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ASYMPTOMATIC CARRIAGE OF COAGULASE POSITIVE STAPHYLOCOCCI IN HUMANS AND ANIMALS

PhD thesis

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LIST OF ABBREVIATIONS

ACKR1 – Atypical Chemokine Receptor 1

adsA – Adenosine synthase A

agr – Accessory gene regulator

BORSA – Borderline oxacillin resistant *S. aureus*

BRIG – BLAST Ring Image Generator

BSI– Blood stream infection

CA-MRSA – Community associated methicillin-resistant *S. aureus*

CC – Clonal complex

CHIPS – Chemotaxis inhibitory protein of *Staphylococcus*

Clf – Clumping factor

CNA – Collagen binding protein

CNS – Coagulase-negative staphylococci

CPS – Coagulase-positive staphylococci

DARC – Duffy antigen receptor for chemokines

DHFR – Dihydrofolate reductase

DHPS – Dihydropteroate synthetase

DIC – Disseminated intravascular coagulation

Dsg1 – Desmoglein 1

Eap – Extracellular adherence protein

Ebp – Elastin binding protein

ECM – Extracellular matrix

egc – Enterotoxin gene cluster

ET – Exfoliative toxin

FAO – Food and Agriculture Organization

FnBP – Fibronectin binding protein

HA-MRSA – Hospital acquired methicillin-resistant *S. aureus*

HVCN1 – Human hydrogen voltage-gated channel 1

ica – Intercellular adhesion

ICAM-1 – Intercellular adhesion molecula-1

LA-MRSA – Livestock associated methicillin-resistant *S. aureus*

MALDI-TOF – Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MAP – MHC analogous protein

MFS – Major facilitator superfamily

MGE – Mobile genetic element

MHC – Major histocompatibility complex

MLS_B – Macrolide-lincosamide-streptogramin B

MLST – Multi-locus sequence typing

MOF – Multiple organ failure

MRSA – Methicillin-resistant *S. aureus*

MRSP – Methicillin-resistant *S. pseudintermedius*

MSCRAMMs – Microbial surface components recognizing adhesive matrix molecules

MSSA – Methicillin-susceptible *S. aureus*

MSSP – Methicillin-susceptible *S. pseudintermedius*

NAM – N-acetylmuramic acid

NGS – Next generation sequencing

NPPc – Non-professional phagocytic cell

OIE – World Organization for Animal Health

pABA – Para-aminobenzoic acid

PBP – Penicillin binding protein

PFGE – Pulsed-field gel electrophoresis

PFT – Pore forming toxin

$PhLOPS_A$ – Phenicol, lincosamide, oxazolidine, pleuromutilin, streptogramin A

PIA – Polysaccharide intercellular adhesin

PMN – Polymorphonuclear leukocyte

PSM – Phenol-soluble modulin

PVL – Panton-Valentine leucocidin

QRDR – Quinolone resistance determining region

SaPI1 – Staphylococcal pathogenicity island 1

SaPIbov – *S. aureus* bovine pathogenicity island

sarA – Staphylococcal accessory regulator A

SCC*mec* – Staphylococcal cassette chromosome *mec*

SE – Staphylococcal enterotoxin

SIET – *S. intermedius* exfoliative toxin

SIG – *S. intermedius* group

spA – Staphylococcal protein A

SSSS – Staphylococcal scalded skin syndrome

ST – Sequence type

TGS – Third generation sequencing

TSST – Toxic shock syndrome toxin

VISA – Vancomycin-intermediate *S. aureus*

VRSA – Vancomycin-resistant *S. aureus*

vWbp – Von Willebrand factor binding protein

WGS – Whole genome sequencing

WHO – World Health Organization

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1. INTRODUCTION

1.1. Background

In the 1880s Alexander Ogston, an English surgeon, first described the disease caused by staphylococci and the role of cocci in sepsis, acute suppurative inflammation and abscess formation (1,2). Moreover the presence of the bacterium can lead to life threatening bacteremia and toxin mediated syndromes (3).

The *Staphylococcus* genus belongs into the Staphylococcaceae family with 81 species and subspecies (4,5). Under the microscope *Staphylococcus* species are Gram positive cocci (1 µm) arranged into clusters (3). They are predominantly facultative anaerobes except a few strict anaerobe species like *Staphylococcus saccharolyticus*. Staphylococci can grow in the presence of 10% NaCl, in a wide temperature range between 18°C-40°C. Regarding biochemical tests, staphylococci are usually catalase positive and based on their coagulase enzyme production we can distinguish between coagulase positive and negative species (3). Several species have human medical or veterinary importance. Coagulase-positive (CPS) staphylococci like *Staphylococcus aureus* and *Staphylococcus pseudintermedius* represents the major pathogens in the genus while coagulase-negative (CNS) staphylococci are relatively minor pathogens causing opportunistic and facultative pathogen infections (6).

If we talk about human infections, the most important species in the genus is no doubt *S. aureus*. The treatment of *S. aureus* infections became more and more challenging because of the emergence of antibiotic resistance throughout the last decades (3). In 1960 the first methicillin-resistant *S. aureus* (MRSA) was found among clinical isolates, carrying the so-called *mecA* gene, conferring resistance to all ß-lactam antibiotics except the 5th generation cephalosporins (7,8) (Figure 1). MRSA became a wide-spread multidrug-resistant nosocomial pathogen and a global health threat which made it into the WHO's list of global priority pathogens in 2017 (9). One of the last resort antibiotics against MRSA was vancomycin. In 1997 the first isolate with reduced vancomycin susceptibility (VISA) was isolated. VISA strains were a product of the accumulation of several spontaneous mutations. In 2002 the first vancomycin resistant

strain (VRSA) was found, which acquired the *vanA*-transposon from Enterococcus species (7) further increasing the threat of *S. aureus* infections .

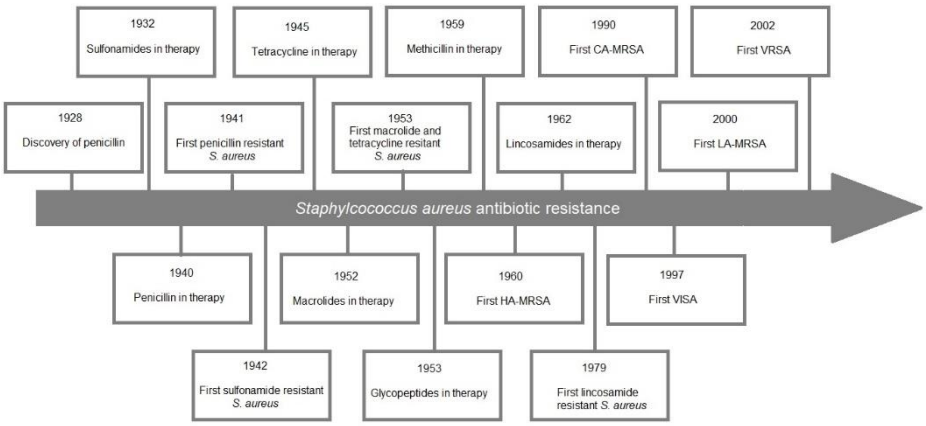

Figure 1. Antibiotic resistance in *S. aureus* throughout of the years (8).

Staphylococci are commensals on the skin and mucosal membranes of humans and animals, as well as they can exist in the environment: in soil, water, air, and food. They are widespread in nature, however, one of their biggest reservoirs is the nasal mucosa of humans. They can be transmitted either via direct contact and fomites or zoonotically (10). Several different infection categories are known with *Staphylococcus* and MRSA based on the source of the bacteria. The hospital acquired (HA) infections are microbial infections contracted in the hospital, during hospitalization. In the 1990s community associated (CA) infections appeared outside the hospital environment, between people who lived in closed groups such as children's institutions, team sport players, military personnel (10). From the mid-2000s, several scientific reports were published which documented animal related infections, which were named livestock associated infections (LA). Therefore, farm animals and pets also play a huge role as reservoirs. *S. aureus* more commonly colonize cows and pigs, but it can survive in pets as well (10). While *S. pseudintermedius* is part of the natural microbiota in dogs, it was documented from human infections sporadically (10–12). In the case of LA infections, the source of the bacteria is a carrier animal, and from there the pathogen colonize people who had close contact with the animal (13).

In the beginning of the 2000s, recognizing the connection between human and animal health and the ecosystem, the WHO, the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) brought to life the One Health approach to deal with health threats in the animal (including both domestic animals and wildlife), human and environment interface to promote global, multisectoral and transdisciplinary responses, policies and researches (Figure 2) (14). The One Health approach also deals with zoonotic diseases and antimicrobial resistance because of the increased risk of their spread into new territories with globalization and climate change (15).

Figure 2. The One Health approach.

1.2. Epidemiology in humans and animals

Staphylococcus infections have a serious effect both on public health and agriculture. Infected or asymptomatic animals can function as reservoirs for bacterial transmission. These host switching among humans and animals and between different animal species are frequent and can lead to spread and development of new antimicrobial resistance determinants. Normally the host switching of *Staphylococcus* species are followed by adaption to the new host population through mobile genetic elements such as plasmids and pathogenicity islands as well as with host specific genome mutations (16,17) (Figure 3).

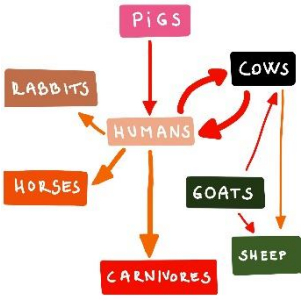

Figure 3. Humans as a hub for host switching (17).

1.2.1. CPS epidemiology in humans

In humans *Staphylococcus* species inhabit the skin, and diverse mucosal surfaces in the intestinal, urinary and upper respiratory tract, and most particularly they can be found in the nasal cavities. 20-30% of the general human population is permanent carrier (13,16), meaning the bacteria is continuously present on their mucus membranes. Staphylococci can cause opportunistic infections if they break through damaged skin or mucosae.

Acute and purulent infections can develop at the colonized body sites such as the skin leading to superficial impetigo and deeper furuncles, carbuncles, cellulitis. If these conditions remain untreated, they may spread deeper into the surrounding tissues and break into the blood vessels leading to bacteremia. Through hematogenic dissemination the infection can lead to pneumonia, osteomyelitis and even to meningitis (18).

Amongst the CPS, *S. aureus* is the most dangerous one, as it can have several various virulence factors, like toxins and can gain methicillin resistance via the mutation of the penicillin binding protein (MRSA) (3). This latter can lead to serious hospital acquired, nosocomial infections like purulent surgical site infections, blood stream infections and ventilation associated pneumonia (18). MRSA carriage rate can be 0% to 21% depending on the screened population (19,20). Humans act as a huge hub for host-switching because people are in continuous contact with pets and farm animals thus promotes the emergence of endemic livestock and animal adapted strains (16).

Another CPS which can also be responsible for sporadic cases in humans is *S. pseudintermedius*. The species itself was first described in 2005 from several different animal hosts (such as horse, dog, cat and parrot), however the colonization in humans is not common (21). With 16S rRNA gene sequence analysis and DNA hybridization techniques they were able to differentiate *S. pseudintermedius* from its closest phylogenetic relatives *S. intermedius* and *S. delphini*. Hence the *S. intermedius* group (SIG) was established (22). The first documented human infection happened in 2006 in Belgium. A 60-year-old male patient’s implantable cardioverter-defibrillator got infected. This was probably a community acquired infection, however, the source was never found (12). More human cases were reported: mainly skin and soft tissue infections (some of them linked to dog bites (23)), and a few invasive infections like prosthetic joint and blood stream infections. The majority of these cases could be linked to animal contact (11,24–26).

Several screenings were made among veterinarians and dog owners to examine their asymptomatic carriage rate and the occurrence of interspecies transmission. During these surveys they found both methicillin susceptible (MSSP) and resistant *S. pseudintermedius* (MRSP) in humans (27–33). The carriage rate in the general

population for *S.pseudintermedius* is 0-12 % (19,28,33) while for MRSP it is 0-4% (32,34).

The CNS - e.g. *S. epidermidis, S. haemolyticus* - can also be a reason for concern, because they can form biofilms on plastic surfaces which can lead to nosocomial infections, especially in immunocompromised and long-term hospitalized patients (18).

1.2.2. CPS epidemiology in animals

1.2.2.1. CPS epidemiology in pets

In dogs and cats *S. pseudintermedius* is the most recognized CPS that can cause opportunistic infections. It is an important cause of canine pyoderma (16), and mainly associated with otitis externa, wound and urinary tract infections. Furthermore it can also acquire methicillin resistance and can spread in small animal hospitals leading to nosocomial surgical site infections (35). The carriage rate is between 1-92% for *S. pseudintermedius* (36) and 0-45% for MRSP (37) in pets.

The *S. aureus* carriage rate in companion animals is 2-36%, while the MRSA is 0-11% depending on the sampled populations (37,38). The strains isolated from pets are frequently associated with humans and normally passed from the owners to their pets (16). The infections linked to *S. aureus* in pets are predominantly skin and soft tissue infections.

1.2.2.2. CPS epidemiology in wildlife

Because *Staphylococcus* species are widely spread in the environment it is crucial to understand their transmission within and between communities (10). However, wildlife represents a special and understudied area in the epidemiology of antibiotic resistance (10). Wildlife can act as a reservoir for antibiotic resistance genes naturally found in the environment or amplified by human activity. This could happen with the excrement of antibiotic treated people who release resistant bacteria into the sewage system which will disperse the bacteria into the environment and natural waters, or from livestock waste used as fertilizer on fields, which if dried can promote the airborne transmission and spread of the microorganism and resistance genes (10). Wild animals have the

potential to harbor new *S. aureus* strains and then transmit it to the human population. However, systemic screening of wild animals and molecular typing of *S. aureus* isolates from them has not been a concern previously, thus these bacterial transmissions between species could have been missed (10). In most studies wildlife is just documented as asymptomatic carrier of the bacteria, but it could lead to infections in them as well, like abscesses, bacteremia, bite wounds, dermatitis.

The systematic reviews show us that MSSA and MRSA were described in many wild animal species from small mammals to primates, from birds to fish. During my work I only focused on a small mammalian species, the Northern white-breasted hedgehog (*Erinaceus roumanicus*).

Hedgehogs increasingly inhabit areas of human activity therefore people are likely to come in contact with them (39). Hedgehogs can asymptomatically carry *S. aureus* on their skin and mucus membranes, and it can also be linked to diseases such as dermatitis and septicemia in these small mammals. The carriage rate of *S. aureus* is not a widely researched area in these animals, however according to some data from New Zealand it can reach up to 40-85% (40,41). Nowadays MRSA strains were mainly examined in hedgehogs and their carriage rate can range from 2% (42) to 64% (43).

1.3. Virulence factors

1.3.1. Virulence factors of *S. aureus*

S. aureus can express a wide range of factors which enhance the virulence of the bacterium, including toxins, immune-evasion factors and enzymes that promote host tissue invasion (Table 1) (Figure 4) (44,45).

Table 1. The most common virulence factors of *S. aureus* (44,45).

