD*. Instead, MRSP from Households B and D possessed another adhesion gene, *spsL*. The MSSP isolates from Households A and B possessed the bacteriocin-encoding gene *bacSp222* and the enterotoxin-encoding gene *sec3*, which are unique to these strains. In addition to the MRSP isolate from Household F, these were the only isolates in possession of the exfoliative toxin-encoding gene *expB* and the surface protein-encoding gene *spsI*. Compared to the MRSP isolate from Household G, the MSSP isolate from the same household lacked the *mecA* gene and the surface protein-encoding genes *spsG* and *spsM*.

Similar to the MRSP/MSSP isolates, the MRSE isolates from Household H possessed a rich variety of virulence genes, including genes encoding adhesins; *aae*, *atlE*, *bhp*, *ebpS*, *fbe*, *gehC*, *gehD*, and *sdrF-H*. Genes involved in the regulation of biofilm production, *htrA*, *sepA*, and *sspA*, were present, but the biofilm-producing genes *icaA-D* were not identified.

3. Discussion

An increasing number of reports state that *S. pseudintermedius* is an opportunistic human pathogen, while *S. epidermidis* can cause infections in several species [18–23]. Considering the close relationship between dogs and owners, we aimed to investigate the transmission of MRS from clinical cases in dogs. By analyzing their locations, the antimi-

crobial resistance properties, and the virulence genes of MRS, we assessed the transmission of MRS to the surroundings, and the severity of potential zoonotic transmission to owners.

The results indicate that clinical MRS are primarily located on the dogs and in their immediate surroundings. Household F was the exception, but a plausible explanation could be that Dog F's movement was confined to an enclosure in the living room. Unlike the other participating dogs, Dog F's infection site was covered by bandages, thus limiting bacterial shedding. Clinical MRS were present in locations that were out of reach for the dogs in half of the households, indicating an indirect transmission route, either by dust particles or mechanical vectors such as cleaning cloths or hands [24,25]. We detected one case of MRSP and one case of MRSE among the owners, both isolated from the owners' nostrils. In the case of MRSP, we can be reasonably certain that the MRSP had been transmitted from the dog to the owner, as it is primarily a canine-associated bacteria. In the case of MRSE, however, the transmission route is less clear. *S. epidermidis* has a broad spectrum of mammalian hosts, including dogs and humans [26]. ST640 has previously been reported in humans and dairy cows, but not in dogs [27,28]. MSSP was likely the primary cause of Dog H's infection, as it is a common bacteria that is isolated from canine pyotraumatic dermatitis [29]. However, we cannot exclude the possibility of the opposite, as no results from previous bacterial culturing were available. Regardless of which bacteria were the primary cause, MRSE was recovered from the perineum of the dog, indicating that the finding of MRSE from the infection site was not temporary contamination.

To better understand the dynamics over time, we continued with follow-up sampling for two periods in Households A and B. Dog B recovered from acute otitis externa at the beginning of Sampling 2. Except on one occasion, we did not detect MRSP from Dog B during Samplings 2 and 3. The absence of MRSP in Dog B could be due to the method's detection limit. However, the isolation protocol included both an enrichment- and a selective culturing step, thus increasing the method's sensitivity. Interestingly, Dog B's samples were dominated by an MSSP strain that possessed the gene encoding the BacSp222 peptide. BacSp222 functions as a bacteriocin that kills Gram-positive bacteria, including related staphylococci [30]. Thus, it is tempting to hypothesize that the domination of MSSP prevented the colonization of MRSP. The dominating strain of MSSP was recovered from the owner's throat/pharyngeal samples on two occasions, but it was not detected over the following days, indicating that the findings were temporary contamination.

As opposed to Dog B, Dog A presented with a more chronic clinical state, with both active and recovering dermal lesions throughout the sampling periods. Consequently, we recovered MRSP from the dog and the home environment throughout the sampling periods. Interestingly, an MSSP strain containing *bacSp222* was also detected twice in owner A. On both occasions, the MSSP could not be recovered the following day, thus supporting the theory that the MSSP was a temporary contaminant.

Despite the MRSP's stable presence over time in Households A and B, the owners remained negative, indicating a species barrier. As the MRSP were selectively enriched, the bacterial load in the home environment could not be quantified. However, as Dog A continuously shed MRSP from the active lesions, we assume the quantity was higher than negligible. Consequently, the owner was continuously exposed to MRSP.

As exemplified in Households B and C, staphylococci can survive in nutrient-poor, dry conditions for weeks to months [31]. The home environment remained positive regardless of Dog B's negative carrier state and the maintenance of regular house cleaning routines until sampling was terminated. It is likely that the MRSP detected in the home environment for the remaining sampling period originated from the initial infection. Dog C had been euthanized shortly after Sampling 1, and the contact dog was no longer present in the household. In the meantime, the owner had implemented several hygienic measures, but a thorough inspection revealed dog hair in various locations. Consequently, we recovered MRSP from the floor and the sofa five weeks after the dog was euthanized. In contrast, we could not detect MRSP from any environmental sample 10 weeks after euthanization, even though dog hairs still were present in the home environment. Hence, the MRSP had

been eliminated or reduced to quantities that were below the detection limit sometime between 5 and 10 weeks after the dog was euthanized. Considering MRSP's resilience and that it is easily transmitted between dogs [25], caution should be taken when introducing MRSP-naïve dogs to home environments that have been previously occupied by an MRSP-infected dog.

Though not a virulence factor, antimicrobial resistance genes offer a competitive advantage for bacteria when they are exposed to antibiotics. The phenotypic resistance analysis established that all the MRSP/MRSE isolates were multi-resistant by the definition proposed by Magiorakos et al. [17]. However, the genetic resistome analysis revealed some MRS carried resistance genes that were not apparent through phenotypic susceptibility testing. This was especially evident for the aminoglycoside resistance genes. The *ant(6′)-Ia*, *aph(3′)-IIIa*, *ant(4′)Ib*, *ant(9)-Ia*, and *sat4* genes encode proteins that are unaffected by gentamicin, the only aminoglycoside antibiotic included in the phenotypic panel. Furthermore, the resistome analysis revealed genes that were not expressed in vitro, including genes encoding trimethoprim- and clindamycin resistance, and the multidrug efflux pump NorA. These findings show that the antimicrobial resistance potential can be underestimated by relying on limited phenotypic susceptibility profiles alone.

Knowledge about *S. pseudintermedius* pathogenesis is still sparse [32]. Overall, we observed few differences in virulence genes among the MRSP and MSSP isolates. Virulence genes associated with adherence to host tissue such as *ebpS* and *lip*, the biofilm-associated genes *icaA-D*, and genes encoding the cytotoxins *lukF-I* and *lukS-I* were present in all of the sequenced isolates. In addition, most MRSP/MSSP isolates possessed *spsD*, the protein of which contains an A domain that is homologous to fibronectin-binding proteins and clumping factors, which are both important adhesins in *S. aureus* [33]. Furthermore, SpsD mediates the adherence to human fibronectin and is associated with the internalization of human osteoblasts in vitro [34]. In contrast to other more virulent staphylococci, such as *S. aureus*, *S. epidermidis* does not possess aggressive virulence properties [7]. As a well-adapted skin commensal, *S. epidermidis* has an arsenal of adhesins that enable it to maintain this lifestyle. The MRSE isolates in this study were no exception, which likely contributed to their ability to colonize both the owner and the dog in Household H.

4. Conclusions

This study has documented that the home environment is an important reservoir for clinical multidrug-resistant MRS that is shed by infected dogs. The locations in direct contact with the infected dogs were most frequently positive for clinical MRS. These locations stayed positive over an extended period, despite infection recovery, cleaning measures, and the absence of dogs. Hence, the human household members are exposed to clinical MRS directly through contact with the dogs, and indirectly through the home environment. The significance of this exposure is debatable. Undoubtedly, MRSP and MSSP can transmit from dogs to humans. However, the findings in this study and previous studies indicate that human carriership is rare and temporary [24,35]. MRSP and MSSP produce a broad range of virulence factors. Yet, many of the virulence factors have not been characterized. Given that a significant part of reported MRSP/MSSP infections in humans has been observed in patients with underlying diseases, host factors such as age and health state seem to be important [5,36]. In the MRSE-positive household, the transmission direction was not clear. Nonetheless, co-carriership in the dog and owner, and the vast presence of MRSE in the home environment indicate that MRSE transmit between dogs, humans, and the environment. Prophylactic measures to reduce the transmission risk of MRS could be considered for implementation in households in which immunocompromised individuals are exposed.