Gene name	Gene product	Function	Location
Adhesion, Biofilm formation			
clfA and clfB	Fibrinogen binding proteins	Adhesion	Core genome
fnbA and fnbB	Fibronectin binding proteins	Adhesion	Core genome
sdrC, sdrD, and sdrE	Ser-Asp repeat proteins	Adhesion	Core genome
ebp	Elastin binding protein	Adhesion	Core genome
eap/map	Extracellular adherence protein	Adhesion	Core genome
cna	Collagen adhesin	Adhesion	Core genome

icaABCD	N-acetylglucoseaminotransferase enzyme	Synthesis of polysaccharide intercellular adhesin (PIA)	Core genom
Immune evasion factors			
cap5 and cap8	Capsular polysaccharide biosynthesis proteins	Immune evasion	Core genome
chp	Chemotaxis inhibitory protein	Immune evasion	Bacteriophage
spa	Immunoglobulin G- binding protein A	Immune evasion	Core genome
adsA	Adenosine synthase A	Immune evasion	Core genome
sbi	Staphylococcal immunoglobulin binding protein	Immune evasion	Core genome
scn	Staphylococcal complement inhibitor	Immune evasion	Bacteriophage
Cytotoxins			
hlA	α- hemolysin	Hemolysis	Core genome
hlB	β-hemolysin	Hemolysis	Core genome
hlgA, hlgB, hlgC	γ-hemolysin	Hemolysis	Core genome
hld	δ- hemolysin	Hemolysis	Core genome
lukAB (lukGH)	Leukotoxins	Immune evasion	Core genome
lukD and lukE	Leukotoxins	Immune evasion	Pathogenecity island
lukS- PV and lukF-PV	Panton–Valentine leucocidin	Leukotoxin	Bacteriophage
PSMα1–PSMα4, PSMβ1 and PSMβ2	Phenol-soluble modulins	Hemolysis, leukotoxins	Core genome
Superantigens			
sea-selv	Enterotoxins, superantigens	Food poisoning	Bacteriophage, Pathogenecity island
tsst	Toxic shock syndrome toxin 1	Superantigen List	Pathogenecity island
Exfoliative toxins			
eta	Exfoliative toxin A (serine protease)	Scalded skin syndrome	Plasmid
etb	Exfoliative toxin B (serine protease)	Scalded skin syndrome	Bacteriophage
etd	Exfoliative toxin D (serine protease)	Scalded skin syndrome	Pathogenecity island
ete	Exfoliative toxin E (serine protease)	Scalded skin syndrome	Pathogenecity island
Enzymes			
aur	Aureolysin	Tissue destruction	Core genome
coa	Staphylocoagulase	Coagulation	Core genome
vWbp	von Willebrand factor binding protein	Coagulation	Core genome
geh	Lipase	Lipid degradation	Core genome
hysA	Hyaluronidase	Tissue invasion	Pathogenecity island

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sak	Staphylokinase (protease III)	Clot dissolution	Bacteriophage
sspA	V8 Serine protease	Tissue destruction	Core genome
sspB and C	Cysteine protease - Staphopain B and C	Tissue destruction	Core genome

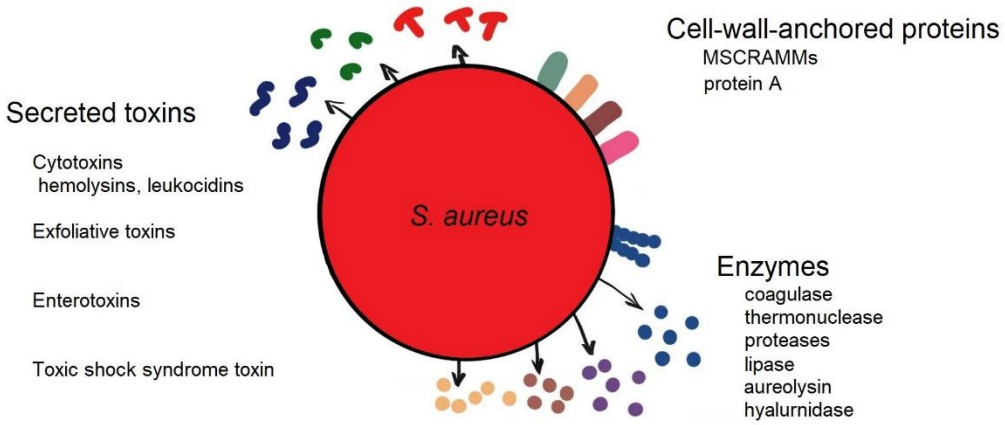

Figure 4. Important virulence factors of *S. aureus*.

1.3.1.1. Adhesion and biofilm production

Staphylococci belongs into those group of microorganisms which can form biofilm very easily (46). Biofilm is a community of bacterial cells producing a massive polymer matrix around themselves which can adhere to inanimate or living surfaces (46). The presence of biofilms are very upsetting in a medical environment where they can contaminate medical devices such as catheters, prosthetic joints, endotracheal tubes (47).

The first step in the biofilm development is the microbial adhesion to a surface and coaggregation (46). Staphylococci have a huge collection of surface adhesins which together are called **microbial surface components recognizing adhesive matrix molecules** (MSCRAMMs) which will bind to the host extracellular matrix proteins (ECM) (46).

One of the adhesins which can bind to these ECM proteins are **the fibronectin binding proteins**, FnBPA and FnBPB, anchored to the cell wall. Most *S. aureus* strains express

either one or both proteins which are coded on closely linked genes (*fnbA, fnbB*) (48). Fibronectin is a large glycoprotein which has functions in tissue repair after injury (48) and it can mediate the adherence to endothelial cells initiating endovascular infections (49).

S. aureus produces serine-aspartate (Ser-Asp) rich fibrinogen binding adhesins, which are in the cell wall and called **Clumping factor** (ClfA, ClfB). The distinct genes encoding these proteins are the *clfA* and *clfB*. The major adhesin, ClfA is a mediator of platelet aggregation (49,50). ClfB can mediate clumping of the bacterial cells and their attachment to immobilized fibrinogen. The Clumping factor can be detected with latex agglutination kits, and very frequently used in diagnostics to identify CPS species (51).

The **Ser-Asp repeat proteins** are also belong to the MSCRAMM family and encoded by the *sdr* locus which contains the *sdrC, sdrD,* and *sdrE* genes (49,52). These sdr proteins bind to bone sialoprotein and fibrinogen (49).

The **collagen binding protein** (CNA) produced by the *cna* gene is not frequently expressed in the *S. aureus* strains (49). CNA mediates adherence to collagenous tissues, and its presence shows association with osteomyelitis and septic arthritis (49,53).

The **elastin binding protein** (EbpS) is not a classic MSCRAMM as it is not a cell wall-associated protein but an integral membrane protein which binds to elastin (54). Organs and tissues that require elasticity (such as blood vessels, skin, lungs) contain elastin (54). EbpS is produced by the *ebp* gene and promotes the bacterial colonization in the host (49).

The **extracellular adherence protein** (Eap) which is also called **MHC analogous protein** (Map) has high homology to the peptide binding groove of mammalian MHC class II molecules. This protein has a high affinity for various ECM proteins and promotes bacterial attachment (49). It is also capable of modulating the inflammatory response in the endothelial cells through its interaction with intercellular adhesion molecula-1 (ICAM-1) (55).

Although *S. aureus* strains possess MSCRAMMs, the key constitutive of the biofilm is the polysaccharide intercellular adhesin (PIA) (46). PIA is a homopolymer of poly-*n*-

succinyl-β-1,6 glucosamine units, encoded by an **intercellular adhesion operon** called *ica*, containing the *icaABCD* genes (46,47,49).

Regardless of where the biofilm exists, its formation begins with attachment of the bacteria followed by aggregation, reproduction, and the secretion of polymeric substances, which form a matrix that encapsulates and protects the bacteria (47). After adhesion every biofilm goes to a maturation process (46). Once the biofilm is matured, it becomes highly difficult to eradicate (47). The mature biofilm is a complex structure where the proper functioning of the individual cells needs a coordinating system. This is called quorum-sensing system (46). The two most important gene regulatory systems are called **accessory gene regulator**—*agr*, and **staphylococcal accessory regulator A**—*sarA*. These systems are based on cell-to-cell communication through the secretion of signal molecules which can lead to gene expression changes (46).

Despite the benefits of the biofilm for the bacteria they are not infinite, bacterial cells will detach disseminating the bacteria in the host (46). Research on staphylococci showed that the *agr* gene is directly involved in this disassembly through the production of pore forming toxins and enzymes like proteases and DNases (46,56). These pore forming toxins are called **phenol-soluble modulins** (PSMs) and they have a detergent-like function while the enzymes solubilize the substances in the biofilm matrix (46). The PSMs have additional roles in the bacterial pathogenesis: they can lyse red and white blood cells and stimulate inflammatory responses (57). In *S. aureus*, PSMs are produced by the following genes: the four PSMα peptides are encoded by the *psmα* operon; the two PSMß, by the *psmβ* operon; while the δ-toxin is produced by the *hld* gene (57).

1.3.1.2. Immune evasion

Staphylococcal protein A (spA) is one of the most famous immune evasion factors (49). It owns tandem repeats of five homologous IgG binding domains (49,58) and binds to the Fc domain of IgG molecules, this way inhibiting the opsonization and the phagocytosis (49,59,60).

The other **staphylococcal immunoglobulin binding protein** (sbi) is a secreted protein that contains two immunoglobulin binding domains and two C3d complement factor binding domains (49). The sbi damages the link between the innate and adaptive immune responses and inhibits the neutrophil cell mediated opsonophagocytosis (49).

Over 90% of *S. aureus* strains can produce **capsule**, which prevent phagocytosis and opsonization (49,61,62). Two capsular serotypes are the most prevalent among the clinical and carried human and animal isolates: type 5 and 8 (49,61,63). Due to the high prevalence of these types, capsular typing on its own is not analytical enough for epidemiological purposes (62). Studies were also made to explore the immunization possibilities with capsular type 5 and 8, however these initial studies did not offer promise that capsular antibodies would protect against staphylococcal infection (61).

The capsule production of *S. aureus* was first described by Gilbert et al. in 1931 (64). In 1985 Karakawa and Vann made a new capsular polysaccharide typing scheme (65). They were the first investigators to report that most of the *S. aureus* strains were encapsulated and they described eight different serotypes (61,65). In 1985, Sompolinsky detected three new serotypes bringing the number of capsular serotypes to 11 (62).

S. aureus is able to synthesize adenosine, an immune signaling molecule with its cell-wall linked **adenosine synthase A** (adsA) enzyme. With adenosine *S. aureus* can weaken the innate and adaptive immune responses during infection (49,66). To survive in the human host *S. aureus* must avoid a wide variety of innate immune mechanisms, such as phagocytes, antimicrobial peptides and the complement system (66). In mammals adenosine regulates these immune responses (67). The balancing of pro- and anti-inflammatory mediators plays a crucial role in the successful immune clearance of microbes from the host. Lots of cytokines have a restrictive effect on inflammation however only adenosine is completely able to suppress immune responses, and this is what *S. aureus* utilizes through adsA (66,68).

The **chemotaxis inhibitory protein of Staphylococcus** (CHIPS) helps in the innate immune evasion (49,69). It is located on the β-hemolysin (*hlb*)-converting bacteriophages (49). It is a protein that binds to the C5a receptor and thereby inhibits C5a complement mediated responses in humans (49).

1.3.1.3. Enzymes

Coagulase (Coa), also called staphylocoagulase, is an extracellular protein responsible for the conversion of fibrinogen to fibrin, encoded by the *coa* gene. This enzyme can be detected with the tube coagulase test which frequently used in the identification of CPS isolates. This gene is located on the chromosome, between the lipase gene (*geh*) and the protein A gene (*spa*).

The **von Willebrand factor binding protein** (vWbp) is a staphylococcal protein which has coagulase activity and able to bind to the von Willebrand factor inhibiting platelet adhesion. The open reading frame for vWbp is located between the *clfA* and *emp* genes.

These coagulase proteins with their respective genes promote pathogenesis of staphylococcal abscesses, bacteremia, and endocarditis, along with the persistence of the disease (70).

In the early stages of the infection the proteins produced by staphylococci act as adhesive factors. In later stages, the cell surface linked proteins are suppressed by the secretion of soluble extracellular enzymes. This change is controlled by global regulatory systems such as the previously mentioned *agr* and *sarA* (71).

Staphylococci also produce **proteases**, which can be categorized into three catalytic groups: serine proteases: V8 protease coded on the *sspABC* operon, serine protease-like proteins (Spl) coded on the *spl* operon, cysteine proteases: staphopain A, B and C coded on the *scp* and *ssp* operon, and metalloproteases like aureolysin (71).

Lipases are water soluble enzymes, they usual activity is the hydrolysis of ester bonds; hence they are responsible for the release of fatty acids. They contribute to virulence by making the bacteria able to survive in the fatty secretions of the human or mammalian skin. The lipase genes like *geh* and *lip* can be found on the core genome of the bacteria (49,72,73).

1.3.1.4. Toxins

Pore forming toxins (PFTs) are a common tool that bacteria can use during an infection to cause cell lysis. There are two major PFT classes: α-PFTs which form α-helix pores,

and β-PFTs which form β-barrel pores. *S. aureus* can produce several ß-PFTs, including α-hemolysin and several bicomponent leucocidins. These leucocidins consist of two subunits (F and S) that are encoded within a single open reading frame. The two subunits are generally secreted as water soluble monomers but must be combined for maximal cytolytic activity. Normally the assembled leukotoxin is an octamer of four F-subunits and four S-subunits in alternating fashion (74).

The **Panton-Valentine leukocidin** (PVL) which consists of the LukF-PV and LukS-PV subunits is coded on bacteriophages. The occurrence of the PVL is linked to human adapted strains (49,74,75). C5aR1 and C5aR2 are the receptors of this leucocidin.

Another leukocidin, called **LukED** is frequent in both human CA-MRSA isolates and in cow mastitis samples where its gene is harbored on the bovine pathogenicity island called SaPIbov (74,76,77). It binds to CCR5, CXCR1 and CXCR2 cell surface receptors on white blood cells and to the Duffy receptor (DARC; or Atypical Chemokine Receptor 1, ACKR1) on human red blood cells (74).

LukGH, also known as **LukAB** is one of the most recently discovered leucocidins of *S. aureus* which binds to the CD11b receptors (74). The *lukAB* locus is part of the core genome. This LukAB toxin has a new variant possessed by *S. aureus* CC30 and CC45 which can kill human phagocytes binding to a new target: the human hydrogen voltage-gated channel 1 (HVCN1) (78).

LukMF' only circulates in non-human hosts such as cow, goat and sheep and most commonly associated with mastitis in these animals (74,76). It will bind to CCR1/2/5 receptors.

The **γ-hemolysin** is also a bicomponent toxin. The hemolysin locus consists of three genes: *hlgA, hlgB, hlgC* which makes up two different S-subints: HlgA and HlgC, and one F-subunit, HlgB. HlgA and HlgB combine to form the classical γ-hemolysin (HlgAB) which is particularly effective in lysing human red blood cells and human leukocytes, as well. HlgAB binds to the CXCR1/2/4 and CCR2 receptors on white blood cells and DARC on red blood cells. On the other hand, HlgC and HlgB together (HlgCB) also called γ-hemolysin, although it primarily acts as a leukotoxin, as a result early studies called it LukSF or LukR (74,79).

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The **α-hemolysin**, which also belongs to the β-barrel pore-forming cytotoxins is encoded on the chromosomal *hla* gene. Its cell receptor is the metalloprotease 10 (ADAM10). The α-hemolysin causes the complete hemolysis of a broad range of mammalian erythrocytes (e.g. rabbit, sheep), leading to a wide zone of complete hemolysis around the bacterial colonies on blood agar. This toxin can cause skin necrosis and lethal infections (80).

The **β-hemolysin** is produced by the chromosomal *hlb* gene. The produced toxin protein shows sphingomyelinase activity. The β-toxin expresses highly haemolytic effects on sheep, ox, and human erythrocytes. On blood agar, the β-hemolysin leads to a wide area of incomplete hemolysis (80) (Figure 5).

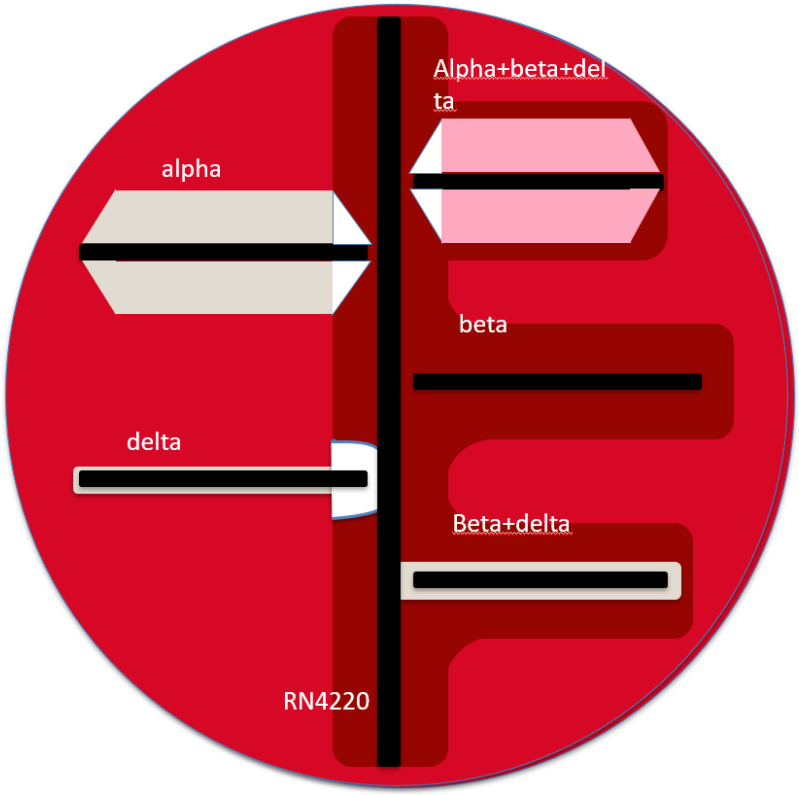

Figure 5. The hemolysins of *S. aureus*.

The hemolytic activity of *S. aureus* can be determined on sheep blood agar by cross-streaking different strains perpendicularly to RN4220, which produces only β-hemolysin. This test can identify two staphylococcal hemolysins: alpha- and delta-hemolysins. The delta-hemolysin is enhanced by the beta-hemolysin, while alpha-hemolysin is inhibited by the beta-hemolysin. Beta toxin is also known as the hot-cold toxin because at 37°C it does not lyse red blood cells on sheep blood agar plates, but placed at 4°C, the cells lyse and complete hemolysis can be seen (81,82).

RN4220: Test strain provided by Richard Novick. RN4220 produces only beta, a hot-cold hemolysin that is synergistic with delta.

black colour: bacterial colonies, dark red colour: effect of ß-hemolysin, weak ß-hemolysis, light brown colour: effect of α-hemolysin, clear ß-hemolytic zone, white colour: amplified, strongest ß-hemolysis, pink colour: the combined effect of α-, ß- and δ-hemolysin

The **δ-hemolysin** with its α-helix structure is able to damage the membrane of a variety of mammalian cells like human, sheep, horse erythrocytes. By acting as a surfactant, δ-toxin can disrupt the membrane structure of the cells rapidly leading to lysis. The δ-hemolysin is produced by the chromosomal *hld* gene. The toxin leads to a narrow zone of complete hemolysis around the bacterial colonies on sheep blood agar (80–82).