5. Materials and Methods

5.1. Participants

Dogs and their owners were recruited to the project from small animal clinics in the surrounding areas of Oslo. Dogs that had recently been diagnosed with an infection from which MRS could be cultured were included. All participants signed individual consent forms and answered a questionnaire regarding their dogs, professions, antimicrobial consumption, and travel habits. Eight households (A–H) participated in the study, of which seven were households with dogs with MRSP infections. The remaining dog had a co-infection with MRSE and MSSP. In addition to the infected dogs, one owner and eventual contact dog(s) from each household were included in the study. A summary of the participating dogs is presented in Table 5. An extended summary of the dogs and the participating households is presented in Table S3.

Table 5. Summary of the participating dogs. Dogs D–F were on or had received antimicrobial (AM) treatment within the past 14 days before sampling.

Dog	A	B	C	D	E	F	G	H
Breed	English Bulldog	Hungarian Vizsla	Chow Chow	English Staffordshire Bullterrier	Rottweiler	Great Dane	Bullmastiff	Rottweiler
Age	4	2	1	1	2	8 months	8	3
Sex	Neutered male	Male	Female	Male	Female	Male	Female	Male
Diagnosis	Interdigital furunculosis	Otitis externa	Pyotraumatic dermatitis	Surgical site infection	Mastitis	Surgical site infection	Surgical site infection	Pyotraumatic dermatitis
Bacteria	MRSP	MRSP	MRSP	MRSP	MRSP	MRSP	MRSP	MRSE, MSSP
Contact dog	-	-	Mixed breed (n = 1)	-	Rottweiler (n = 10)	Rottweiler (n = 1)	-	-
AM at time of sampling	-	-	-	Cefalexin	Amoxicillin Trimetho-prim	Amoxicillin Ampicillin Cefalexin Enrofloxacin	-	-

5.2. Sampling

The samples were collected during the period from January 2020 to November 2021. Samples of the infection site, oral mucosa, and perineal samples were collected from the infected dogs using nylon flocked swabs (Eswab™ 480C, Copan group, Brescia, Italy). Samples were taken from the perineum and the oral mucosa from the contact dogs by a veterinarian. According to the veterinarian's instructions, the owners collected swab samples from their nostrils and throat. The home environment was sampled using moist cloths (Sodibox® Swab cloth, Nevez, France). Samples were collected from the pets' food bowl and sleeping place, floor (living room and kitchen), bathroom (sink faucet and hand towel), and the kitchen (kitchen counter, dish towel, cloth, and sink faucet). The samples from the two latter locations were taken in areas that were out of reach from the pets. All households were sampled once.

Households A, B, and C participated in further sampling, as outlined in Figure 1. Households A and B were sampled over two periods for five subsequent days, with a four-week break in between. The first follow-up sampling was performed two weeks after Sampling 1. Both households were told to maintain their regular cleaning routines during this period. Only the floor, bathroom, and kitchen were included for the follow-up environmental samples.

The dog in Household C was euthanized approximately one week after Sampling 1. The owner and home environment were sampled 5 and 10 weeks after the dog was euthanized. Before the first follow-up sampling, the owner had cleaned and disinfected the floor with a disinfecting agent containing 58% ethanol and 0.1% alkyl dimethyl benzyl ammonium saccharinate. The carpets and curtains had been dry cleaned, and the dog bed had been removed. The environmental samples were then taken from the floor, sofa, bathroom, and kitchen.

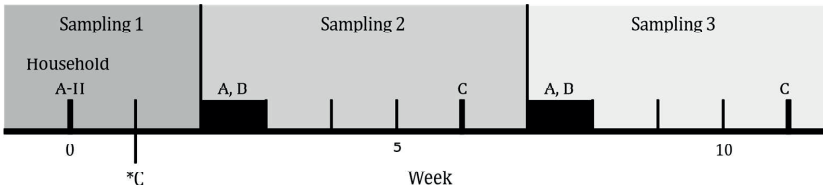

Figure 1. Schematic timeline of sampling days in Households A–H. All households were sampled once (Week 0). Households A and B were resampled two and seven weeks after Sampling 1. The follow-up samplings in these households lasted for five days. Household C was sampled five and 10 weeks after Dog C was euthanized (*C).

5.3. Culturing and Species Identification

Swabs and cloths were analyzed individually. The swabs were vortexed for a minimum of 10 s before 10 µL was plated on 5% bovine blood agar and incubated overnight at 37 °C. Additionally, 100 µL of the liquid Amies were transferred to 7 mL of Mueller Hinton broth containing 6.5% NaCl. One hundred milliliters of MH broth was added to each cloth. All samples were incubated overnight at 35 °C before 20 µL of the MH broth was inoculated on Oxacillin Resistance Screening Agar Base (ORSAB, Oxoid, Basingstoke, Hampshire, UK) supplemented with 2 mg/L of oxacillin and incubated for 24 h at 35 °C. In cases of no growth after 24 h, the plates were re-incubated for 24 h before reading.

Presumptive staphylococcal colonies growing on ORSAB plates were subcultured on 5% bovine blood agar overnight and identified to the species level by using a combination of standard laboratory techniques such as colony morphology, tests for coagulase, catalase, ONPG, mannitol, and Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) (VITEK® MS, bioMérieux, Craponne, France).

5.4. Susceptibility Testing

Verified staphylococcal isolates were susceptibility tested according to CLSI guidelines against 10 antibiotics, using the disk diffusion method (Rosco Diagnostica, Taastrup, Denmark). The panel consisted of: Trimethoprim + sulfa (1.25/23.75 µg), tetracycline (30 µg), fucidic acid (100 µg), enrofloxacin (5 µg), gentamicin (10 µg), clindamycin (2 µg), oxacillin (1 µg), cefoxitin (30 µg, MRSE only), chloramphenicol (30 µg), and erythromycin (15 µg). Phenotypically oxacillin/cefoxitin-resistant isolates were confirmed as being methicillin-resistant by *mecA* PCR [37]. Isolates were evaluated for multidrug resistance using the definition proposed by Magiorakos et al. and Sweeney et al. [17,38]

5.5. DNA Extraction and Whole-Genome Sequencing

We selected a subset of 36 MRS and MSSP from the different households for whole-genome sequencing (Table S1). DNA extraction was performed using a modified version of the MasterPure™ Gram Positive DNA Purification Kit protocol (Appendix A) (Lucigen Corporation, Middleton, WI, USA). The DNA quality control and quantification were performed using a NanoDrop® ND-1000 (ThermoScientific, Wilmington, CA, USA) and Qubit fluorometer with the dsDNA Broad Range Assay kit (Invitrogen, Eugene, OR, USA), respectively. The Norwegian Sequencing Centre (NSC) (Oslo, Norway) performed the library prep in two batches using the Swift Turbo 2S flex DNA library prep and Nextera DNA Flex prep protocols for Batches one and two, respectively. The change in the protocol was due to the Swift Turbo 2S flex prep having been phased out. The paired-end sequencing reads (300 bp) were obtained using the Illumina MiSeq platform v3 (NSC).

5.6. Bioinformatical Analysis

The raw sequencing reads were processed by adapter clipping and quality trimming with Trim Galore version 0.6.7 [39]. Quality-controlled reads were then used for genome assembly using SPAdes version 3.15.3 [40]. The STs was determined by scanning the

assembled genomes against a default PubMLST typing scheme using MLST v 2.19.0 [41]. SCC*mec* Finder v. 1.2 was used with default settings to identify SCC*mec* elements [42]. We characterized the resistomes and virulence genes using ABRicate version 1.0.1. [43]. The resistome analysis was run against the CARD database with default cutoff values of 80% nucleotide identity and 80% coverage. We performed a supplemental Blast search on fluoroquinolone-resistant isolates against point mutations in the *gyrA* gene (Accession: AM262968.1) [44]. For the virulome analysis, we used an in-house database on the MRSE sequences consisting of nucleotide sequences for 27 virulence genes (Table S4). The *S. pseudintermedius* isolates were run against the database made by Zukancik et al. [45], consisting of 69 gene sequences. The cutoff values were set to the same level as for the resistome analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics11050637/s1, Table S1: Summary of all isolates, Table S2: Virulence genes MRSP, MRSE, MSSP, Table S3: Metadata of households; Table S4: DB virulence genes of *S. epidermidis*.