The **exfoliative toxins** are serine proteases which bind and hydrolyze specifically to a single peptide bond in the extracellular segment of desmoglein 1 (Dsg1), which is a desmosomal cell to cell adhesion molecule. This enzymatic lysis leads to the disintegration of keratinocytes in human and animal skin. So far, four ET serotypes are known: ETA, ETB, ETD, ETE and they are associated with Staphylococcal scalded skin syndrome (SSSS) or bullous impetigo in humans. Nonetheless, exfoliation caused by exfoliative toxins is described in many more different host species, with different susceptibility, which indicates host specificity. In animals, ET producing isolates were most commonly detected from severe or mild ovine mastitis cases (83,84).

The *tsst* gene is present on the staphylococcal pathogenicity island 1 (SaPI1) and the **TSST-1** toxin is responsible for the toxic shock syndrome. Furthermore, TSST acts as a superantigen: it stimulates the T cells by cross-linking the T-cell receptor with MHC-I molecules. As a result, nonspecific T-cell activation will happen. The presence of the TSST (with CNA) shows association with the clonal complexes (49,85).

The most frequently examined **enterotoxins** are the classical five SEs: SEA to SEE, meanwhile 27 different enterotoxins have been described to date from SEG to SEl27. Moreover, due to the mutation, and recombination in their encoding genes, new SEs (such as SELV and SELU), and pseudogenes (such as Ψent1 and Ψent2 and SE variants (such as SEC1−4, SECbovine, SECovine, SELU2, and others) are continuously appearing (86). After consuming the contaminated food, SEs act through two pathways: the first is a neurotoxic effect which triggers vomiting by the activation of the vagus nerve and the emetic center of the brain, the second is a superantigenic activity leading to enhanced T-cell activation and strong fever. All SEs function as a superantigen, but not all have emetic effects (87).

SE genes are localized on various mobile genetic elements (MGEs), such as plasmids, prophages or pathogenicity islands (SaPIs). Sometimes several SE genes can be found

within the same MGE (87). For example the enterotoxin gene cluster (*egc*) frequently harbors five SE genes coding for five different toxins: SEG, SEI, SEM, SEN, and SEO and two pseudogenes Ψent1 and Ψent2 localized between the *sei* and *sen* genes (88). Some variants of this *egc* have the gene coding for SElV instead of the *sei* and *sem* genes (87). Some other variants contains a novel putative enterotoxin called SEU instead of the two pseudogenes Ψent1 and Ψent2 (88). Phylogenetic analysis of enterotoxin genes indicated that they all potentially derived from this *egc* cluster by duplication, transposition and mutation, identifying *egc* as a putative nursery of enterotoxin genes (87,89). Thus, these mechanisms have led to several different enterotoxin genetic clusters (89,90).

1.3.2. Virulence factors of *S. pseudintermedius*

Although not so widely studied, but *S. pseudintermedius* can also express a wide range of virulence factors (Table 2) (Figure 6).

Table 2. The most common virulence factors of *S. pseudintermedius* (49,91–93).

Gene name	Gene product	Function	Location
Adhesion, Biofilm formation			
icaABCD	N-acetylglucoseaminotransferase enzyme	Synthesis of polysaccharide intercellular adhesin (PIA)	Core genom
Immune evasion factors			
spsQ "spa"	Immunoglobulin G- binding protein A	Immune evasion	Core genome
psbi	*S. pseudintermedius* immunoglobulin binding protein	Immune evasion	Core genome
Cytotoxins			
hlA	α- hemolysin	Hemolysis	Core genome
hlB	β-hemolysin	Hemolysis	Core genome
hlgA, hlgB, hlgC	γ-hemolysin	Hemolysis	Core genome
hld	δ- hemolysin	Hemolysis	Core genome
lukS- I and lukF- I	Leucocidin	Leukotoxin	Core genome
psmε	phenol-soluble modulin	Hemolysis, leukotoxins	Core genome
Superantigens			
sec_{canine}	Enterotoxin, superantigen	Food poisoning	Pathogenicity island
sec-int	Enterotoxin related gene	Food poisoning	Pathogenicity island
Exfoliative toxins			

siet	*S. pseudintermedius* Exfoliative toxin	Exfoliation	Core genome
expA "exi"	*S. pseudintermedius* Exfoliative toxin	Exfoliation	Core genome
expB "orf"	*S. pseudintermedius* Exfoliative toxin	Exfoliation	Core genome
Enzymes			
coa	Staphylocoagulase	Coagulation	Core genome
lip	Lipase	Lipid degradation	Core genome
pta	Phosphoacetyltransferase	Acetyl coenzyme A synthesis	Core genome

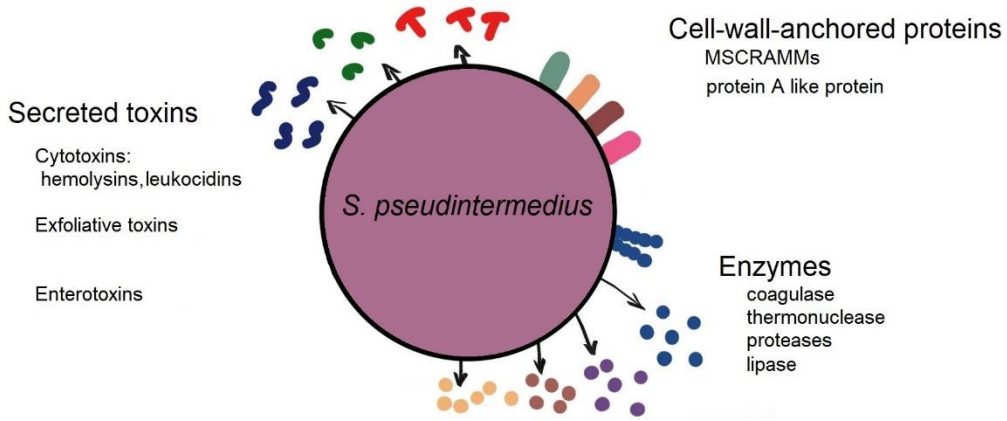

Figure 6. The most common virulence factors of *S. pseudintermedius* (36).

1.3.2.2. Adhesion and biofilm production

Biofilm formation is an ability which is variable between bacterial species, and this ability of *S. pseudintermedius* has not been deeply characterized compared to *S. aureus*. Nevertheless, it was found that *S. pseudintermedius* can also possess the *ica* operon to produce PIA (91).

1.3.2.3. Immunevasion

S. pseudintermedius has a protein which 40% identical with the *S. aureus* Sbi, it is called **pSbi**. Sbi proteins of *S. aureus* and *S. pseudintermedius* have very similar structures, but pSbi binds to only canine, equine, and feline IgG. This binding pattern correlates with the host specificity of *S. pseudintermedius*, which naturally infect dogs,

cats and horses. Moreover, pSbi is able to bind to canine B cells suggesting that it may modify the adaptive immune responses of the host organisms (94).

S. pseudintermedius possesses a ***spsQ*** gene seemingly analogous to the *S. aureus spa* gene. The Fc region of canine IgG bounds to this *S. pseudintermedius* protein A in a similar manner than the human Ig with the protein A from *S. aureus*, thus helps the bacteria to hide from the immune system of the hosts, leading to more persistent infections (95).

1.3.2.4. Enzymes

The enzyme production of *S. pseudintermedius* strains is not so commonly studied compared to *S. aureus*. Nevertheless, it can produce **coagulase, thermonuclease, DNase and lipase** as well (*coa*, *nuc*, *lip* respectively). The **phosphoacetyltransferase** (*pta*) enzyme have a role in acetyl coenzyme A production and the PCR of *pta* and *nuc* genes can be used in the species identification of *S. pseudintermedius* (96–99)

1.3.2.5. Toxins

S. pseudintermedius has the ***lukS-I* and *lukF-I* leucocidin** genes (100,101). This leucocidin has a cytotoxic activity on the PMN cells. Its receptor is the CXCR2. *S. pseudintermedius* has two types of **PSM**: *psmε* and *hld*. These PSMs are cytotoxic for non-professional phagocytic cells (NPPc). Furthermore PSMs have a receptor-independent, direct action on the cell membranes, therefore, human PMNs are also sensitive to them (92).

S. pseudintermedius has its own **exfoliative toxins**. One of them is called SIET. This protein caused rounding of the cells in cell cultures and exfoliation in animal experiments (100,102). ExpA and ExpB also listed as exfoliative toxins of *S. pseudintermedius* (30,103,104). ExpA and ExpB selective only to canine Dsg1 and causes intraepidermal cell junction splitting in dog skin (104)

S. pseudintermedius produces its own variant of **enterotoxin** coded by the sec_{canine} gene (100,105) It also has an enterotoxin-related gene, *se-int* (106). *S. pseudintermedius* is also able to produce hemolysins. The most frequently detected hemolysin is the ß-

hemolysin in this species, coded by the *hlb* gene. However the species specific *hla*, *hlc*, *hlg* genes can also be found in their genome (92,107).

1.4. Clinical disease

1.4.1. Clinical diseases caused by *S. aureus*

S. aureus is a major cause of skin, soft-tissue, respiratory, orthopedic, and bloodstream infections in humans. *S. aureus* can be responsible for bacteremia which most commonly develops related to catheter-associated infections. Factors associated with increased mortality include old age, nonremovable source of bacterial infection, and serious underlying co-morbidities. *S. aureus* one of the most frequent reasons behind endocarditis as well. It occurs more frequently in intravenous drug users, in patients with prosthetic valves, or in pateints who are hospitalized. Metastatic infections of *S. aureus* have a tendency to spread to particular body sites, including parenchymal organs, bones and joints. Suppurative inflammations at these sites serve as potential source for recurrent or persisting infections. Bacteremia or local infections can even progress to sepsis. Severe cases of sepsis lead to multiple organ failure (MOF), disseminated intravascular coagulation (DIC), acidosis, and even to death. Risk factors for sepsis include age, immunodeficiency, and invasive medical procedures (3).

S. aureus infections can be toxin mediated diseases, as well. Staphylococcal toxic shock syndrome came to be in the public eye in 1980s, when numerous cases were associated with the superabsorbent tampons. The main characteristic of toxic shock syndrome is the rapid onset of symptoms, in previously healthy people. The diagnosis is mainly based on the clinical findings which include very high fever, erythematous rashes all over the body with subsequent desquamation, hypotension, and multiorgan disfunction. This syndrome more often develops from an asymptomatic colonization rather than an infection. The cases of the syndrome were associated with menstruation and superabsorbent tampon usage. These were taken off the market, so nowadays important to consider other sources like localized infections, surgeries, or insect bites (3).

The exfoliative toxins of *S. aureus* are responsible for the staphylococcal scalded skin syndrome which was first observed by a German physician in young children in 1878.

SSSS is a skin disorder with desquamation that particularly affects infants and young children, although rarely it can occur in adults with an underlying disease. The spectrum of the affected areas ranges from localized blisters to extensive exfoliation (84).

Staphylococcal food poisoning, caused by the staphylococcal enterotoxins, is a very common, self-limiting food-borne disease worldwide. Symptoms appear rapidly within a few hours after ingestion of the toxin and include nausea, vomiting, diarrhea and abdominal pain. The disease is usually mild and resolves within a day or two from the onset of symptoms. The main sources of staphylococcal contamination of the food are humans, via either manual contact or from the respiratory tract, by coughing and sneezing. However, in foods such as raw meat, sausages, raw milk, and dairy products, contamination from livestock animals is more frequent, due to animal carriage or infections (most frequently from mastitis) with enterotoxin producing strains. The most common factors contributing to food poisoning outbreaks include inadequate temperature control and heat treatment, infected food handlers, contaminated raw ingredients (108,109).

Pet dogs can be colonized by enterotoxigenic *S. aureus* which gives possibility to zoonotic transmission of such strains to human contacts; thus, pet animals may have their part in staphylococcal food poisoning (110).

1.4.2. Clinical diseases caused by *S. pseudintermedius*

In companion animals, especially in dogs, *S. pseudintermedius* is a more frequent colonizer than *S. aureus*, and it can also be the cause of infections. It is one of the most common etiological agent in skin and soft tissue infections like wound infections, abscesses, otitis externa in dogs (111,112).

Moreover, *S. pseudintermedius* can also cause diseases in humans, especially in people who had known contact with dogs, which suggests zoonotic transmission. The bacterium was isolated from skin and soft tissue infections, dog bite wounds, invasive cases like, bloodstream infection, prosthetic joint infection, implantable cardioverter defibrillator infection, pneumonia. Moreover humans can get colonized with *S. pseudintermedius* , as well (11,12,112,113).

1.5. Diagnostic methods, laboratory identification

Diagnosis of infections caused by Staphylococci is most commonly made by the clinical presentation and history of the patient, with the isolation of *S. aureus* or *S. pseudintermedius* (114).

From certain types of clinical samples Staphylococci can be identified directly with microscopy: Gram-positive cocci arranged into clusters, (however they can appear as single cells when directly examined in clinical specimens). Moreover, commercial nucleic acid amplification tests are available for the direct detection and identification of *S. aureus* in clinical specimens (115).

However, specimens most commonly are inoculated onto agar media supplemented with 5% of sheep blood where they grow rapidly producing large, smooth colonies within 24 hours. *S. aureus* colonies produce golden pigmentation while *S. pseudintermedius* produces white pigmentation. Almost all isolates of *S. aureus,* and *S. pseudintermedius* cause ß-hemolysis on sheep blood agar, and *S. pseudintermedius* commonly causes double zone hemolysis (116). Furthermore, they can be isolated selectively on a variety of special media including commercially available chromogenic agar (where different staphylococcal species have characteristically colored colonies) or mannitol-salt agar, which is supplemented with mannitol (and colonies will have different color based on their mannitol fermentation) and 7.5% sodium chloride (inhibits the growth of other organisms) (117).

After cultivation and the examination of colony morphology, relatively simple biochemical tests can be used to identify *S. aureus* which shows a positive catalase and coagulase test, its protein A can be detected and it ferments mannitol while *S. pseudintermedius* is also catalase and coagulase positive, but can not ferment mannitol (116). Colonies resembling *S. aureus* are identified in most laboratories by mixing the organism with latex co-agglutination kits and observing clumping of the organisms (positive coagulase test). Alternatively, plasma placed in a test tube can be inoculated with the organism and examined at 4 hours for formation of a clot (positive tube coagulase test). More recently mass spectrometry (MALDI-TOF) has been used to identify pathogens such as bacteria with a high level of accuracy and rapid time to

results. Specific genes from the genomic DNA can be detected with PCR however, whole genome sequencing is rapidly becoming the preferred tool of molecular methods (114–116).

1.6. Antibiotics used against Staphylococci

1.6.1. Antibiotics used in localized infections

Impetigo and folliculitis are localized, superficial infections where topical treatment can be enough with mupirocin or retapamulin. Alternatively, fusidic acid can be used as well. For extensive cases systemic treatment is necessary with cephalexin or dicloxacillin, or erythromycin (118). In the case of cellulitis first and second generation cephalosporins are recommended for oral therapy and cefazolin, nafcillin, clindamycin as parenteral treatment for extensive infections. To treat skin abscesses, furuncles, carbuncles first drainage is recommended than administration of antibiotics (119). Here clindamycin, doxycycline, or trimethoprim-sulfamethoxazole can be used as oral therapy and cefazolin, ceftaroline, ceftriaxone, dalbavancin, daptomycin, vancomycin, tedizolid, linezolid can be tried as parenteral treatment (120).

Oral antibiotic therapy is recommended for localized MRSA skin and soft tissue infections. The recommended drugs are the following: trimethoprim-sulfamethoxazole, tetracyclines like doxycycline or minocycline, or the novel representative omadacycline, clindamycin, alternatively oxazolidinones or delafloxacin from the fluoroquinolone group can be used. In immunocompromised patients or when extensive soft tissue infections are present, or the symptoms are progressing rapidly, parenteral treatment is necessary where vancomycin or daptomycin can be the first choice (121).

1.6.2. Antibiotics used in systemic infections

Bacteremia caused by MSSA is treated with vancomycin, daptomycin, or nafcillin, oxacillin, cefazolin based on antibiotic susceptibility testing (122).

For systemic MRSA infections the initial treatment is either vancomycin or daptomycin (teicoplanin where available) in monotherapy. For those patients who are not responding to these drugs, combination regimens can be applied: daptomycin with

ceftaroline which is a fifth-generation cephalosporin, or with a ß-lactam, vancomycin with a hydrophilic ß-lactam. There are other antibiotic agents which need further study about their efficacy against MRSA bacteremia like the lipoglycopeptides (telavancin, dalbavancin, oritavancin), linezolid, tedizolid (123). Infections caused by VISA and VRSA strains are difficult to treat, there is no optimal regimen: daptomycin or vancomycin with ceftaroline, or combined with trimethoprim-sulfamethoxazole, or telavancin, fifth generation cephalosporins (ceftaroline) or linezolids can be tried (124).

The development of antibiotic resistance in *S. aureus* has had two major ways: the first is the acquisition of resistance determinants by horizontal gene transfer, the second is by mutations of the bacterial genome which can lead to the alteration of the drug bindings sites or to the increase of the expression of efflux pumps (125) (Table 3).

Table 3. Resistance genes in Staphylococci**.**

Antibiotics	**Target**	**Resistance mechanism**	**Genes determining resistance**
Cell wall synthesis inhibitors			
Betalactams	PBP	Beta-lactamase production	*blaZ*
		Changing the target to PBP2a	*mecA, mecB, mecC*
Glycopeptides	D-Ala-DAla dipeptide	Prevention of transglycosylation and transpeptidation (VRSA)	*vanA*
		Increased cell wall thickness and reduced crosslinking (VISA)	chromosomal gene mutations: *fdh2, sle1, rpoB, rpoC, walK…*
Cell membrane inhibitor			
Cycliclipopeptide	Cell membrane	Changing surface charge	chromosomal gene mutations*: mrpF*
Protein synthesis inhibitors			
Tetracyclines	30S ribosome	Efflux pump	*tet(K), tet(L)*
		Protection of the ribosome	*tet(O), tet(M)*
Aminoglycosides	30S ribosome	Aminoglycoside modifying enzymes	*aacA-aphD, aphA, aadD*
Macrolides	50S ribosome	Methylation of rRNA	*erm, cfr*
		Ribosomal protection	*vga, lsa, sal, msr, optrA*
Lincosamides	50S ribosome	Methylation of rRNA	*erm, cfr*
		Ribosomal protection	*vga, lsa, sal, msr, optrA*
Streptogramin	50S ribosome	Methylation of rRNA	*erm, cfr*
		Ribosomal protection	*vga, lsa, sal, msr, optrA*

Mupirocin	Isoleucyl t-RNA synthetase	Changing of target	*ileS*
		Alternation of target	*mupA*
Fusidic acid	Elongation factor G	Changing of the target	chromosomal gene mutation: f*usA*
		Alternation of target	*fusB, fusC*
Nucleic acid synthesis inhibitors			
Fluoroquinolones	DNA gyrase	Chromosomal mutations changing the target	*gyrA, gyrB*
	Topoismerase IV	Chromosomal mutations changing the target	*grlA, grlB*
		Efflux pumps	norA, norB, norC
Folate synthesis inhibitors	DHPS, DHFR	Changing the target	*dfrA, dfrB, dfrC, dfrD*

1.6.3. Antibiotics acting on cell wall and cell membrane and resistance to them

1.6.3.1. β-lactams

The main inhibitory target for the ß-lactam antibiotics is the transglycolase-transpeptidase PBP2 enzyme (125).

Staphylococci have two main mechanism to overcome ß-lactam antibiotics: one is antibiotic degrading enzyme production, the other is the alteration of the binding site of the ß-lactam antibiotics (126).

In the mid-1940s, shortly after the beginning of penicillin usage in the clinical setting, penicillin-resistant *S. aureus* appeared in hospitals. These isolates produced a penicillinase by the plasmid-encoded *blaZ* gene, that hydrolyzed the beta-lactam ring of penicillins, although cephalosporins could be used against these strains (126)

The clinical introduction of a new, semisynthetic, ß-lactamase resistant antibiotic, the methicillin triggered a novel type of resistance. The first reports of this methicillin resistant *S. aureus* were published in 1961. Although the specific gene, *mecA*, was identified years later, it was suspected early on that the resistance mechanism was different from the penicillinase production and drug inactivation. *MecA* and its newer homologue genes like *mecB* and *mecC* are the methicillin resistance determinants which encode the low affinity penicillin binding protein, PBP 2a (also referred to as PBP 2′). Unlike the penicillinase enzyme mediated resistance, which only inactivates penicillins,

the spectrum of methicillin resistance is broad: resulting in resistance against all ß-lactam antibiotics except the 5th generation cephalopsorins, which were developed specifically against MRSA (125–127).

The *mecA* gene is located on the staphylococcal cassette chromosome *mec*, which is a mobile genetic element. The *SCCmec* element is built from three structural parts: the first is the *mec* gene complex, which contains the *mec* gene accompanied by its transducer and repressor genes. The second element is the *ccr* genes, encoding cassette chromosome recombinases. The last element includes every remaining regions of the cassette and they are called joining regions (J1, J2, J3) (128,129).

Resistance to oxacillin (a ß-lactam antibiotic) in *S. aureus* can be low-level, non-*mec* gene mediated, known as borderline oxacillin-resistant *Staphylococcus aureus* (BORSA), Typically, this borderline phenotype results from the overproduction of ß-lactamase enzyme which partially hydrolyze oxacillin and these strains become fully susceptible to oxacillin in the presence of ß-lactamase inhibitors (130).

The MRSA strains spread around the world first leading to a worldwide pandemic in hospitals and later they adapted to community and livestock, therefore MRSA became an important pathogen (14,126).
S. aureus and MRSA infections are increasingly reported in small animals worldwide. Among the clinical infections, postoperative wound infections are most reported, with fewer occurrences of intravenous catheter site or urinary tract infections, or pneumonia, and skin infections. So far both household and nosocomial infection sources have been documented (131).

Although not all animals who got in contact with *S. aureus* will develop clinical disease. Presumably, most of the pets eliminate the bacteria before infection can happen, and only a small percentage will show symptoms. Colonization means the survival of MRSA/ *S. aureus* at body sites, without any inflammation and immune response to the bacterial presence. Although colonized animals are clinically asymptomatic, as reported in humans, they may be at increased risk for the development of clinical infection. Colonized pets could also serve as a source of infection for other animals and their owners. Colonized household animals have been implicated as bacterial reservoirs when in humans had recurrent MRSA infection or colonization. Presumably humans were the

original source of the bacterium in these cases, and the pets were only carriers and reservoirs after they acquired *S. aureus* from their owners (131).

European hedgehogs are most commonly asymptomatically colonized with *S. aureus*, however, the bacterium was isolated from infections, like septicemia and severe dermatitis, as well (39,42,43,132).

1.6.3.2. Glycopeptides

Glycopeptide antibiotics, vancomycin and teicoplanin are used against Gram-positive bacteria in case of multidrug resistance or allergy to other drugs (133). In the Gram-positive bacteria, vancomycin interferes with the cell wall synthesis. It forms bonds with the D-Ala4-D-Ala5 residues of the newly synthesized pentapeptide side chains of N-acetylmuramic acid (NAM), this way vancomycin-pentapeptide complexes accumulate within the cell thereby disrupting peptidoglycan assembly and synthesis(134).

The increasing number of MRSA infections led to heavy usage of vancomycin, and under this intensive selective pressure new strains emerged: like the vancomycin intermediate *S. aureus* (VISA), which can grow in vitro in the presence of 4 to 8 μg/ml vancomycin concentrations and the vancomcyin-resistant *S. aureus* (VRSA), which are only inhibited at antibiotic concentrations of 16 μg/ml or more (126).

VISA strains have differently regulated cell wall biosynthesis, reduced cross-linking of peptidoglycans and changes to growth characteristics which will lead to increased cell wall thickness. Although the complete molecular basis of the VISA phenotype is unknown, several chromosomal gene mutations are identified as a contribute to the development of VISA through the alteration of cell wall biosynthesis and cell physiology (134).

VRSA strains first have been isolated in the United States in 2002 (134). This high level of vancomycin resistance in Staphylococci is luckily rare because the presence of the *van* gene carrying plasmid significantly reduce the fitness of the bacterium (125). The staphylococcal species acquired the *van* genes from Enterococci species. Six types of glycopeptide resistance genes have been described so far (*vanA, vanB, vanC, vanD,*

vanE, and vanG), but *vanA* is the most frequent in Staphylococci. (133). These *van* genes are located on mobile genetic elements (125). *VanA*-type resistance is characterized by high-level resistance to both vancomycin and teicoplanin. The effect of the *vanA* operon: first leads to the hydrolysis of the dipeptide D-Ala-D-Ala, and then to the synthesis of D-Ala-D-lactate instead, which cannot bind anymore to vancomycin (134).

New semisynthetic lipoglycopeptides, as oritavancin and telavancin cause membrane damage and inhibit the cell wall synthesis. These drugs are bactericidal and work against MSSA, MRSA and VISA strains, as well.

1.6.3.3. Lipopeptide antibiotic

Daptomycin is a cyclic peptide antibiotic, which is active against multi-resistant Gram-positive bacteria, including MRSA. It disrupts cell membrane with depolarization, leakage of ions, leading to permeabilization and cell death. Resistance to daptomycin is uncommon in *S. aureus*, it may develop during prolonged antibiotic therapy, especially with vancomycin. The suspected pathomechanism of resistance is changing the charge of the bacterial surface, leading to repulsion of the drug from the cell surface, preventing the drug reaching the cytoplasmic membrane. Several mutations occur in the chromosomal genes of the bacteria which will cause an increase in the MIC values. Interestingly however, MRSA strains which became resistant to daptomycin paradoxically become sensitive to ß-lactams. Therefore, combinations of ß-lactams and daptomycin act synergistically on MRSA. On the other hand, there is an overlap between the VISA and daptomycin resistant phenotype, because there is an overlap between the mutated gene of interest here (125,135).

1.6.4. Protein synthesis inhibitor antibiotics and resistance to them

1.6.4.1. Antibiotics that inhibit protein synthesis on the 30S subunit of the ribosome

Tetracyclines

Tetracyclines have been widely used for decades. These antibiotics inhibit protein synthesis on the 30S subunit of the bacterial ribosome and prevent the attachment of tRNA to its recognition site. A third generation semisynthetic derivative, a glycylcyline antibiotic, tigecycline can have even a 100- fold higher affinity for its binding site on the 30S subunit so it has enhanced potency compared to tetracyclines (125).

Bacteria may become resistant to tetracyclines and doxycycline by producing tet(K) or Tet(L) efflux pumps encoded on plasmids, whereas tigecycline remains active in efflux pump producing strains (125,136).

Another resistance mechanism is the protection of the ribosome by the GTPase protein produced by the chromosomally coded TetO/M genes. This protein dislodges tetracyclines from the ribosome (137). Tigecycline overcomes resistance based on ribosomal protection, as well. There are only a few reports so far on tigecycline resistance in *S. aureus*. Resistance is thought to be the result of mutation of chromosomal genes, but the exact mechanism is not clear (125).

Aminoglycosides

Aminoglycoside antibiotics are the only ribosome targeting drugs which have bactericidal effect. They cause high rate of misreading during translation, resulting in faulty proteins. The most common resistance mechanism to aminoglycosides is enzymatic modification of the drugs by various enzymes. The most important enzymes in the case of Staphylococcal species are the following: aminoglycoside-acetyltransferase-phosphotransferase (*aacA-aphD*), phosphotransferase (*aphA*), and adenyltransferase (*aadD*). These aminoglycoside-modifying enzymes are encoded on mobile genetic elements, usually plasmids (125,138).

1.6.4.2. Antibiotics that inhibit protein synthesis on the 50S subunit of the ribosome

Macrolides, lincosamides, pleuromutilins, oxazolidinone drugs, chloramphenicol and florfenicol, streptogramins and ketolids prevent peptid bond formation and polypeptid exit and elongation on the 50S subunit of the ribosome (125).

Macrolides, as erythromycin and clarithromycin; and clindamycin are not widely used to treat staphylococcal infections, but may have a role in the therapy of MSSA infections.

Lincosamides are important drugs against MSSA (125). Pleuromutilins were used in veterinary medicine for decades. Recently new derivatives of the class were approved for human use like repamutilin, which is a topical agent to treat skin infections. Oxazolidinones, as linezolid inhibit the binding of tRNA to the ribosome and linezolid is approved to treat nosocomial MRSA infections. Linezolid and quinupristin/dalfopristine combination are the most relevant in combating MRSA Chloramphenicol is only used topically to treat conjunctivitis, florfenicol is used only in veterinary medicine. From streptogramins, quinupristin/dalfopristine combination is used in the treatment of *S. aureu*s and MRSA infections (125).

The most important mechanisms of resistance here act on several different antibiotic group simultaneously: the Erm rRNA methyltransferase changes the target site and confers the MLS_B (macrolide-lincosamide-streptogramin B) resistance, the Cfr methyltransferase determinant codes resistance against $PhLOPS_A$ (phenicol, lincosamide, oxazolidine, pleuromutilin, streptogramin A), the ribosomal protection ABC-F portein domain with the Vga, Lsa, Sal, Msr, OptrA determinants codes resistance against lincosamides, streptograminA, macrolide, pleuromutilin, oxazolidinone, phenicol antibiotics. Furthermore, several other mechanisms exist, like efflux of the drugs, target site modifications and mutations (8,125).

1.6.4.3. Other antibiotics that inhibit protein synthesis

Fusidic acid, which targets the elongation factor G is a commonly used topical drug against *S. aureus* skin infections. When used in monotherapy, resistance is common to

it. *FusA* resistance happens due to spontaneous chromosomal mutations, *FusB* is coded on plasmids while *FusC* is located within SCC elements.

Mupirocin is widely used intranasally, as a topical agent to eradicate colonization of MRSA or locally to treat skin infections. This drug inhibits protein synthesis through binding to isoleucyl t-RNA synthetase (IleRS). Low level mupirocin resistance develops because of mutations in the IleRS target. High-level resistance is transmissible by a plasmid encoded MupA, an alternative IleRS enzyme, to which the drug cannot bind (125).

1.6.5. Nucleic acid synthesis inhibitors

1.6.5.1. Fluoroquinolones

Fluoroquinolones target the DNA gyrase and topoisomerase IV enzymes and their effect lead to double-stranded DNA breaks that cannot be fixed by the bacteria. Fluoroquinolones have an extensive activity against both Gram-negative and Gram-positive bacteria (125).

Resistance to fluoroquinolones quickly emerged in MRSA strains due to the frequent exposure to these antibiotics. Resistance against fluoroquinolones can be the consequence of two main mechanisms: chromosomal mutations in topoisomerase genes that reduce drug binding efficacy and elevate expression of antibiotic efflux pumps. Mutations in the quinolone resistance determining region (QRDR) result in amino acid substitutions at the drug binding sites. In staphylococci the *parC* topoisomerase gene is the most important. Exposure to low levels of fluoroquinolones allow selection of resistant mutants and induce higher mutation rates (125,139).

High-level flouroquinolone resistant isolates often overexpress chromosomally encoded efflux pumps, namely NorA, NorB and NorC. These Nor proteins are members of the major facilitator superfamily (MFS) therefore related to Tet efflux pumps (125,140).

1.6.5.2. Antibiotics inhibiting folate synthesis

Sulphonamides inhibit dihydropteroate synthetase (DHPS) by competing with para-aminobenzoic acid (pABA) and trimethoprim inhibits dihydrofolate reductase (DHFR),

both being essential enzymes in the folate synthesis of prokaryotic bacteria. Sulfamethoxazole is only used in combination with trimethoprim called co-trimoxazole in the treatment of skin and soft tissue infections. Resistance mostly results from mutations in the chromosomal DHFR gene or by horizontal gene transfer of one of the *dfr* gene (s*dfrA, dfrB, dfrC, dfrD*) that encode modified DHFR enzymes that are resistant to inhibition (125).

1.7. Typing methods

Historically bacteriophage typing was used to type isolates of *S. aureus*. However, studies revealed that this typing method had poor reproducibility and failed to type a high percentage (approximately 20%) of isolates (141).

Therefore, laboratories started to use other typing methods to distinguish epidemiologically related strains from unrelated strains and to study the local and global epidemiology of Staphylococci (141).

Pulsed-field gel electrophoresis (PFGE) is also an old method however it has a greater discriminatory power and a more universal typeability. PFGE is a method where the complete bacterial DNA is treated with rare-cutting restriction enzymes and the digested fragments are separated by gel electrophoresis so the banding patterns can be compared. The major disadvantage of PFGE is the difficulty of comparing the results from different laboratories, because this method depends on the comparisons of DNA fragment patterns on gels. For this reason, the genetic relatedness of *S. aureus* clones described by different laboratories with these methods can not be defined and there was a need for typing methods which are more objective and less ambiguous (141,142).

Therefore the multi locus sequence typing (MLST) scheme has been developed for *S. aureus* and *S. pseudintermedius*, which can be performed and evaluated the same way and compared by different laboratories via the Internet (142–144).

MLST is a highly discriminatory method which is based on the sequencing of approximately 450-bp long parts of seven housekeeping genes. For each gene fragment, every different sequence is assigned to a different allele, and each isolate has its own sequence type (ST) based on the allele combinations of the seven housekeeping loci.

Closely related STs that match at four or more loci are grouped into clonal complexes (CCs). As there are many alleles at each locus, it can be assumed that isolates with identical allelic profiles are not randomly identical, but because they are members of the same clone (45,142).