Author Contributions: Conceptualization, M.R., A.M.B., Y.W. and A.H.H.; methodology, M.R.; software, S.I. and M.R.; formal analysis, M.R. and S.I.; investigation, M.R.; data curation, M.R. and S.I.; writing—original draft preparation, M.R.; writing—review and editing, M.R., A.M.B., Y.W., A.H.H. and S.I.; visualization, M.R.; supervision, A.M.B., Y.W. and A.H.H.; project administration, M.R. and A.M.B.; funding acquisition, A.M.B., Y.W., A.H.H. and M.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Norwegian National Research Ethics Committee (REK Sør-Øst) (Protocol code: 2019/97, 3 April 2019).

Informed Consent Statement: Written informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in Tables S1 and S2. Whole-genome sequence data are available at http://www.ncbi.nlm.nih.gov/bioproject/820295.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Appendix A.1 DNA Extraction Protocol

This protocol is developed for the extraction of DNA from *Staphylococcus* spp.

1. Pellet via centrifugation 4.0 mL of an overnight *Staphylococcus* bacterial culture. (4600× *g*, 15 min, 15 °C) Growth media: TSB or BHI. Discard the supernatant.
2. Resuspend the pellet in 1.0 mL PBS. Pellet via centrifugation. (100,00× *g*, 10 min, 22 °C) Discard the supernatant.
3. Repeat Step 2.
4. Add 460 µL TE buffer and 20 µL lysozyme (100 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). Vortex for 10 s.
5. Incubate at 37 °C overnight.
6. Add 150 µL MasterPure™ Gram Positive Cell Lysis solution (Lucigen Corporation, Middleton, MA, USA) and 20 µL Proteinase K (20 mg/mL) (Qiagen, Hilden, Germany)
7. Incubate at 65–70 °C for 15 min, vortexing briefly every 5 min.
8. Cool the samples to 37 °C.
9. Place the samples on ice for 5–7 min.
10. Add 175 µL of MPC Protein Precipitation Reagent (Luicgen Corporation, Middleton, MA, USA), and vortex vigorously for 10 s.

11. Pellet the debris via centrifugation at 4 °C for 10 min at 15,000× *g*.
12. Transfer the supernatant to a clean Eppendorf tube and discard the debris pellet.
13. Add 2 µL of RNase A (17,500 units) (Qiagen, Hilden, Germany) and vortex for a couple of seconds.
14. Incubate at 37 °C for 30 min.
15. Add 500 µL of isopropanol. Invert the tube 40 times. DNA should now be visible in the suspension.
16. Pellet the DNA via centrifugation at 4 °C for 10 min at 15,000× *g*.
17. Discard the supernatant.
18. Rinse the pellet with 1000 µL of 70% ethanol. Leave the ethanol for 2–3 min before centrifuging at 4 °C for 2 min at 10,000× *g*.
19. Discard the ethanol and leave the tubes open to air-dry the pellet, or incubate at 42 °C for ~15 min.
20. Resuspend the DNA in the desired volume of elution buffer.

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Paper III

The home environment is a reservoir for methicillin-resistant coagulase-negative staphylococci and mammaliicocci

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Abstract

Coagulase-negative staphylococci (CoNS) and mammaliicocci are opportunistic human and animal pathogens, often resistant to multiple antimicrobials, including methicillin. Methicillin-resistant CoNS (MRCoNS) have traditionally been linked to hospitals and healthcare facilities, where they are significant contributors to nosocomial infections. However, screenings of non-hospital environments have linked MRCoNS and methicillin-resistant mammaliicocci (MRM) to other ecological niches, including community and wildlife sources. This study explores the home environment's role as a reservoir for MRCoNS and MRM. Furthermore, we characterize the resistance and virulence properties, SCC*mec* elements, sequence types, and the mobilome of MRCoNS and MRM recovered from different households in Norway.

Importance

This study shows that home environments make up reservoirs for methicillin- and multidrug-resistant coagulase-negative staphylococci and mammaliicocci. The study further demonstrates that oxacillin is more reliable than cefoxitin for detecting methicillin resistance.

Introduction

Coagulase-negative staphylococci (CoNS) and *Mammaliicoccus* spp. (formerly known as the *Staphylococcus sciuri* group) are a heterogeneous group of skin and mucous membrane commensals but are also opportunistic pathogens responsible for various infections in humans and animals (Piette & Verschraegen, 2009; Becker *et al.*, 2014). They are considered to have a lower pathogenic potential than the more virulent *Staphylococcus aureus* and *Staphylococcus pseudintermedius*. Still, CoNS cause a substantial number of infections in immunocompromised but also in otherwise healthy patients (Becker *et al.*, 2020). For instance, *Staphylococcus epidermidis, Staphylococcus hominis,* and *Staphylococcus haemolyticus* are significant contributors to septicemia in neonates, while *Staphylococcus saprophyticus* is one of the most common causative agents in urinary tract infections (Hovelius & Mårdh, 1984; Klingenberg *et al.*, 2005; Kranz *et al.*, 2018).

Methicillin resistance (MR) is widely disseminated in CoNS (MRCoNS) and mammaliicocci (MRM) (Becker *et al.*, 2020; Schwendener & Perreten, 2022). The *mecA* gene encodes the transpeptidase penicillin-binding protein 2a (PBP2a) with a lower affinity to beta-lactam antibiotics than other PBPs (Shalaby *et al.*, 2020). In addition to resistance to beta-lactams, resistance to non-beta-lactam antibiotics is common in MRCoNS (May *et al.*, 2014). The multidrug-resistance properties of MRCoNS offer a competitive advantage in hospital- and healthcare settings where antimicrobials are widely used. (Becker *et al.*, 2020).

As opposed to human and animal infections caused by MR *S. aureus* (MRSA) and *S. pseudintermedius* (MRSP), MRCoNS infections are not monitored in Norway. Hence, the knowledge of the prevalence of MRCoNS is largely unknown. Based on reports of high rates of MRCoNS in cases of neonatal septicemia and MRCoNS-carrying health care personnel, we can assume that Norwegian healthcare facilities make up important reservoirs for MRCoNS (Klingenberg *et al.*, 2001; Klingenberg *et al.*, 2005). However, non-hospital environments may also serve as reservoirs for these bacteria (Xu *et al.*, 2015). High rates of MRCoNS have been reported in public transportation systems and humans without previous exposure to health care systems (Stepanović *et al.*, 2008; Barbier *et al.*, 2010). When studying the transmission of MR *Staphylococcus* spp. (MRS) from infected dogs to their owners, we detected several species of MRCoNS and MR *Mammaliicoccus* spp. (MRM) in the home environments in these households (Røken *et al.*, 2022). These observations made us wonder whether the presence of MRCoNS and MRM in the home environments was related to the infection status of the dogs or if MRCoNS are commonly present in all types of households.

Thus, this study aims to gain more insight into the home environment's role as a reservoir for MRCoNS by screening the environment, humans, and pets in different households for MR bacteria. Further, we examine the distribution of MRCoNS and MRM by identifying the species, sequence types, and SCC*mec* elements in the home environment. Finally, we screen the bacteria for virulence genes, antimicrobial resistance genes (ARGs) and mobile genetic elements.

Materials and methods

This project was approved by the Regional Committee for Medical and Health Research Ethics Southeast, approval number: 2019/97.

Participants

Participants were recruited through social media and small animal clinics in Oslo and the surrounding areas. All participants signed individual consent forms and completed questionnaires regarding their professions, antimicrobial consumption, and hospital admissions within the past 12 months. Thirty-three households participated in the study. Of these, eight were households with dogs diagnosed with an MRS infection; eight were households with clinically healthy dogs, six with clinically healthy cats, and 11 were households without pets. One human and one pet from each home participated in the

study. The inclusion criteria for healthy pets were: Clinically healthy pets without symptoms of infection when examined by a veterinarian.

Sampling

The samples were collected in the period from October 2019 to October 2021. The same veterinarian was responsible for sampling all the household environments and the participating pets. Pets diagnosed with an MRS infection were sampled from the infection site, the perineum, and the oral mucosa, using nylon flocked swabs (Eswab™ 480C, Copan group, Brescia, Italy). These dogs participated parallelly in another study (Røken *et al.*, 2022). Healthy dogs and cats were sampled from the oral mucosa and perineum. Human participants collected swab samples from their nostrils and throats according to the instructions of the veterinarian present at the time of sampling. The home environments were sampled using cloths (Sodibox® Swab cloth, Nevez, France) and were collected from the pets' food bowls and sleeping areas, living room floors, bathrooms (sink faucet and hand towel), and kitchens (kitchen counter, dish towel, cloth and sink faucet). In the households without pets, the three latter locations were sampled.