Spa typing can be also used as an accurate and reliable method for *S. aureus* typing. During this method the repeat regions of Staphylococcus protein A gene (*spa*) are sequenced (145). Each repeat is assigned a numerical code, and the spa type is determined through the order of these repeats. To ensure a universal and uniform code terminology, the Ridom StaphType software was developed with an accompanying SpaServer that functions as source for all new spa-repeat and -types codes (146). This method can be used for *S. pseudintermedius* typing as well, however so far there has not been developed a SpaServer to collect these spa types, hence this is not a widespread tool with this species (147,148).

Finally, the *SCCmec* typing method has to be mentioned, which compares the MRSA isolates based on differences in the *SCCmec* mobile genetic element, encoding the *mec* resistance genes and its regulatory genes. So far 13 different *SCCmec* types and several subtypes were described, each labelled by Roman numbers.

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1.8 Clonality and clonal lineages

1.8.1. Clonality of *S. aureus* in humans

In Europe between 1961 and 1999 the most common MRSA types circulating on the continent were the following: ST5-MRSA-I, ST5-MRSA-II (also called the New York-Japan GISA), ST5-MRSA-IV, ST8-MRSA-II, ST8-MRSA-III, ST8-MRSA-IV, ST22-MRSA-IV (EMRSA-15), ST30-MRSA-IV, ST36-MRSA-II, ST45-MRSA-IV (also called Berlin), ST239-MRSA-III (Hungarian/Brazilian clone), ST228-MRSA-I (South-German clone), ST241-MRSA-III, ST247-MRSA-I, ST250-MRSA-I, ST254-MRSA-I, ST254-MRSA-IV (149,150) (Figure 7, 8 and 9).

According to an overview published in 2016 on the topic, the most common types circulating in Europe were ST5, ST8, ST22 HA-MRSA, ST80 CA-MRSA (150).

In the interval between 1999 and 2017 the most prevalent spa types were t032, t008 and t002 in Europe, followed by t044, t003, t067, t018, t004. in frequency. In this study the CC22: ST22, CC8: ST8, ST247, CC5: ST5 were the most detected HA-MRSA MLST types, while ST80 was the most frequent CA-MRSA (151).

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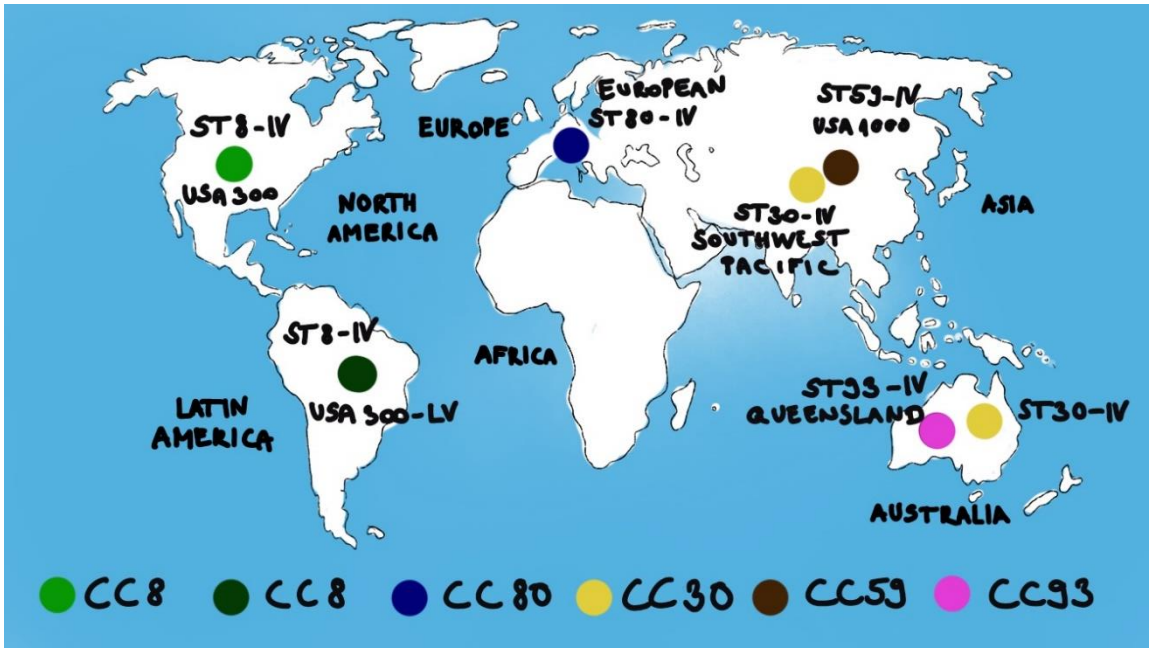

Figure 7. Major HA-MRSA clones in each continent. (150)

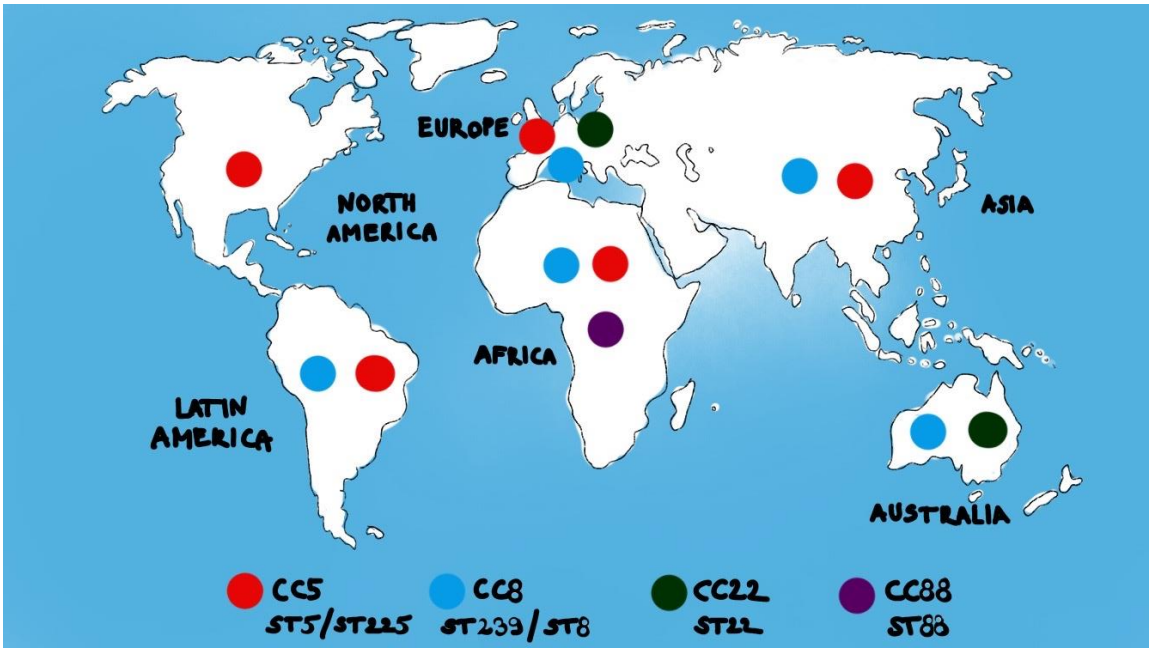

Figure 8. Major CA-MRSA clones in each continent. (150)

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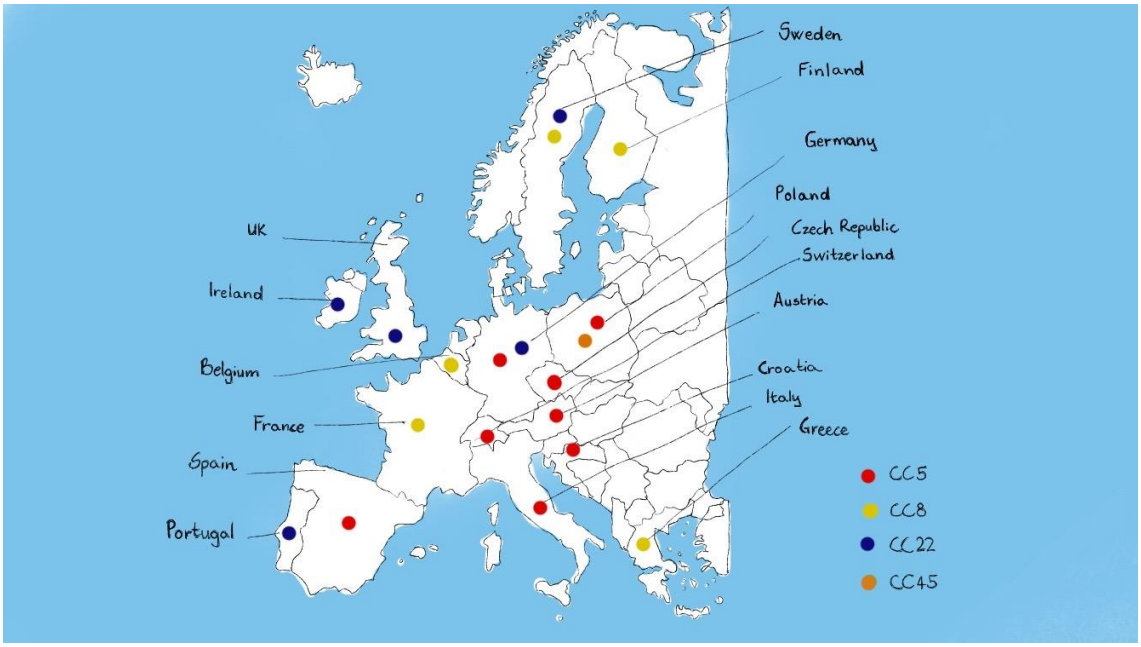

Figure 9. HA-MRSA lineages in Europe. (150)

The *mecC*-MRSA isolates have already been identified from humans and they belong to diverse clonal lineages such as CC130, CC49, CC599 or CC1943 and sequence types ST151, ST425 (152).

MRSA strains from Hungary were studied on isolates collected between 1994 and 1998, and between 2001 and 2004. The ST239-MRSA-III clones was predominant in 1994–1998 which had almost disappeared by 2003–2004, and was replaced by the ST228-MRSA-I (Southern German) clone and the ST5-MRSA-II (New York /Japan) clone, which represented approximately 85% of the 2001–2004 MRSA isolates (153).

In Hungary, the most prevalent HA-MRSA clones from blood stream infection samples were ST22-MRSA-IV and ST5-MRSA-II isolated between 2011-2016 (154).

In a study in 2012, Hungarian children and university students were screened for asymptomatic carriage of *S. aureus* and the MRSA strains isolated from them mostly belonged to the ST45-MRSA-IV and ST7-MRSA-V type (82,155).

1.8.2. Clonality of *S. aureus* in animals

In a Portuguese study clinical pet samples were examined in the time period between 1999-2004 and the isolated MRSA strains belonged to ST22-MRSA-IV, ST105-MRSA-II, ST398-MSA-V and ST5-MRSA-VI (156).

In 2009-2011, veterinary clinical isolates of *S. aureus* were examined in Spain. The only MRSA isolate was t011-ST398-MRSA-V, the MSSA isolates were t045-ST5, t10576-ST1660, and t005-ST22 (112).

According to a 2016 overview, animal clonal types correlate with the ones infecting humans living in the same geographic region. Therefore ST22 was the most frequent in Europe, although ST398 was also a common MLST type (157).

Since the 1960s hedgehogs have been known as carriers of *S. aureus* although we do not know the clonality of these early isolates (40,41,158). From Swedish hedgehogs presented with clinical symptoms (in 2003 and 2001) ST130-MRSA-XI isolates were isolated from lesions and these were identical to previously described human isolates (132). In 2012, a t3256-ST130-MRSA-XI was isolated from an Austrian hedgehog (159). A CC599-MRSA-XI strain was found in a road-killed hedgehog from Thuringia in 2014 (42). In a study carried out between 2012-2015 in Spain, a *mecA*-MRSA isolate was found in a hedgehog and typed as t386-ST1-MRSA-IV (160). In a Swedish screening among hedgehogs in 2015, *mecC*-MRSA isolates were found with the following spa-types: t843, t978, t3391, t9111, t10751 t10893, t11015 and t15312. The MLST typing was not carried out, however, these spa-types are most commonly associated with CC130 and CC2361 (43). In a 2016 study carried out in Denmark, *mecC*-MRSA was found in hedgehogs belonging into two genetic lineages: CC130 (spa-types: t528, t843, t1048, t3256, t3570, t6220, t17133) and CC1943 (spa-types: t978, t2345, t3391, t8835, t16868) (10,39) (Figure 10).

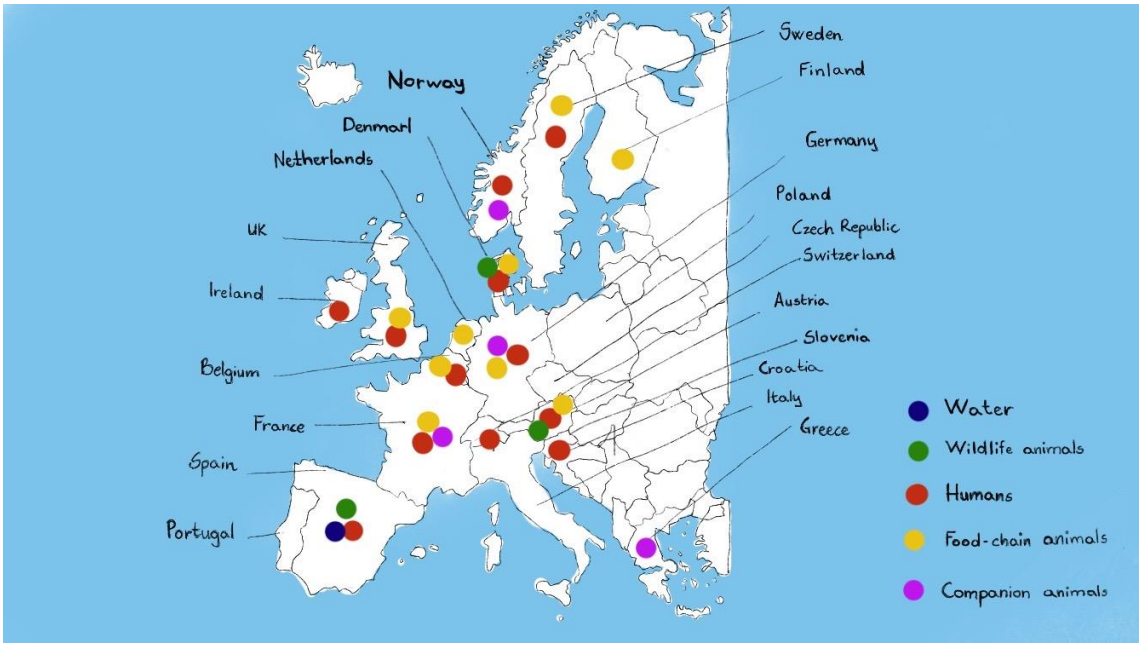

Figure 10. Distribution of *mecC*-MRSA isolates in Europe. (150)

There is no previous data about the circulating *S. aureus* and MRSA clonal types in companion animals or in hedgehogs in Hungary.

1.8.3. Clonality of *S. pseudintermedius* in humans

During a survey for asymptomatic nasal colonization in companion animals and owners in Germany in 2009 one human MRSP isolate was found and that was typed as ST41. The MLST analysis of the remaining human *S. pseudintermedius* isolates revealed a mixed distribution of already known (ST44, ST33) and new (ST119 ST120, ST123) sequence types (30).

In 2012 a German small animal hospital was screened and two MRSP isolates were found among staff members. These MRSP strains had two different spa types, namely t02 and t06 (161).

In 2009-2011, households were screened for CPS in Spain. Three owners were positive for MSSP, but no MRSP was obtained from humans. Concerning these human MSSP isolates, two were ascribed to ST21 and ST100, and one to the novel ST142 (28)

There are no reviews on the clonality of *S. peudintermedius* in humans, however, it can be established that the STs of *S. pseudintermedius* isolates in humans are identical or

closely related to commensals found in dogs. This suggests the zoonotic transmission of the bacteria(162).

1.8.4. Clonality of *S. pseudintermedius* in animals

During a study on MRSP isolates from diseased and healthy dogs between 2004-2009, the most widespread and common lineage in Europe was the t02-ST71-MRSP-II/III (163).

According to a systematic review which analyzed MRSP isolates published in articles between 2007-2015, the CC71 clonal complex (previously described as the epidemic European clone), was found widespread worldwide. In Europe, the isolation of CC258 was more increasingly reported and the previously described epidemic North American clone, CC68, was increasingly reported in Europe as well (164).

In a more limited Portugal study performed during the same period (2007 to 2014), clinical and carriage isolates of MRSP and MSSP were studied. The MRSP isolates belonged to ST71, ST203 (CC71), ST196 (CC196), ST213 (CC196), and ST195 (CC71). ST196 and ST213. The MSSP isolates were typed ST17, ST197, ST198, ST199, ST200, ST201, ST202, ST204, ST205, ST206, ST207, ST208, ST209, ST210, ST211, ST212, ST214, ST215, ST216, ST217, ST379 (96).