Culturing and identification

The samples were cultured as described by Røken *et al.*(2022). Briefly described, all samples were enriched overnight in Müller Hinton (MH) broth supplemented with 6.5 % NaCl. Then, 20 µl of MH broth were inoculated on Oxacillin Resistance Screening Agar Base (ORSAB) supplemented with 2 µg oxacillin and incubated for 24 hours at 35°C. Blue, blue-white, and white colonies growing on the ORSAB agar were sub-cultured on 5 % bovine blood agar overnight. The species were identified using Matrix-assisted laser desorption/ionization (MALDI-TOF MS) (VITEK® MS, bioMérieux, Craponne, France). Isolates were tested for the presence of the *mecA* gene by PCR (Stegger *et al.*, 2012).

Susceptibility testing

Isolates were susceptibility tested against 12 antibiotics using the agar-disk diffusion method (EUCAST, 2019). The test panel included aminoglycoside (gentamicin 10 µg), amphenicol (chloramphenicol 30 µg), beta-lactams (amoxicillin-clavulanic acid 20/10 µg, oxacillin 1 µg, cefoxitin 30 µg, cefalexin 30 µg), fluoroquinolone (enrofloxacin 5 µg), fusidane (fusidic acid 100 µg), folate pathway antagonist (trimethoprim/sulfamethoxazole 1.25/23.75 µg), macrolide (erythromycin 15 µg), lincosamide (clindamycin 2 µg), and tetracycline (tetracycline 30 µg). When testing resistance to oxacillin, we used Müller Hinton agar supplemented with 4 % NaCl. Müller Hinton plates were incubated at 35 °C for 18-20 hours before reading the zone diameters. As there are no official breakpoints for amoxicillin-clavulanic acid and cefalexin, we used the breakpoints ≥ 25 mm for susceptible and ≤ 24 for resistant. Isolates displaying intermediate resistance to antimicrobial agents

were registered as resistant in the phenotypic analysis. Isolates expressing resistance to three or more classes of antimicrobials were defined as multidrug-resistant (Magiorakos *et al.*, 2012).

Selection of isolates for the different analyses

One MRCoNS/MRM from each species was included from each sampling location. Based on the phenotypic resistance profiles, species, and households, isolates were selected for whole-genome sequencing (WGS). All WGS isolates went through an additional species identity check identification check using the Microbial Genomes Atlas (MiGA) webserver against the TypeMat database (Rodriguez-R *et al.*) (Table 1). If the species identities differed between MALDI-TOF and TypeMat, we used the TypeMat output. Further, all sequenced isolates were included in the resistome-virulence gene-and SCC*mec* analyses. However, isolates that turned out to be redundant were excluded when presenting the results (isolates from the same household, identified to the same species with identical resistance genes, virulence genes, SCCmec elements, and sequence types (STs). Non-redundant, sequenced *S. epidermidis*, *S. hominis*, *S. haemolyticus*, and *S. saprophyticus* isolates were included in an additional mobilome analysis.

DNA extraction, library preparation, sequencing, and assembly

DNA was extracted using a modified version of the Master Pure™ Gram Positive DNA Purification protocol (Lucigen Corporation, Middleton, WI, USA) (Røken *et al.*, 2022). The DNA quality was assessed by NanoDrop® ND-1000 (Thermo Scientific, Wilmington, USA), and the DNA quantity was determined using a Qubit fluorometer with the dsDNA Broad Range Assay kit (Invitrogen, Eugene, OR, USA). Quality controlled DNA was submitted to The Norwegian Sequencing Centre for library preparation and sequencing. The library prep was performed in two batches using Swift Turbo 2s flex DNA library prep on batch one and Nextera DNA Flex Prep on batch two. The samples were sequenced on the Illumina MiSeq platform v3, resulting in 300 bp paired-end reads. The fastq files were quality checked using FastQC version 0.11.9. Adapters and low-quality sequences were removed using Trim Galore version 0.6.7 (Krueger, 2015). We used SPAdes version 3.15.3 for genome assembly (Bankevich *et al.*, 2012).

Bioinformatical analyses

We used ABRicate version 1.0.1 for the resistome analysis (Seemann, 2018). The assembled sequences were run against the CARD database with cutoff values of 80 % nucleotide identity and 80 % coverage (Alcock *et al.*, 2020). We used SCC*mec*Finder v. 1.2 with default settings (nucleotide identity 90 % and minimum sequence length of 60 %) to type SCC*mec* elements (Kaya *et al.*, 2018). *S. epidermidis, S. hominis*, and *S. haemolyticus* assemblies were run against a default PubMLST scheme using MLST version 2.19.0 (Seemann).

For the virulence gene analysis, we aligned a custom database containing amino acid sequences of staphylococcal virulence factors (Naushad *et al.*, 2019) against assembled staphylococcal and

mammaliicoccal genomes using tblastn (v. 2.5.0) with the default settings except for high-scoring segment pair (HSP) = 1 and the culling limit of 1. The resulting sample/VF matrix values were expressed as *Ha* scores ranging from 0 to 1 (Naushad *et al.*, 2019). Briefly, the scores were calculated using the following formula: *Ha* = (pident × length) /qlen/ 100
where "**pident"** represents the proportion of amino acid sequence identities between the VF query and translated proteins from the bacterial genomes in this study, "**length"** represents the alignment length of a hit, and **"qlen"** is the length of the query sequence drawn for each VF.

The mobilome analysis was conducted using Anvi'o bioinformatics suite version 7.1. (Eren *et al.*, 2015). Before the pangenome analysis, we excluded one *S. saprophyticus* from the dataset due to a high number (>2000) of partial genes. We created the Anvi'o contigs database with the "anvi-gen-contigs-database" program using Prodigal to identify open reading frames (Hyatt *et al.*, 2010). The resulting genes were associated with the functions from the NCBI's Clusters of Orthologous Groups (COGs) database (Tatusov *et al.*, 2000). The pangenome was computed by the core Anvi'o program "anvi-pan-genome" (default settings), which in turn utilized DIAMOND (Buchfink *et al.*, 2015) and MCL (van Dongen & Abreu-Goodger, 2012). Metadata was integrated into the pangenome results with the "anvi-import-misc-data" program. After the pangenome visualization, we extracted all gene clusters annotated with the "Mobilome" COGs category with the "anvi-split" program for further manual inspection. We used COG annotations or an *ad hoc* protein web-blast search to characterize the gene clusters of the staphylococcal mobilome.

Statistical analysis

We used one-way ANOVA to compare the number of different MRCoNS and MRM species between the households with infected pets, healthy pets, and without pets. Tukey's Honest Significant Difference (HSD) test was applied to test the pairwise difference between the three household groups. The significance level was set to 0.05.

The virulence factor (VF) matrix was transferred to R version 4.1.0 for further principal component analysis (PCA) using "prcomp" function of the "stats" package (v. 4.0.1), followed by a visualization using the "fviz_pca" function of the "factoextra" package (v. 1.0.7).

Data availability

The sequences included in the analyses are available at
https://www.ncbi.nlm.nih.gov/bioproject/PRJNA856113
Susceptibility profiles, SCC*mec* data, sequence types, and antimicrobial resistance genes, and isolation sources are available in Table S1 for the sequenced isolates.
Complete virulence gene matrix with *Ha* scores is available in Table S2.

Results

Isolates

A total of 118 verified MRCoNS and MRM isolates constituted the sample material for the occurrence analysis. Of these, 103 isolates were submitted for whole-genome sequencing, of which 75 are presented in the resistome-, virulence-, and SCC*mec* results. Thirty-nine *S. epidermidis*, *S. haemolyticus,* and *S. hominis* isolates were included in the *in silico* MLST analysis, while 57 *S. epidermidis, S. haemolyticus, S. hominis,* and *S. saprophyticus* are presented in the mobilome analysis.