In 2009-2011 veterinary clinical isolates of *S. pseudintermedius* were also examined in Spain, here the MRSP isolates belonged to ST71-MRSP-II/III, ST68-MRSP, ST258-MRSP-IV, the MSSP isolates were not typed (112)**.** In a household carriage survey in 2012 in Spain the identified MRSP isolates belonged to ST71-MRSP-II/III and ST92-MRSP-V (28).

Clinical MRSP isolates from 1999-2004 period included ST45-NT, ST71-MRSP-II/III, ST195-MRSP-III, ST196-MRSP-V, ST339-NT, ST342MRSP-IV and the new ST400-MRSP-III (156).

In Slovenia from the period 2008–2018, clinical MRSP isolates were studied from companion animals. Five sequence types were identified, with ST71 and ST551 being the predominant types and one-one isolate belonged to ST25, ST307 and a novel ST1095 (165).

Only few studies from the USA and from the UK provided typing information on canine MSSP isolates. In these studies only three STs (ST15, ST17 and ST19) were documented (36).

There is no previous data about the circulating *S. pseudintermedius* and MRSP clonal types in companion animals in Hungary.

1.9. Whole genome sequencing (WGS)

The genome is the complete genetic material of an organism. It can be a single or double stranded nucleic acid in a linear or in a circular form. To determine this nucleic acid sequence, more efficient technologies have been developed in recent years which are characterized by increased accuracy, throughput and speed. Sequences from microbial species can be aligned and compared to study their genetic and molecular evolution. In the beginning of the sequencing era, available technologies only allowed to focus on relatively small genomes. Now novel platforms, known as Next Generation Sequencing (NGS), and Third Generation Sequencing (TGS) have been developed to work with larger genomes, in a method called Whole Genome Sequencing (WGS) (166).

All these sequencer machines can generate sequences known as reads, which have different ranges of lengths, but normally all shorter than the size of the genomes sequenced. After sequencing the complete genome has to be build up from the overlaps of these fragments, during a process which is called *de novo* genome assembly. After the *de novo* assembly, the genomes can be annotated to understand the function of genes. Finally we can look for certain genes in these annotated and assembled genomes, important virulence and resistance genes, novel gene mutations, single nucleotide polymorphism with the help of internet-based searching tools or manually (166–168).

WGS data can be also used for MLST and *SCCmec* type determination, even with the publicly available servers MLST 2.0 and SCCmecFinder (169,170).

Traditionally spa typing is done by PCR amplification of the target gene region followed by Sanger sequencing. There were efforts to use WGS to assess spa types, but it was found that traditional spa typing had better performance compared to the WGS-

based techniques in this specific occasion. Because of these discrepancies between the two approaches WGS-based spa typing is not recommended. Although it was suggested that the *de novo* assembly should be optimized before the extraction of spa types from WGS and with this approach nearly full compatibility can be reached with the Sanger sequencing-based *spa* typing. Furthermore, the longer read producing sequencers may improve WGS-based *spa* typing, because with these there is a lower chance for misassemble compared to the short Illumina NGS read data (148,171).

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2. OBJECTIVES

The objectives of our study were the following:

- To assess CPS prevalence in dogs and their owners in dog parks in Hungary.
- To conduct a survey in veterinary hospitals in Budapest to establish CPS carriage among veterinarians, owners and their pets.
- To examine the presence of staphylococci in wildlife in the country and explore the Hungarian hedgehog population as a possible reservoir for *mecC*-MRSA strains.
- To compare human and animal strains based on their resistance and virulence genes.
- To gain further information on transmission dynamics between humans and animals.

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3. MATERIALS AND METHODS

3.1. Sample collection

3.1.1. Dogs and their owners in dog parks

In total we have screened 102 dogs and 84 pet owners in Budapest (61%) and 14 other towns (39%) in Hungary (Figure 11) in 2015 to assess the prevalence of CPS at dog parks. Only healthy pet owners and their dogs were sampled. One specimen was collected with the same swab from both nostrils of the humans, whereas animals were sampled at their nose, mouth and skin of the head with individual sterile swabs. All samples were stored in transport media (Sigma Transwab Stuart's Charcoal, Medical Wire & Equipment, Corsham, UK) and transported to the laboratory (172).

Figure 11. Towns where dog park samples were collected in Hungary (2015).

3.1.2. Dogs, owners and veterinarians in veterinary hospitals

For the prevalence of CPS at veterinary hospitals 101 humans (72 owners and 29 veterinarians) and 81 animals (65 dogs, 13 cats, and one dwarf hamster, a rabbit and

parrot each) were screened between October 2016 and March 2017 at nine different animal hospitals, eight in Budapest and one near Budapest (Figure 12). Samples were taken from the skin of the lower arm and from both nostrils of the owners and veterinary staff, and from the nose and perianal area in pets with individual sterile swabs. All samples were carried into the research facility in transport culture media (Sigma TSB with 6.5% NaCl, Medical Wire & Equipment, Corsham, UK).

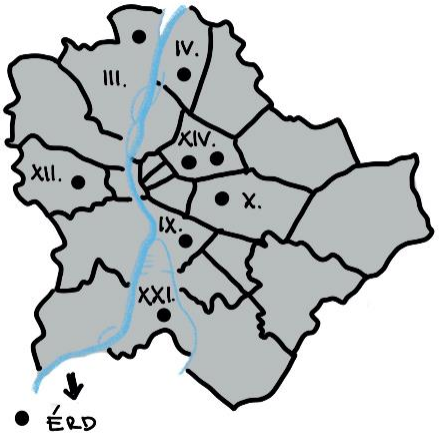

Figure 12. Location of the sampled veterinary hospitals in the Budapest area (2016-2017).

3.1.3. Hedgehogs

Between February and June 2020, 200 hedgehogs were sampled for *S. aureus* carriage. 125 of them were alive and screened in the Budapest region following their admission to the Wildlife Rescue Center at the Budapest Zoo and Botanical Garden. 75 carcasses were also sampled. These dead hedgehogs were either road kills found in Budapest and its suburbs or from wildlife centers where they succumbed to their injuries. *Post-mortem* microbiological sampling was done at the Department of Parasitology and Zoology, University of Veterinary Medicine, Budapest.

Samples were collected from the following sites: one mini-tip swab from both nostrils transported in liquid Amies transport medium (Sigma Transwab ENT, Medical Wire & Equipment, Corsham, UK), and another taken with a traditional swab from the skin between the spikes and toes (Sigma Transwab Stuart's Charcoal, Medical Wire & Equipment, Corsham, UK) (173).

The study and its methodology were approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (reference numbers: SE RKEB 180/2020, SE RKEB 181/2020).

3.2. Cultivation, identification, and antibiotic susceptibility testing

All samples belonging to these studies were transported within 48 hours to the Institute of Medical Microbiology, Semmelweis University, Budapest for cultivation and further analysis.

Swab samples were streaked onto blood agar plates (5% of sheep blood added) and incubated overnight at 37°C in 5% CO_2. CPS isolates were identified based on their colony morphology (gold or white pigmented colonies with strong ß-hemolytic zones). Suspicious colonies were further investigated by catalase and coagulase latex agglutination test (Pastorex Staph-Plus Kit, Bio-Rad, Marnes-la-Coquette, France) and subcultured for PCR based species identification .

3.2.1. Identification of *S. aureus*

The catalase and coagulase-positive isolates were assumed to be *S. aureus* and a PCR was developed at our laboratory which can be used to detect simultaneously the *S. aureus* species-specific thermonuclease *nucA* gene, and the *mecA*, the most common methicillin resistance gene (174). For the detection of the rare *mecC* gene, we used the primers from the manuscript of Paterson et al. (2012) (175) with modified PCR conditions. As positive controls ATCC 33591 (*mecA*+) and ATCC BAA-2312 (*mecC*+) strains were used (Table 4)

3.2.2. Identification of *S. pseudintermedius*

The catalase positive and Pastorex negative ß-hemolytic, white pigment producer isolates were suspected to be *S. pseudintermedius*. At the beginning of the study, for the dog park samples, *pta* PCR-RFLP was used to differentiate *S. intermedius* and *S. pseudintermedius*: the PCR product of the *pta* gene in *S. pseudintermedius* could be digested by the *MboI* enzyme, whereas the *pta* gene of *S. intermedius* did not have a *MboI* recognition site. Therefore after the digestion the *pta* gene of *S. pseudintermedius* ended up in two fragments, while the gene of *S. intermedius* had only one product (99). Later, for the veterinary hospital samples we developed an in-house multiplex PCR to differentiate between *S. intermedius* and *S. pseudintermedius* based on the work of

Sasaki et al 2010 (176). ATCC29663 (*S. intermedius)* and Q81 *(S. pseudintermedius)* strains were used as positive controls (Table 4).

Table 4. Primers used for the identification of *S.aureus, S. pseudintermedius, S. intermedius* and methicillin resistance.

gene	primer sequnece (5' -3')	amplicon size (bp)	source	positive control
SA nucA for	ATGGACGTGGCTTAGCGTAT	193	Laub et al 2011[a]	ATCC 33591
SA nucA rev	TGACCTGAATCAGCGTTGTC			
mecA for	CCCAATTTGTCTGCCAGTTT	538	Laub et al 2011[a]	ATCC 33591
mecA rev	ATCTTGGGGTGGTTACAACG			
mecC for	CATTAAAATCAGAGCGAGGC	188	Paterson et al 2012 [b]	ATCC BAA-2312
mecC rev	TGGCTGAACCCATTT TTGAT			
pta for	AAAGACAAACTTTCAGGTAA	320	Bannoehr et al. 2009[c]	ATCC 29663, Q81[e]
pta rev	GCATAAACAAGCATTGTACCG			
SP nucA for	GTTGCGTAATGAACCCACCT	966	this work[d]	Q81
SP nucA rev	TTCAAGCGCTCATTGATACG			
SI nucA for	AAAAGGGGGAGTTGCAAAGT	731	this work[d]	ATCC 29663
*SI nuc*A rev	TTGGCTCACGGTCATATTCA			

[a] The cycling parameters were as follows: 3 minutes at 94ºC, then 30× 94ºC 60 s, 54ºC 60 s, 72ºC 30 s, and finally an extension of 72ºC for 10 minutes

[b] The cycling parameters were as follows: 3 minutes at 94°C, then 30x 94°C 60 s, 57°C 60 s, 72°C 30 s, and finally an extension of 72°C for 10 minutes

[c] The cycling parameters were as follows: 2 minutes at 95°C, then 30x cycles of 95°C 60s, 53°C 60s, 72°C 60s, and finally an extention of 72°C for 7 minutes

[d] The cycling parameters were as follows: 2 minutes at 95°C, then 30x 95°C 30s, 56°C 35s, 72°C 60s, and finally an extension of 72°C for 2 minutes

[e] Q81 is a *S. pseudintermedius* specimen collected during the dog park screening study. It was whole genome sequnced and in default of ATCC or National Center for Epidemiology control strains we used this as an in-house positive control.

3.2.3. Antibiotic susceptibility testing

For the dog park samples antibiotic sensitivity to penicillin, erythromycin, clindamycin, tetracycline, gentamicin, ciprofloxacin, mupirocin, oxacillin, was determined by agar dilution, while disc diffusion was used in case of cefoxitin. Inducible resistance to clindamycin was performed by D-test with the strains where it was necessary. In

addition to these drugs the following antibiotics were tested for the veterinary hospital samples: levofloxacin, sulfamethoxazole-trimethoprim, vancomycin by dilution method.

For the hedgehog samples MIC test strips (Liofilchem®, Roseto degli Abruzzi, Teramo, Italy) were used to perform an E test to determine the antibiotic susceptibility to penicillin, erythromycin, clindamycin, gentamicin, ciprofloxacin, tetracycline and mupirocin. Disk diffusion was used to test the susceptibility to cefoxitin and microdilution was performed with vancomycin, respectively .

CPS strains were streaked onto cation-adjusted Mueller-Hinton plates to have a bacterial lawn and the EUCAST breakpoints were used for interpretation during each study (177). As a quality control strain for the MIC determinations *S. aureus* ATCC 29213 was included and tested.

3.3. Detection of resistance and virulence genes with PCR methods

The presence of the *tetK*, *tetM*, *ermA*, *ermB*, *vanA*, *mupA*, (133,178–181) resistance genes were examined only in the veterinary CPS isolates (Table 5).

Table 5. Primers used for the identification of antibiotic resistance genes in CPS isolates.

gene	primer sequnece (5' -3')	amplicon size (bp)	source	positive control
tetK for	GTAGCGACAATAGGTAATAGT	360	Strommenger et al. 2003[a]	Q37[e]
tetK rev	GTAGTGACAATAAACCTCCTA			
tetM for	AGTGGAGCGATTACAGAA	158	Strommenger et al. 2003[a]	-
tetM rev	CATATGTCCTGGCGTGTCTA			
ermA for	AAGCGGTAAACCCCTCTGA	190	Strommenger et al. 2003[a]	-
ermA rev	TTCGCAAATCCCTTCTCAAC			
aacA-aphD for	TAATCCAAGAGCAATAAGGGC	228	Strommenger et al. 2003[a]	-
aacA-aphD rev	GCCACACTATCATAACCACTA			
ermB for	GAAAARGTACTCAACCAAATA	639	Sutcliffe et al. 1996[b]	-
ermB rev	TTCGCAAATCCCTTCTCAAC			
vanA for	GGGAAAACGACAATTGC	732	Depardieu et al. 2004[c]	ATCC 51559
vanA rev	GTACAATGCGGCCGTTA			
mupA for	TATATTATGCGATGGAAGGTTGG	458	Anthony et al. 1999[d]	-
mupA rev	AATAAAATCAGCTGGAAAGTGTTG			

[a] The cycling parameters were as follows: 3 minutes at 94ºC, then 30× 94ºC 30 s, 55ºC 30 s, 72ºC 30 s, and finally an extension of 72ºC for 4 minutes.

[b]The cycling parameters were as follows: 3 minutes at 93ºC, then 35× 93ºC 60 s, 52ºC 60 s, 72ºC 60 s, and finally an extension of 72ºC for 5 minutes.

[c]The cycling parameters were as follows: 3 minutes at 94ºC, then 30× 94ºC 60 s, 54ºC 60 s, 72ºC 60 s, and finally an extension of 72ºC for 7 minutes.

[d] The cycling parameters were as follows: 3 minutes at 95ºC, then 35× 95ºC 30 s, 57ºC 60 s, 72ºC 30 s, and finally an extension of 72ºC for 5 minutes.

[e] Q37 is a *S. aureus* specimen collected during the dog park screening study. It was whole genome sequnced and in the ansence of ATCC or National Center for Epidemiology control strains we used this as an in-house positive control.

In the dog park isolates we tested the presence of the following *S. aureus* toxin genes: *tsst, sea, seb, sec, eta, etb,* (154,182–184), and the *S. pseudinteremdius* enterotoxin gene *siet* (185).

The *icaD, cna, fnbA, fnbB* genes are responsible for adhesion and biofilm formation and the *pvl, tsst, sea, seb, sec, eta, etb, hla, hlb, hlg, hlg-v* genes are responsible for toxin production in *S. aureus*. These genes were examined in all veterinary *S. aureus* isolates (154,182–184,186–191). The virulence genes, *icaD, siet, lukF-I, lukS-I, hld, psmε* were examined in all veterinary *S. pseudintermedius* by PCR (101,185,192,193) (Table 6).

The presence of the adhesion genes *cna, icaD, fnbA* and *fnbB* was investigated in all hedgehog related *S. aureus* isolates by PCR (186,187). The presence of the toxin genes *sea, seb, sec, tsst, eta, etb* and *pvl* was also examined by PCR in each hedgehog isolate (154,182–184,188) (Table 6).

Table 6. Primers used for the detection of adherence and toxin genes in CPS isolates.