Occurrence of MRCoNS and MRM

All but three households (30/33) tested positive for minimum one species of MRCoNS or MRM (Table 2). Most of the MRCoNS/MRM isolates were detected in the home environments (n=107/118). Overall, MR *S. saprophyticus*, *S. haemolyticus, S. hominis,* and *S. epidermidis* were the most prevalent species in the households. Additionally, some species tended to be more prevalent in specific households: MR *S. epidermidis* (MRSE) in homes with infected dogs and healthy cats and MR *S. hominis* in homes with infected and healthy dogs. Households with infected dogs had a higher mean number of different species (3.13) compared to those with healthy pets (1.79) and without pets (1.55). A one-way ANOVA revealed that there was a statistically significant difference in the mean numbers of species between the households ($F(2,30) = [3.487]$, $p=0.04$). The Tukey's HSD Test found that this difference was between the homes with infected pets and those without pets ($p=0.03$).

As shown in Table 2, six humans and three dogs carried MRCoNS. These three dogs suffered from clinical MRS infections (two with MRSP and one with MRSE) in addition to carrying MRSE. Their three owners tested positive for MRSE. Two owners carried MR *S. haemolyticus*, one owner of an infected dog and one owner of a healthy cat. One owner of a healthy dog carried MR *S. warneri.* None of the humans in the household without pets tested positive for MRCoNS, while all but one of the eleven home environments tested positive for at least one species of MRCoNS.

Antimicrobial resistance

A slight majority (41/75) of the isolates were multi-resistant, expressing resistance to three or more classes of antimicrobials. *S. cohnii* ssp. *cohnii, S. hominis*, and *S. haemolyticus* were the most resistant species, expressing resistance to means of 4.3, 3.7, and 3.6 classes, respectively (Figure 1). Following resistance to anti-staphylococcal beta-lactams, resistance to macrolides (erythromycin) and fusidanes (fusidic acid) was most frequently observed (Table 3). The resistome analysis supported the phenotypical resistance profile, displaying a high prevalence of genes conferring resistance to erythromycin and fusidic acid among the MRCoNS and MRM (Table 4**Error! Reference source not found.**) and further confirmed that all sequenced MRCoNS and MRM possessed the *mecA* gene. Despite the presence of *mecA,* we observed a variable phenotypic expression of resistance to beta-

lactams among the isolates (Table 3). This was particularly evident for *S. hominis,* with 1/11 isolates being phenotypic susceptible to oxacillin and 5/11 isolates being susceptible to cefoxitin. Furthermore, we observed phenotypic susceptibility to amoxicillin-clavulanic acid among six MRCoNS and MRM species: *S. cohnii* ssp. *cohnii* (2/3), *S. epidermidis* (8/12), S. haemolyticus (2/16), *S. hominis* (6/11), *S. warneri* (1/5) and *M. vitulinus* (n=1). In addition to *mecA*, the beta-lactamase encoding *blaZ* gene was present in 16 of the 20 amoxicillin-clavulanic acid-susceptible isolates.

SCC*mec* cassettes and sequence types

Table 5 shows the predicted SCC*mec* cassettes based on detected genes and best homology in the sequenced MRCoNS and MRM isolates. For four isolates (one *S. epidermidis*, one *S. haemolyticus*, and two *S. hominis*), the *SCCmec* prediction based on genes deviated from the prediction based on homology. In these cases, the SCC*mec* cassettes were reported based on the prediction of the genes. The *S. epidermidis* isolates were assigned three types (II (2a), III (3A), and IV (2b)) in addition to one non-typeable (NT) isolate. In just one household, the same SCC*mec* cassette was predicted in isolates of different species: an *S. epidermidis* and an *S. saprophyticus* isolate with type III (3A). The *SCCmec* elements of a substantial number of isolates (43/75) were non-typeable. Eleven of the 14 NT *S. haemolyticus* isolates showed best homology with SCC*mec* type V but missed either *ccr*C1, *mec* class C2 or both. Three of the NT *S. saprophyticus* isolates had predicted best homology to SCC*mec* III (3A) but missed either *ccr*A3 or both *ccr*A3 and *ccr*B3, while two additional *S. saprophyticus* isolates shared best homology with SCC*mec* I (1B) but missed *ccr*A1 and *ccr*B1.

The sequence types (ST) were predicted by *in silico* MLST for 25 of the 39 *S. epidermidis*, S. *haemolyticus,* and *S. hominis* isolates. The 12 *S. epidermidis* isolates were assigned to 10 different STs (5, 35, 57, 81, 130, 218, 224, 332, 640 (n=2), and 679) in addition to one non-typeable isolate. The two MRSE ST640 were from different households. Nine of 16 *S. haemolyticus* were typed to seven STs (1, 3 (n=2), 30, 42, 49, 52 (n=2), and 56). Isolates with identical STs were from different households. The remaining *S. haemolyticus* isolates had combinations of allelic profiles not reported earlier. This was also the case for six of the 11 *S. hominis* isolates, while the remaining five were typed to two different STs (1 and 18), of which all were from different households.

Household analysis of human and dog isolates

In households with infected pets (C, F and H), the dogs and owners concurrently tested positive for MRSE (Table 1). In household H, the dog, owner, and home environment tested positive for MRSE ST640 with identical susceptibility profiles, resistance genes, and SCCmec elements (Røken et al., 2022). In contrast, the dog and owner in household C carried two different STs (MRSE ST679 and ST130), presenting different resistance genes and SCCmec types. (Table S1). MRSE isolates with identical susceptibility profiles to the dog isolate were detected in the bathroom and kitchen. However, no isolates similar to the MRSE found on the owner were recovered from the home

environment. We observed similar findings in household F, in which the owner and dog carried MRSE with different STs (ST218 and ST5) and SCCmec cassettes (III (3A) and IVa (2B)). Contrary to household C, we recovered only isolates with identical susceptibility profiles to the human isolate from the home environment. In household D, MR S. haemolyticus ST42 and ST1 were recovered from the owner while the dog tested negative. Only the MR S. haemolyticus ST42 was detected in the home environment.

Two owners of healthy pets carried MRCoNS. The first owner tested positive for a MR S. warneri possessing a SCCmec cassette V (5C2&5). An MR S. warneri with an identical SCCmec cassette was recovered from the household's kitchen. The second owner carried an MR S. haemolyticus with an NT SCCmec cassette and ST. An MR S. haemolyticus ST52 with different resistance genes was recovered from the home environment.

Virulence genes

The hits in the virulence gene database are presented in Table S2 and show the respective *Ha* scores for each virulence gene. The exfoliative toxin encoding gene *etc,* was detected in all sequenced isolates. Furthermore, we observed a high frequency of phenol soluble modulin-encoding genes, thermonuclease-encoding *nuc* gene, and siderophore-encoding genes in most MRCoNS isolates. Overall, the MRCoNS and MRM showed a high degree of species specificity in the virulence gene analysis, apart from *S. haemolyticus* and *S. hominis* that clustered (Figure 2). We detected a high occurrence of genes involved in adherence in the *S. epidermidis* isolates (*atl*, *ebh,* and *sdr* genes). In addition, we observed two subpopulations of *S. epidermidis* isolates based on the presence of Type VII secretion-associated genes (Table S2). The tendency of two subpopulations was also evident among the *S. haemolyticus* isolates. Based on *Ha* scores of several capsular polysaccharide synthesis enzymes involved in immune evasion (*cap* genes). The group with high *Ha* scores consisted of six isolates, of which three were recovered from humans, and two were of environmental origin. Isolates with low *Ha* scores for *cap* genes were solely detected in the environment.
Subgrouping based on the *Ha scores* of *cap* genes was also observed among the *S. hominis* and *S. saprophyticus.* The MRM had, in general, few hits with high *Ha* scores apart from the *etc* gene and the *capO* and *capP* genes.

Mobilome analysis

The four most prevalent species found in the households, *S. saprophyticus, S. epidermidis, S. haemolyticus,* and *S. hominis,* were included in the mobilome analysis. Three common gene clusters encoding an IS6 family transposase, the competence protein ComGC, and an uncharacterized SPBc2 prophage-derived protein YoqJ (annotated "Common" in Figure 3) were identified in all the isolates. Otherwise, the mobilomes were mainly species-specific (Figure 3). We observed a few examples of similar gene sequences in different species at the household level. For instance, *S. haemolyticus* and *S.*

saprophyticus isolates from the same household carried phage major capsid protein-encoding genes and phage portal protein-encoding genes with 76.8 % and 80 % amino acid identities, respectively. In addition, a site-specific tyrosine-type recombinase/integrase was shared by *S. epidermidis* and *S. saprophyticus* in two households, and an IS256-like transposase was shared by *S. haemolyticus* and *S. hominis* in two other homes.

Discussion

MRCoNS are opportunistic pathogens prevalent in hospital environments, often due to their hardy nature, ability to form biofilms, and resistance to antimicrobials (Weiß *et al.*, 2013). The newly described genus *Mammalicoccus* shares many properties with staphylococci, like habitat and methicillin resistance (Nemeghaire *et al.*, 2014). In a former study of the dissemination of clinical MRS in households with infected pets (Røken *et al.*, 2022), we detected a broad range of MRCoNS and MRM in the home environments and from pets and their owners. To follow up on this observation, we decided to screen different categories of households for the presence of MRCoNS and MRM. To our surprise, MRCoNS and MRM were nearly ubiquitous in the home environments regardless of the presence of pets or health status. The finding of *S. epidermidis, S. haemolyticus, S. hominis,* and *S. saprophyticus* as the predominant species in the households is reasonable since these species are known as skin commensals in humans. However, they can also cause infections, and the frequent occurrence of methicillin-resistant isolates in home environments is noteworthy. To our knowledge, the home environment has previously not been described as a reservoir for MRCoNS and MRM.

The skin, skin glands, and mucous membranes of mammals are considered the main habitat for CoNS (De Vos *et al.*, 2009). However, in most of the households, both the human and the pet tested negative for MRCoNS/MRM while the bacteria were present in the home environment. The absence of MRCoNS/MRM in humans and pets may reflect that the sampling sites in the humans and pets were not optimal for detecting some of the CoNS/mammaliicoccal species. For instance, *S. saprophyticus* is a frequent colonizer of the perineal region, rectum, and urethra in humans, and *S. hominis and S. haemolyticus* are often isolated from axillae and pubic areas high in apocrine glands (Becker *et al.*, 2014). These sites were not included in the sampling procedures. On the other hand, *S. epidermidis* is a common human, canine, and feline nasal mucosa colonizer (Han *et al.*, 2016; Bierowiec *et al.*, 2019). Therefore, we find it peculiar that we identified relatively few carriers of MRSE, considering that MRSE was present in around one-third of the households. An explanation may be that we only sampled one human member in each household, thus missing possible carriers of the MRCoNS and MRM. Another factor contributing to the high number of MRCoNS/MRS in the households could be that the bacteria had been introduced via visitors, soil, or other external sources.

Carriage of MRCoNS in pets was exclusively found in infected dogs. The owners of the three MRSE-positive dogs all tested positive for MRSE. Interestingly, the isolates from the dogs and owners differed in two of the cases, indicating a diversity of MRCoNS not only between households but also within the household. Moreover, we observed that homes with infected pets had a large diversity of MRCoNS species recovered from the home environment. Five of the eight dogs in this group had been treated with beta-lactam antimicrobials within the past three months before sampling, of which two had undergone antimicrobial treatment several times during the past year. The carriership and the diversity may reflect the MRCoNS and MRM's competitive advantage when exposed to beta-lactam antimicrobials. Furthermore, five dogs in this group had been hospitalized within the past twelve months, and two owners were human health care workers. Hence, it is not unlikely that the pets or owners have been exposed to MRCoNS/MRM in these environments and transmitted them further to their home environment. Still, MRCoNS and MRM were present in many households where neither humans nor dogs had been in contact with health care facilities, again emphasizing that MRCoNS and MRM indeed are outside clinical environments.

The phenotypic resistance analysis revealed that the slight majority of the MRM and MRCoNS were multidrug-resistant. This is consistent with previous reports on CoNS and MRCoNS in non-clinical settings (Xu *et al.*, 2015; Xu *et al.*, 2018). Mobile genetic elements play a central role in spreading ARGs among bacteria (Malachowa & DeLeo, 2010). Considering that MRCoNS constitute reservoirs for ARGs, we conducted a mobilome analysis primarily to investigate whether the most prevalent species in the households had mobile genetic elements in common, which could indicate genetic exchange at the household level. Nonetheless, the detected mobile genetic elements displayed mainly a species-specific profile rather than a household-related pattern. This could indicate that mobile genetic elements are not easily transmitted between different staphylococcal species.

The inconsistent phenotypic expression of resistance to cefoxitin among the MRCoNS isolates was noteworthy. EUCAST and CLSI operate with different zone diameters when assessing cefoxitin resistance. By following the CLSI breakpoints rather than the EUCAST breakpoints, eight of the ten cefoxitin susceptible CoNS isolates would have been classified as resistant. On the other hand, two of the MRSE isolates would have been reported susceptible to cefoxitin. According to the EUCAST guidelines, cefoxitin should be used when screening for methicillin resistance in CoNS (EUCAST, 2022). However, CLSI emphasizes that the cefoxitin disk diffusion test may not perform reliably in detecting methicillin resistance for all CoNS species (e.g., *S. haemolyticus*) (CLSI, 2022). Although cefoxitin is the recommended agent for most CoNS when screening for methicillin resistance, our results show that oxacillin is more reliable than cefoxitin for the purpose.

MRS are considered resistant to other beta-lactam agents, i.e., penicillins, beta-lactam combination agents, cephems (except for ceftaroline) and carbapenems. However, we observed a high frequency

(20/75) of amoxicillin-clavulanic acid-susceptible isolates. Sixteen of the susceptible isolates carried *blaZ,* which encodes a beta-lactamase that inactivates amoxicillin. Admittedly, this could be due to the lack of official breakpoints for amoxicillin clavulanic-acid disk diffusion. Still, six of these isolates were susceptible to cefoxitin, thus demonstrating that even if the *mecA* gene is present, it is not necessarily expressed towards cefoxitin and amoxicillin-clavulanic acid *in vitro.*

We could not predict STs or SCC*mec* cassettes for most MRCoNS/MRM isolates. The pubMLST database only contains data for *S. epidermidis, S. haemolyticus, S. hominis*, and *S. chromogenes,* and the missing ST identification may be due to a lack of characterized environmental genomes in the database. The high proportion of non-typeable SCC*mec* cassettes is consistent with previous reports (Chen *et al.*, 2017; Gómez-Sanz *et al.*, 2019). In many cases, the cassettes shared homology with previously described SCC*mec* but lacked identifiable *ccr* genes to be type determined. This was especially evident for the NT *S. haemolyticus* cassettes that had the best homology with SCC*mec* type V. The combination of NT SCC*mec* elements combined with non-identifiable STs demonstrates the large diversity among the staphylococcal and mammaliicoccal isolates in the home environments and the gaps in knowledge about the epidemiology/ecology of staphylococci from environmental reservoirs.

In general, CoNS and MRM are considered less virulent than *S. aureus.* Still, CoNS and MRM cause a substantial number of infections, presumably possessing virulence genes enabling them to do so. Virulence genes in CoNS and mammaliicocci are far less studied than the virulence genes of *S. aureus*. Consequently, we used a database mainly consisting of amino acid sequences from putative and known virulence factors in *S. aureus* to characterize virulence genes in our MRCoNS and MRM isolates (Naushad *et al.*, 2019). Admittedly, this is not optimal and will cause uncertainty around the hits with low and medium *Ha* scores. We focused on the highest scores within each species. However, we cannot be certain that hits with lower scores are of limited importance. Overall, the MRCoNS and MRM displayed species-specific virulence gene patterns, apart from the ubiquitous *etc* gene. The virulence gene patterns revealed subgroups within the *S. epidermidis, S. haemolyticus, S. hominis* and *S. saprophyticus* isolates based on the presence of type VII secretion-associated genes for the former and *cap* genes for the three latter. The isolation source seemed to matter for multiple *cap* genes only in the *S. haemolyticus* isolates, as all the human isolates were in this subgroup.

In conclusion, we have documented that the home environment is a reservoir for MRCoNS and MRM regardless of the type of household and the carrier status of humans and pets. However, homes with infected pets had a larger diversity in MRCoNS and MRM species than households without pets which might be due to the recent use of antimicrobials and contact with human- and veterinary hospitals. The large diversity in SCC*mec* elements and sequence types among and within the

households indicates no clonal spread of specific strains. The restricted common virulome and mobilome indicate a high degree of species specificity rather than exchanging genetic elements between species in the home environment.