***S. aureus* adherence genes**				
gene	**primer sequnece (5' -3')**	**amplicon size (bp)**	**source**	**positive control**
cna for	TTCACAAGCTTGGTATCAAGAGCATGG	452	Campbell et al. 2008[a]	ATCC 25923
cna rev	GAGTGCCTTCCCAAACCTTTTGAGC			
icaD for	ACCCAACGCTAAAATCATCG	211	Atshan et al. 2013[c]	Q37[b]
icaD rev	GCGAAAATGCCCATAGTTTC			
fnbA for	AAATTGGGAGCAGCATCAGT	120	Atshan et al. 2013[c]	Q37
fnbA rev[d]	AGCTGAATTCCCATTTTCTTC			
fnbB for	ACGCTCAAGGCGACGGCAAAG	197	Atshan et al.	Q37

fnbB rev	ACCTTCTGCATGACCTTCTGCACCT		2013[c]	
***S. aureus* toxin genes**				
gene	**primer sequnece (5' -3')**	**amplincon size (bp)**	**source**	**positive control**
eta for	AAAAACCATGCAAAAGCAGAA	372	Horváth et al. 2020[e]	NCE[f]-13-50001-TX
eta rev	ACCTGCACCAAATGGTTCTT			
etb for	CAGCGCAGAAGAAATCAGAA	609	Horváth et al. 2020[e]	NCE-13-50001-TX
etb rev	CCGCCTTTACCACTGTGAAT			
sea for	TTATCAATGTGCGGGTGGTA	265	Horváth et al. 2020 [e]	NCE-08-50024-TX
sea rev	CCTCTGAACCTTCCCATCAA			
seb for	GTATGGTGGTGTAACTGAGC	164	Mehrotra et al. 2000[g]	NCE-08-50044-TX
seb rev	CCAAATAGTGACGAGTTAGG			
sec for	CTCAAGAACTAGACATAAAAGCTAGG	271	Becker et al. 1998[g]	NCE-08-50036-TX
sec rev	TCAAAATCGGATTAACATTATCC			
tsst for	GCTTGCGACAACTGCTACAG	560	Monday et al. 1999 [e]	NCE-15-50026-TX
tsst rev	TGGATCCGTCATTCATTGTTAA			
pvl for	CAGGAGGTAATGGTTCATTT	151	Al-Talib et al. 2009 [h]	ATCC 25923
pvl rev	ATGTCCAGACATTTTACCTAA			
hla for	AAA GGT ACC ATT GCT GGTC	600	Aarestrup et al. 1999[i]	ATCC 19095
hla rev	CAA TTG GTA ATC ATC ACG AAC			
hlb for	TGTGGATTCGATAATGATAGC	500	Aarestrup et al. 1999 [i]	ATCC 25178
hlb rev	ACGTAGTAATATGGGAACGCT			
hlg for	GCCAATCCGTTATTAGAAAATGC	937	Lina et al. 1999 [j]	CCUG 26215
hlg rev	CCATAGACGTAGCAACGGAT			
hlg-v for	GACATAGAGTCCATAATGCATTYGT	370	Jarraud et al. 2002 [k]	CCUG 25926
hlg-v rev	ATAGTCATTAGGATTAGGTTTCACAAAG			
***S. pseudintermedius* adherence genes**				
gene	**primer sequnece (5' -3')**	**amplicon size (bp)**	**source**	**positive control**
icaD for	CGTTAATGCCTTCTTTCTTATTGCG	166	Casagrande Proietti et al. 2015[l]	Q81[m]
icaD rev	ATTAGCGCACATTCGGTGTT			
***S. pseudintermedius* toxin genes**				
gene	**primer sequnece (5' -3')**	**amplincon size (bp)**	**source**	**positive control**
siet for	ATGGAAAATTTAGCGGCATCTGG	359	Lautz et al. 2006[n]	Q81
siet rev	CCATTACTTTTCGCTTGTTGTGC			
lukF-I for	CCTGTCTATGCCGCTAATCAA	573	Futagawa-Saito et al. 2004[o]	Q81
lukF-I rev*	AGGTCATGGAAGCTATCTCGA			
lukS-I for*	TGAGGATGTAAGCAGCAGAA	509	Futagawa-Saito et al. 2004[o]	Q81
lukS-I rev	GCCCGATAGGACTTCTTACAA			
hld for	CTTAGTCATTGTATTCTTCGCTCA	251	Maali et al. 2018[p]	Q81
hld rev*	GCTTCAATATCGAAACCTGATT			

psmε for	ATTTCGGCATGCACAACTG	230	Maali et al. 2018[p]	Q81
psmε rev	GCCTCAAACTCAGTCAAACGA			

[a] The cycling parameters were as follows: 15 minutes at 95°C, then 32x 95°C 60s, 60°C 90s, 72°C 60s, and finally an extension of 72°C for 10 minutes.

[b] Q37 is a *S. aureus* specimen collected during the dog park screeening study. It was whole genome sequnced and contains icaA, icaD, fnbA, fnbB, in the lack of ATCC or National Center for Epidemiology control strains we used this as an in-house positive control.

[c] The cycling parameters were as follows: 5 minutes at 95°C, then 40x 95°C 20s, 60°C 20s, 72°C 20s, and finally an extension of 72°C for 5 minutes.

[d] The reverese primer's sequnece was modified by our research group.

[e] The cycling parameters were as follows: 4 minutes at 94°C, then 35x 94°C 60s, 54°C 45s, 72°C 50s and finally an extesion of 72°C for 10 minutes.

[f] NCE, National Center for Epidemiology

[g] The cycling parameters were as follows: 4 minutes at 94°C, then 35x 94°C 60s, 51°C 45s, 72°C 50s and finally an extesion of 72°C for 10 minutes.

[h] The cycling parameters were as follows: 3 minutes at 94°C, 30x 94°C 30 s, 60°C 30s, 72°C 30s, followed by an extra cycle of annealing at 60°C for 30 s, and a final extension at 72°C for 5 minutes.

[i] The cycling parameters were as follows: 4 minutes at 94°C, then 35x 94°C 60s, 51°C 45s, 72°C 50s and finally an extesion of 72°C for 10 minutes.

[j] The cycling parameters were as follows: 4 minutes at 94°C, then 35x 94°C 60s, 54°C 45s, 72°C 50s and finally an extesion of 72°C for 10 minutes.

[k] The cycling parameters were as follows: 4 minutes at 94°C, then 35x 94°C 60s, 53°C 45s, 72°C 50s and finally an extesion of 72°C for 10 minutes.

[l] The cycling parameters were as follows: 5 minutes at 94°C, then 35 × 94°C 60s, 57°C 60s, 72°C 60s and finally an extension of 72°C for 7 minutes.

[m] Q81 is a *S. pseudintermedius* specimen collected during the dog park screening study. It was whole genome sequnced and in the lack of ATCC or National Center for Epidemiology control strains we used this as an in-house positive control.

[n] The cycling parameters were as follows: 3 minutes at 94°C, then 30x 94°C 30s, 56°C 30s, 72°C 60s and finally an estension of 72°C for 5 minutes.

[o] The cycling parameters were as follows: 5 minutes at 94°C, then 35x 94°C 60s, 57°C 60s, 72°C 60s and finally an extension of 72°C for 7 minutes.

[p] The cycling parameters were as follows: 5 minutes at 94°C, 25x 94°C 30s, 58°C 30s, 72°C 60s and finally an extension of 72°C for 5 minutes.

*The primer was designed by our research group.

3.4. Genotyping

Pulsed-field gel electrophoresis was used with *SmaI* enzyme digestion to define the genetic relatedness of the CPS strains. A previously published method was used (174,194). Cluster analysis and dendograms were executed with the Fingerprinting II software (Bio-Rad, Marnes-la-Coquette, France).

Selected *S. aureus* isolates were shipped to the Biomi Ltd. laboratory (Gödöllő, Hungary) for *spa*-typing by Sanger sequencing. When new repeat combinations were detected, they were submitted to the Ridom Spa Server (https://spa.ridom.de/submission.shtml).

Selected *S. aureus* and *S. pseudintermedius* strains were genotyped with multi-locus sequence typing following the PubMLST protocol (142,143) and sequence types were defined based on the MLST online database (144). *SCCmec* typing of the *mecA*-MRSA isolates were performed by PCR as described previously (154,195,196) (Table 7).

Table 7. Primers used for *SCCmec* typing.

primer number	*SCCmec* type	primer name	primer sequnece (5' -3')	amplicon size (bp)	source	positive control
1	I	CIF2 F2	TTCGAGTTGCTGATGAAGAAGG	495	Oliveira et al. 2002 [a]	in-house [b]
		CIF2R2	ATTTACCACAAGGACTACCAGC			
2	II	kdp F1	AATCATCTGCCATTGGTGATGC	284	Oliveira et al. 2002 [a]	in-house [b]
		kdp R1	CGAATGAAGTGAAAGAAAGTGG			
3	III J1 region	III J1 F	CATTTGTGAAACACAGTACG	243	Milheirico et al. 2007 [a]	NA [c]
		III J1 R	GTTATTGAGACTCCTAAAGC			
4	II and IV ccr complex	ccrB2 F2	ATTTTCTCAGAATTCGAACG	311	Milheirico et al. 2007 [a]	in-house [b]
		ccrB2 R2	CCGATATAGAAWGGGTTAGC			
5	I, II, IV and VI J3 region	dcs F1	CATCCTATGATAGCTTGGTC	342	Milheirico et al. 2007 [a]	in-house [b]
		dcs R1	CTAAATCATAGCCATGACCG			
6	V J1 region	V J1 F	TTCTCCATTCTTGTTCATCC	377	Milheirico et al. 2007 [a]	in-house [b]
		V J1 R	AGAGACTACTGACTTAAGTGG			

[a] The cycling parameters were as follows: 3 minutes at 94°C, then 35x 94°C 45s, 53°C 45s, 72°C 60s, and finally an extension of 72°C for 5 minutes

[b] positive samples from previous study was used as positive control Horváth et al. 2020

[c] not available

3.5. Whole genome sequencing and data analysis

3.5.1. Whole genome sequencing of the dog park isolates

From the dog park samples two *S. aureus* (Q37, Q38) and two *S. pseudintermedius* (Q81, Q82) isolates derived from owner and dog co-carriage, and two other *S. aureus* isolates (which shared the same PFGE pattern (Q47, Q85)) were whole genome sequenced at the Institute for Veterinary Medical Research, Centre for Agricultural Research, Budapest, Hungary.

Nextera XT kit and Nextera XT Index Kit v2 Set A (Illumina, San Diego, CA, USA) were used for the DNA library preparation. The genome sequencing itself was performed on Illumina NextSeq 500 equipment (Illumina, Inc. San Diego, CA USA) using NextSeq 500/550 Mid-Output Kit, resulting in paired end reads.

The de-novo assembly of the raw reads was performed by the SPAdes software (http://cab.spbu.ru/software/spades/), the annotation of the de-novo draft genome was performed by RAST (https://rast.nmpdr.org/) (197). MAUVE (http://darlinglab.org/mauve/mauve.html) was used to rearrange and align the annotated scaffolds.

The assembled genome sequences were uploaded to NCBI GenBank, under the following accession numbers: Q82: JAEEAO000000000, Q81: JAEDAV000000000, Q85: JAEDAU000000000, Q47: JAEDAT000000000, Q38: JAEDAS000000000, Q37: JAEDAR000000000 (172).

3.5.2. Whole genome sequencing of a hedgehog isolate

The *mecC*-MRSA isolated from the hedgehog survey was sequenced at Biomi Ltd., Gödöllő, Hungary. This genome sequencing was performed on an Illumina Miseq (Illumina, Inc. San Diego, CA USA) equipment.

The genome coverage was 162x. The de-novo assembly was done with the Velvet Assembler software (https://github.com/dzerbino/velvet/tree/master), the annotation with RAST (https://rast.nmpdr.org/) (197). The raw Illumina paired end read was submitted to the NCBI SRA database under the BioProject accession number

PRJNA690008. The assembled genome sequence was introduced into the NCBI GenBank, under the following accession number: JAEQMS000000000 (173).

3.5.3. Data analysis

The identification of resistance genes was accomplished with CARD (https://card.mcmaster.ca/) and ResFinder 2.3 (https://cge.cbs.dtu.dk/services/ResFinder/) (using the default settings). MLST 2.0 was used (https://cge.cbs.dtu.dk/services/MLST/) for WGS based MLST typing. The alleles of the examined housekeeping genes of the isolates were compared with the PubMLST database (https://pubmlst.org/). The *SCCmec* type of the sequenced isolates was identified by the SCCmecFinder 1.2 (https://cge.cbs.dtu.dk/services/SCCmecFinder/) Virulence and toxin genes were recognized by VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/) (using the default settings) and by VFanalyzer (http://www.mgc.ac.cn/cgi-bin/VFs/v5/main.cgi). Genomic islands were recognized by using Geneious 11.1 (Biomatters Ltd, New Zealand) (172,173).

When used, the statistical analyses were done with the Fischer exact test. (https://www.medcalc.org/calc/fisher.php).

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4. RESULTS

4.1. Carriage rate

4.1.1. CPS carriage rates in the dog park study

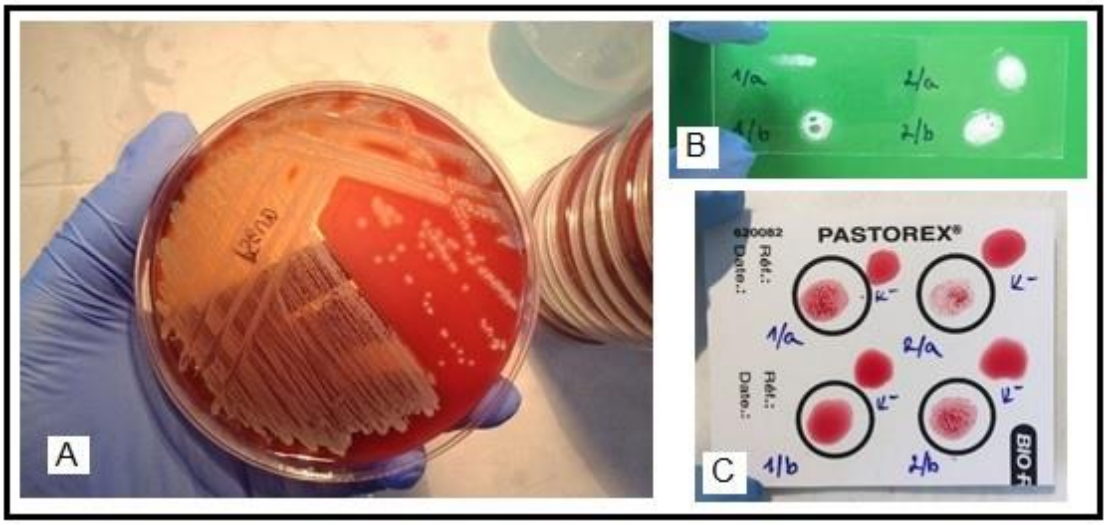

Figure 13. Cultivation and identification of CPS isolates. A) S. aureus culture on sheep blood agar plate B) Catalase test C) Latex agglutination test (Pastorex)

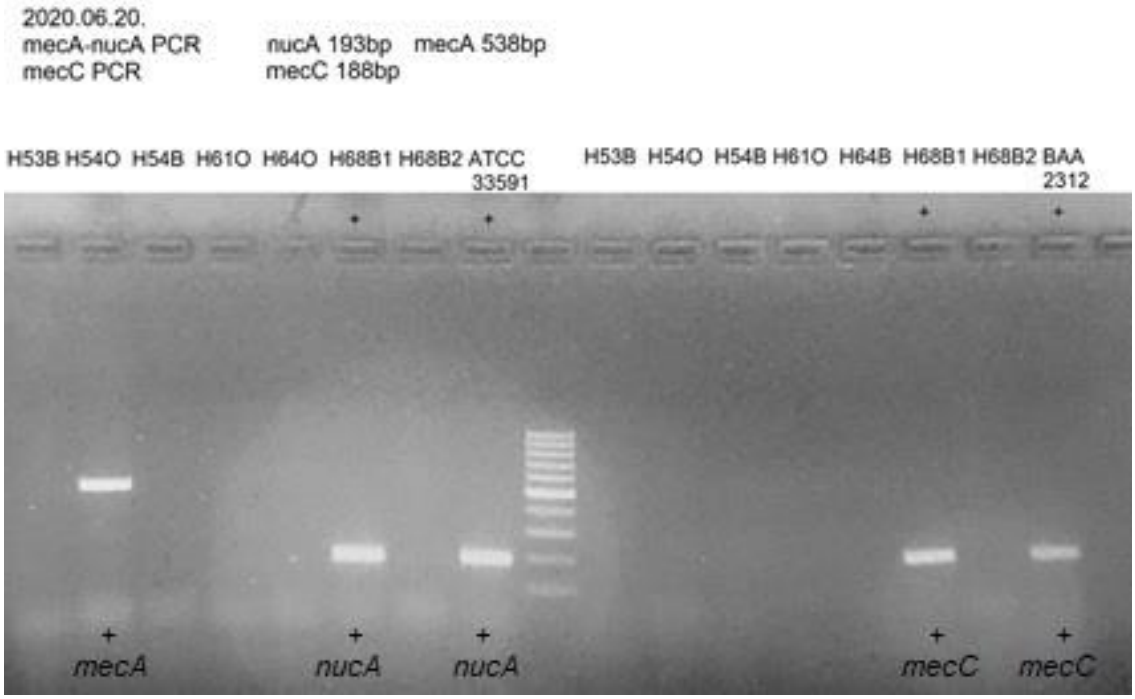

Figure 14. Results of the *nucA*, *mecA*, *mecC* PCR reactions on agar gel. The molecular weight marker ranges from 100 bp to 1000 bp (Thermo Scientific GeneRuler 100 bp DNA Ladder)