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513 *Table 1. Criteria for including isolates in the different analyses.*

Analysis	**Isolates included in the analysis**	**Isolates presented in the result section**
mecA PCR	All cultured isolates	
Species identification (MALDI-TOF MS)	All cultured isolates	
Susceptibility testing	All cultured isolates	Non-redundant WGS isolates
Whole-genome sequencing (WGS)	Non-redundant isolates based on phenotypical resistance profiles, species, and household	
Additional species identification (*in silico,* TypeMat)	All WGS isolates	
Resistome analysis	All WGS isolates	Non-redundant WGS isolates
Virulence gene analysis	All WGS isolates	Non-redundant WGS isolates
SCC*mec* typing	All WGS isolates	Non-redundant WGS isolates
Sequence typing (*in silico* MLST)	All WGS *S. epidermidis*, *S. hominis,* and *S. haemolyticus* isolates	Non-redundant WGS *S. epidermidis*, *S. hominis,* and *S. haemolyticus* isolates
Mobilome analysis	All whole genome-sequenced *S. epidermidis*, *S. haemolyticus, S. hominis*, and *S. saprophyticus* isolates	Non-redundant *S. epidermidis*, *S. haemolyticus, S. hominis*, and *S. saprophyticus* isolates

514

Table 2. Overview of antimicrobial use (AM), hospital admission, and location of methicillin-resistant coagulase negative Staphylococcus *spp. (MRCoNS) and methicillin-resistant* Mammaliicoccus *spp. (MRM) in the 33 households. FB= Food bowl, SP= Sleeping place, FL= Floor, BA= Bathroom, KI= Kitchen.*

Household	Status	Pet	Pet AM treatment	Pet AM agent	Human AM treatment within 12 months	Hospital admission within 12 months	Work in health care	Home environment										Locations home environment	Human carriage	Pet carriage	Species ID Human/pet isolates
								S. arlettae	*S. cohnii ssp. cohnii*	*S. epidermidis*	*M. fleurettii*	*S. haemolyticus*	*S. hominis*	*S. pasteuri*	*S. saprophyticus*	*M. sciuri*	*M. vitulinus*	*S. warneri*				
A	Infection	Dog				No	Yes					+	+		+				FB BA KI			
B	Infection	Dog				Pet	No						+						BA			
C	Infection	Dog	0-3 months, 3-6 months	Cefalexin, Polymyxin B	Did not recall AM agent	No	No			+		+	+					+	FB SP FL BA KI	+	+	*S. epidermidis*
D	Infection	Dog	0-3 months	Cefalexin		Pet	No			+		+			+	+			SP FL BA KI	+		*S. haemolyticus*
E	Infection	Dog	0-3 months	Amoxicillin, Trim/sulfa		Pet	No		+			+			+	+			FB FL BA KI			
F	Infection	Dog	0-3 months, 3-6 months	Amoxicillin, Cefalexin, Enrofloxacin		Pet. Human	Yes			+	+	+	+		+			+	FB SP FL BA KI	+	+	*S. epidermidis*
G	Infection	Dog				Pet	No		+										FL			
H	Infection	Dog	0-3 months, 6-12 months	Amoxicillin, Fusidic acid		No	No			+					+				FB SP FL BA KI	+	+	*S. epidermidis*
1	Healthy	Dog				No	No					+	+		+				FB SP FL KI			
2	Healthy	Dog				No	No															
3	Healthy	Dog			Clindamycin	Human	No						+					+	SP KI	+		*S. warneri*
4	Healthy	Dog	0-3 months	Amoxicillin-clavulanic acid	Penicillin	No	No	+											SP			
5	Healthy	Dog	0-3 months	Fusidic acid	Chloramphenicol	Pet	Yes								+				SP FL BA			
6	Healthy	Dog				No	No															
7	Healthy	Dog				Human	No						+		+				FB FL BA			
8	Healthy	Dog			Erythromycin	Human	No			+			+	+	+				FB SP FL BA KI			
9	Healthy	Cat			Pivemecillinam	No	No			+		+	+						FB SP FL BA KI			
10	Healthy	Cat	0-3 months	Amoxicillin		No	No			+					+				FB FL			
11	Healthy	Cat				No	No			+					+				FB SP			
12	Healthy	Cat				No	No								+				FL			
13	Healthy	Cat				No	No					+							SP FL			
14	Healthy	Cat				No	No			+		+			+				FL BA	+		*S. haemolyticus*
I	No pet	n/a	n/a			No	Yes								+				BA		n/a	
II	No pet	n/a	n/a			No	Yes										+		FL KI		n/a	
III	No pet	n/a	n/a			No	No								+	+			BA KI		n/a	
IV	No pet	n/a	n/a		Penicillin	No	No		+						+				FL BA KI		n/a	
V	No pet	n/a	n/a			No	No								+				FL		n/a	
VI	No pet	n/a	n/a			No	Yes					+							BA		n/a	
VII	No pet	n/a	n/a		Penicillin	No	No														n/a	
VIII	No pet	n/a	n/a			Human	No					+					+		FL BA		n/a	
IX	No pet	n/a	n/a		Did not recall AM agent	Human	No						+						KI		n/a	
X	No pet	n/a	n/a			No	No						+						FL KI		n/a	
XI	No pet	n/a	n/a		Tetracycline	No	No			+		+	+			+		+	FL BA KI		n/a	

519 *Table 3. Percentage of phenotypic expressed resistance in the 75 sequenced MRCoNS and MRM isolates Worth noticing is*
520 *the large proportion of* S. hominis *isolates that expressed no phenotypic resistance to cefoxitin and amoxicillin clavulanic*
521 *acid. Gen: gentamicin, Chl: Chloramphenicol, Oxa: Oxacillin, Cfox: Cefoxitin, AmCl: Amoxicillin-clavulanic acid, Clex:*
522 *Cefalexin, Enr: Enrofloxacin, T/S: Trimethoprim sulfamethoxazole, Fus: Fusidic acid, Cli: Clindamycin, Ery: Erythromycin,*
523 *Tet: Tetracycline.*

		Antimicrobial agent											
Species		Gen	Chl	Oxa	Cfox	AmCl	Clex	Enr	T/S	Fus	Cli	Ery	Tet
All isolates	n=75	20 %	5	99	88	73	95	12	21	51	21	51	20
S. arlettae	1			100	100	100	100					100	100
S. cohnii ssp. cohnii	3		33	100	100	33	100		33	33	67	100	67
S. epidermidis	12	8		100	100	33	83	8	17	50	17	50	17
M. fleuretti	1			100	100	100	100			100	100		
S. haemolyticus	16	50	6	100	88	88	100	38	25	25	31	50	31
S. hominis	11	46		91	55	46	82	18	46	55	27	64	27
S. pasteuri	1			100	100	100	100					100	
S. saprophyticus	19		11	100	95	100	100		16	53	5	58	11
M. sciuri	4			100	100	100	100			100	25		
M. vitulinus	2			100	50	50	100			100			
S. warneri	5	20		100	100	80	80		20	80	20	20	

524

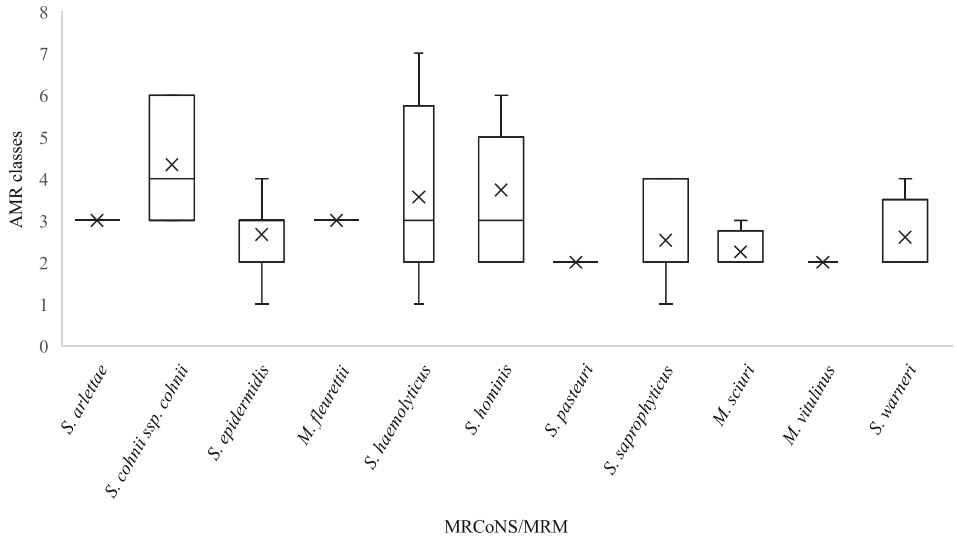
Table 4. Percentage of the 75 isolates testing positive for antimicrobial resistance genes (ARGs)

Antimicrobial class		Aminoglycoside				Amphenicol		Beta-lactam			Folate pathway antagonist		Fosfomycin		Fusidane				Macrolide, lincosamide, streptogramin							Multidrug		Pseudomonic acid	Quaternary ammonium compounds		Tetracycline	
ID	ARG	*aac(6')-Ie/ aph(2'')-Ia*	*ant(4')-Ib*	*aph(3')-IIIa*	*sat4*	*cat*	*catA*	*ar1*	*blaZ*	*mecA*	*dfrC*	*dfrG*	*fosB6*	*fosD*	*fusB*	*fusC*	*fusD*	*fusF*	*ermC*	*lnuA*	*mphC*	*msrA*	*salA*	*vgaA*	*vgaALC*	*mgrA*	*norA*	*mupA*	*qacA*	*qacB*	*tetK*	*tetL*
All isolates	n=75	20 %	13	4	5	4	1	1	63	100	27	8	4	1	29	7	25	4	13	15	29	43	5	4	5	61	24	5	41	3	20	3
S. arlettae	1							100		100												100									100	
S. cohnii ssp. cohnii	3		67			33			33	100								100		67	100	100				33			33	33	67	
S. epidermidis	12	8	25		8				100	100	92	8			50	8			8		17	42				17	100		42		17	
M. fleurettii	1									100																						
S. haemolyticus	16	56	31	19	19		6		94	100		31			25	6			31		38	38			19	100		13	69		38	13
S. hominis	11	46							82	100	9		27		36	27			18	27	9	42		8		92	8	17	91		18	
S. pasteuri	1									100											100	100				100	100			100		
S. saprophyticus	19					11			32	100	21				21		100		5	32	47	46		9					11		11	
M. sciuri	4									100				25									100									
M. vitulinus	2									100																						
S. warneri	5								80	100	80				80				20			20				100	80		40			

526 *Table 5. Predicted SCC*mec *elements based on detected genes in the sequenced MRCoNS and MRM*
527 *isolates.*

Species ID	II (2A)	III (3A)	IV (2B)	IVa (2B)	IVd (2B)	IVc (2B)	V (5C2&5)	VIII (4A)	Non-typeable
S. arlettae			1						
S. cohnii ssp. cohnii									3
S. epidermidis	1	1	2	6	1				1
M. fleurettii									1
S. haemolyticus							1	1	14
S. hominis								4	7
S. pasteuri									1
S. saprophyticus		10							9
M. sciuri									4
M. vitulinus									2
S. warneri						3	1		1

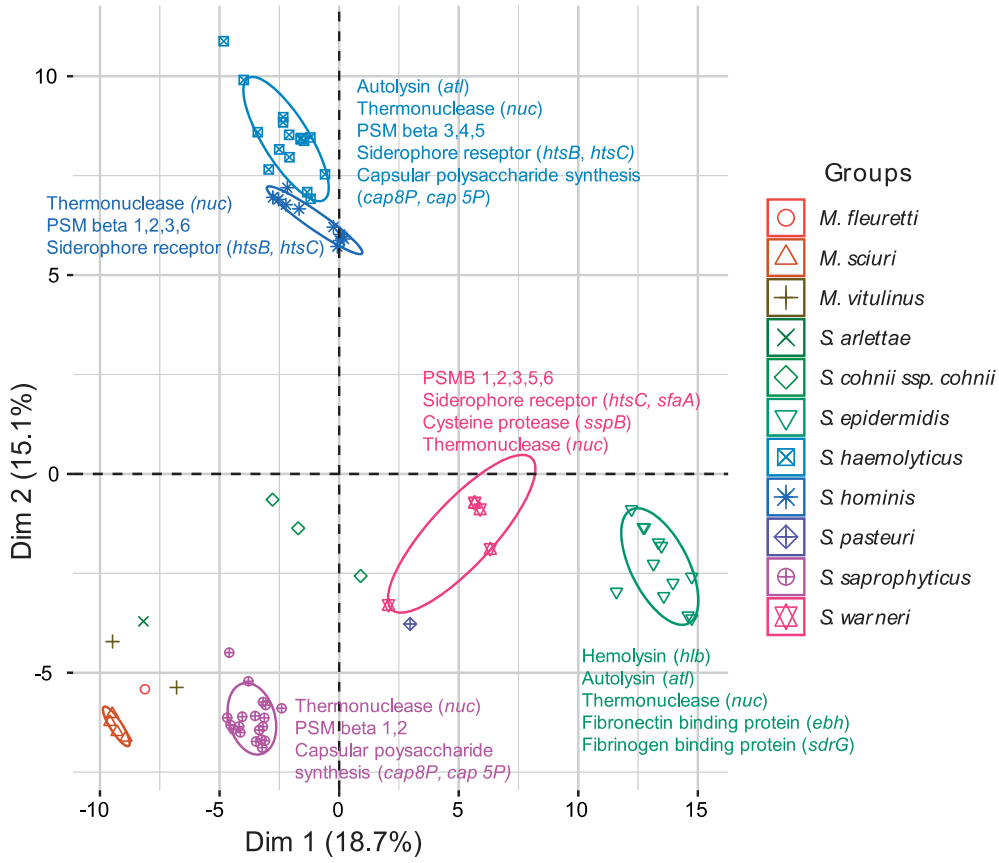
528



529

530 *Figure 1. Number of phenotypic resistance classes in the MRCoNS and MRM isolates n=75. The crosses represent the mean*
531 *number of antimicrobial resistance (AMR) classes, while the horizontal lines represent the median number.*

532



533

534 *Figure 2 Principal Component Analysis plot of virulence gene similarity in MRCoNS and MRM isolates. The exfoliative*
535 *toxin gene* etc *was present in all isolates. The annotations refer to the genes with the highest* Ha *scores among the isolates*

536

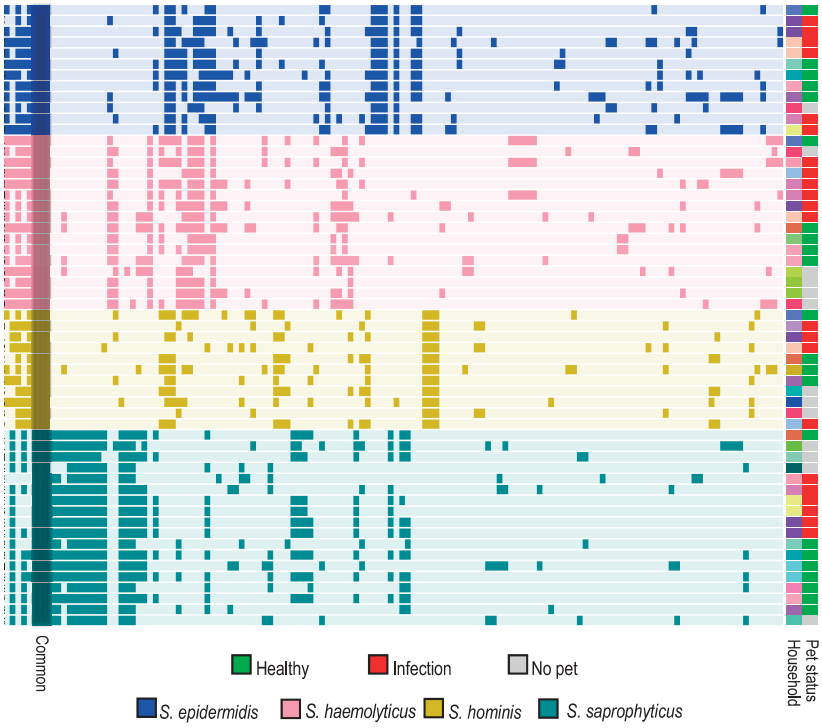


Figure 3. Mobilome analysis of S. epidermidis, S. haemolyticus, S. hominis, *and* S. saprophyticus, *sorted by species. Gene clusters identified in all isolates are annotated as "Common" and include genes encoding an IS6 family transposase, the competence protein ComGC, and an uncharacterized SPBc2 prophage-derived protein YoqJ.*

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