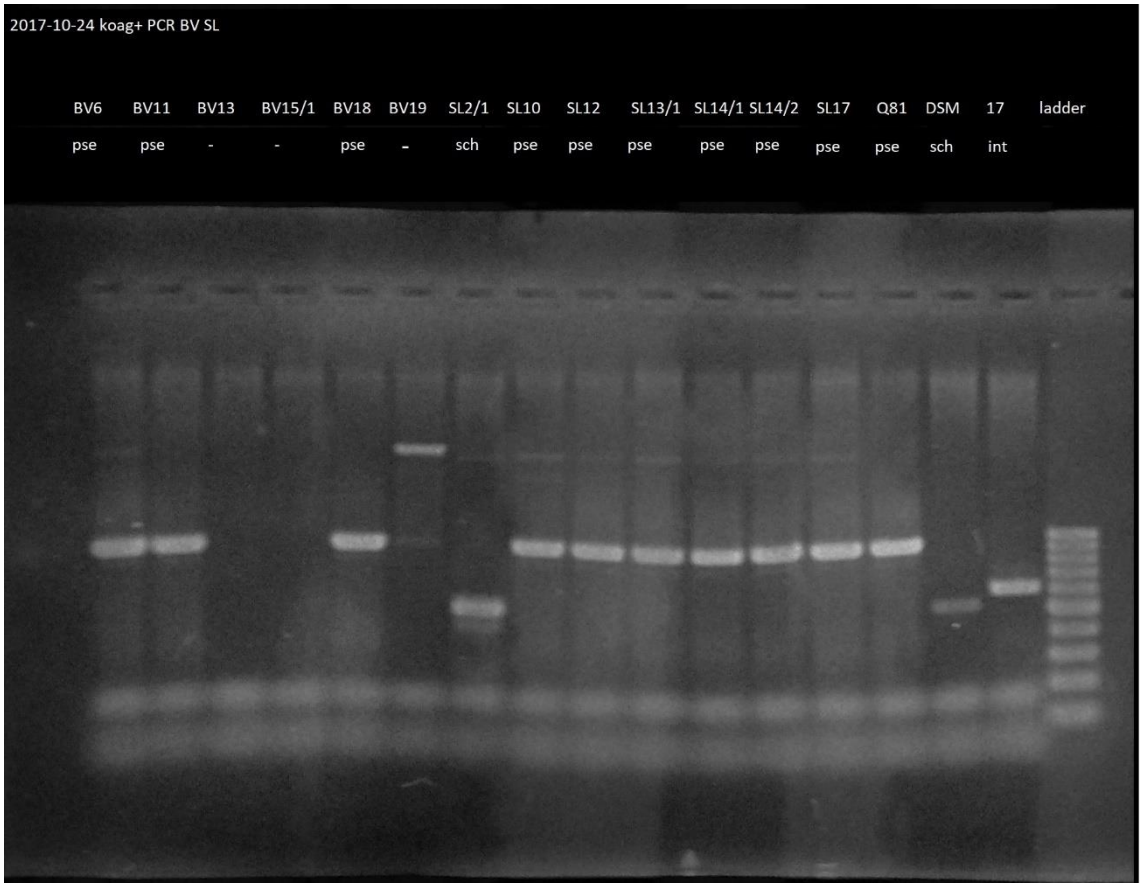

Figure 15. The result of the multiplex PCR used to differentiate between *S. pseudintermedius* and *S. intermedius* based on the species specific *nucA* gene amplification. The *nucA* of *S. pseudintermedius* has a product of 966bp, while the *nucA* of *S. intermedius* has a product of 731bp. The molecular weight marker ranges from 100bp to 1000bp. (Thermo Scientific Gene Ruler 100bpDNA Ladder)

The carriage rate of *S. aureus* was 23.8% (20/84) among humans and 4.9% (5/102) in dogs. While the *S. pseudintermedius* carriage showed an inverse pattern with 2.4% (2/84) prevalence in humans and 34.3% (35/102) in dogs (Figure 13-15). Only two cases (samples: Q37-Q38, Q84-Q85) were found where dogs and their owners both carried *S. aureus* and one single co-carriage was identified with *S. pseudintermedius* (sample Q81 and Q82).

No *S. intermedius* was found, but 27 *S. aureus* and 58 *S. pseudintermedius* strains were identified. In humans only the nose was used for the screening, whereas in dogs three different body sites were sampled. The distribution of the sites where CPSs were isolated from dogs was the following: *S. pseudintermedius* were mainly detected from the nose (23/56) and the mouth (23/56), the least number of isolates were found on the skin of the head (10/56). *S. aureus* was not isolated from the skin at all, only from the

nasal (4/7) and oral (3/7) specimens (Figure 16). Only one isolate per host was included in the final study although the same bacterial species could be isolated from two or three different sampling sites of the same dog in 18 cases. These strains were treated as duplicates, as they had similar types of virulence genes and identical PFGE patterns Consequently the final number of strains examined in this study was n=25 *S. aureus* and n=37 *S. pseudintermedius* (172).

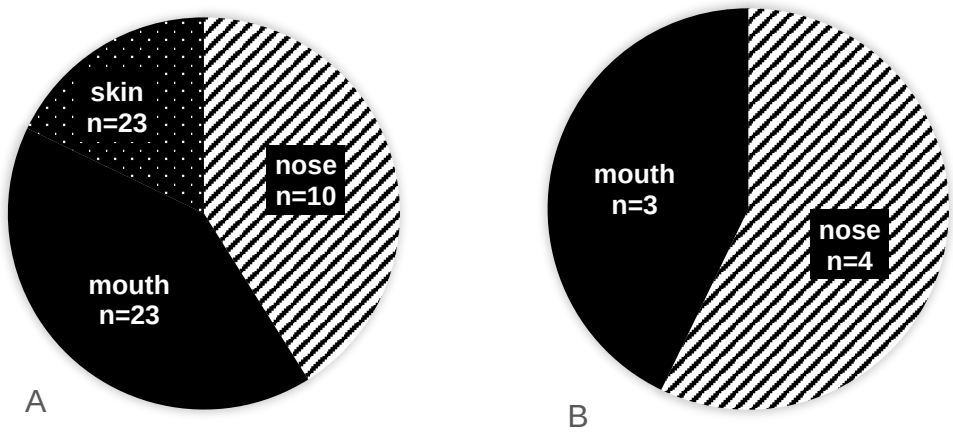

Figure 16. The distribution of CPS isolates collected from different body sites from dogs. A) *S. pseudintermedius* distribution B) *S. aureus* distribution.

4.1.2. CPS carriage rates in the veterinary hospital study

S. aureus carriage rate was 14.8% (12/81) in pets, and 34.6% (35/101) in humans. However human carriers could be divided into two sub-groups: pet owners 33.3% (24/72) and veterinary staff 37.9% (11/29). MRSA was only detected from pet samples, hence the prevalence was 1.2%, because only one dog isolate (CS20/1) proved to be MRSA (1/81). Six cases of *S. aureus* co-carriage were found between pets and their owners.

Altogether 51 *S. aureus* isolates were detected. In pets the isolates derived from the nose (7/13) and from the skin of the perineum (6/13) almost in the same proportion. The distribution of the human samples was the following: *S. aureus* was detected in larger proportions from the nose (29/38) compared to the skin (9/38) (Figure 17). In four cases (three humans and one cat) *S. aureus* was isolated from more than one sampling site.

After further investigations we concluded that these strains are duplicates therefore only one of the duplicates were included in the final study, where 47 isolates were used.

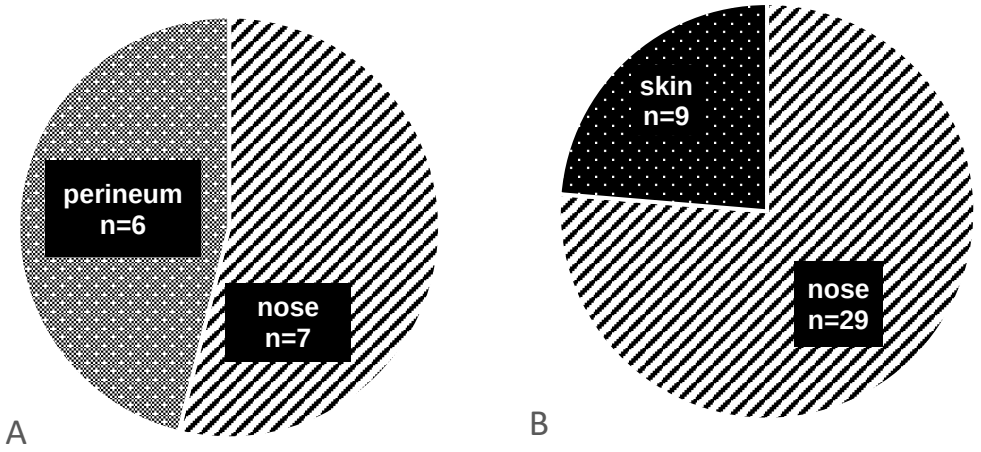

Figure 17. Distribution of *S. aureus* isolates from different sampling sites. A) Distribution of *S. aureus* in companion animals B) Distribution of *S. aureus* in humans.

S. *pseudintermedius* carriage rate was 58.0% (47/81) in pets, and 19.8% (20/101) in humans. Here the human carriers could also be divided into the same sub-groups as in the case of *S. aureus*. The carriage rate in the owner group was 15.3% (11/72), in the veterinarian group 31.0% (9/29). In total six MRSPs were isolated, three from humans and three from pets, hence the prevalence in animals was 3.7% (3/81), in humans 2.9% (3/101). From the human isolates one originated from a nasal sample of a veterinarian and two from nose samples of owners. All three of the animal isolates were found in dogs. Ten cases of co-carriage were found.

In total 85 *S. pseudintermedius* were isolated. A slightly larger proportion of the isolates came from the nose (35/62) than from the skin of the perineum (27/62) in pets. In humans there were no major difference in distribution between the skin (13/23) and nose samples (10/23) (Figure 18). In 18 cases (two humans and 16 pets) *S. pseudintermedius* was isolated from more than one body site. After further tests we assumed that these were duplicates therefore only one of these were included in the final study, hence we used 67 isolates.

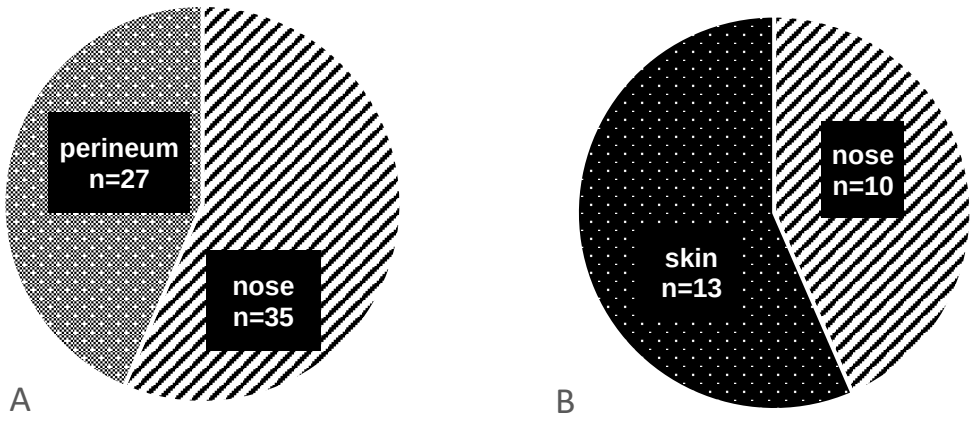

Figure 18. Distribution of *S. pseudintermedius* from different body sites. A) Distribution in dogs B) Distribution in humans.

4.1.3. *S. aureus* carriage rate in the hedgehog study

S. aureus strains were isolated from 13 hedgehogs (13/200), which indicates a 6,5% carriage rate. Of the 13 hedgehogs, seven animals carried the *S. aureus* only in their nose and four on their skin (Figure 19). Two animals had *S. aureus* at both locations, from which only one isolate was selected because they shared identical PFGE and virulence gene patterns, hence 13 strains were analyzed further in this study. Two MRSA isolate were identified (2/200), indicating a 1.0% MRSA prevalence.

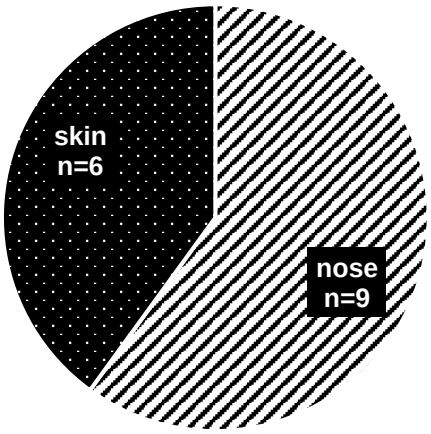

Figure 19. Distribution of *S. aureus* strains between different sampling sites in hedgehogs.

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4.2. Antibiotic susceptibility and resistance genes

4.2.1. Antibiotic sensitivity in the dog park samples

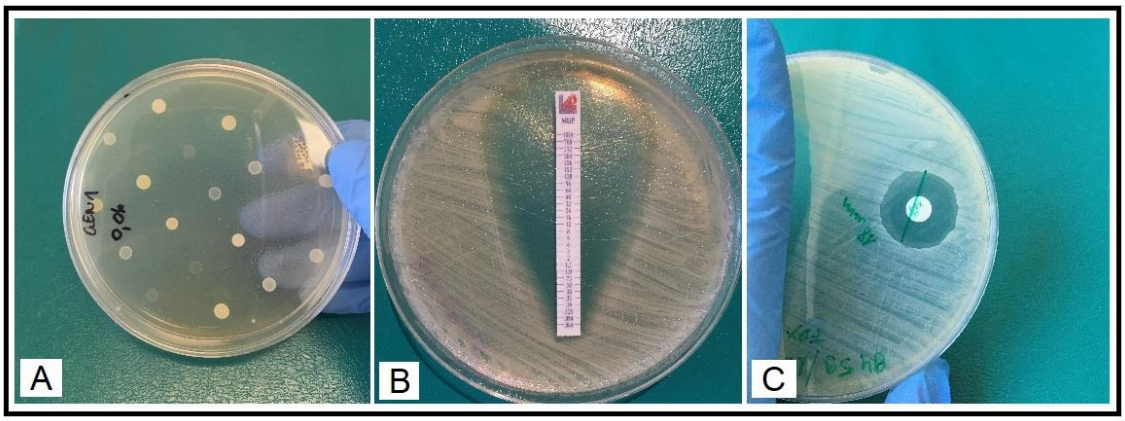

Figure 20. Different methods for antibiotic susceptibility testing. A) agar dilution method B) MIC test strip C) disc diffusion method.

We found that the CPS isolates were susceptible to the majority of the examined antibiotics (Figure 20). All *S. aureus* isolates were sensitive to cefoxitin, oxacillin, mupirocin, gentamicin, and ciprofloxacin. 60.0% (15/25) of the *S. aureus* strains were penicillin resistant. Only 8.0% (2/25) were resistant to tetracycline, 4.0% (1/25) to clindamycin and erythromycin (Figure 21) and only one *S. aureus* proved to be multi-resistant (i.e. resistant to at least three different antibiotic classes) (172).

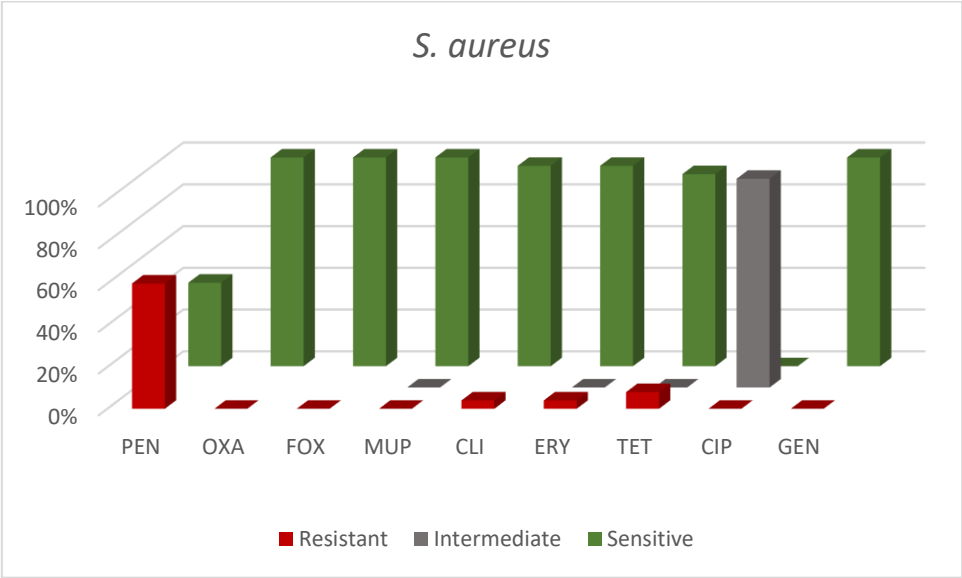

Figure 21. Antibiotic susceptibility in the *S. aureus* strains isolated from humans and dogs from dog parks. PEN -penicillin, OXA-oxacillin, FOX-cefoxitin, MUP-mupirocin, CLI-clindamycin, ERY-erythromycin, TET-tetracycline, CIP-ciprofloxacin, GEN-gentamicin

All *S. pseudintermedius* isolates were sensitive to mupirocin which is locally administered, and to oxacillin and cefoxitin. Surprisingly, only 32.4% (12/37) of the isolates were resistant to penicillin, however 56.7% (21/37) of them showed resistance to tetracycline. A small percentage of the samples showed resistance against further antibiotic drugs (13.5% (5/37) to erythromycin, 10.8% (4/37) to clindamycin and gentamicin each and 2.7% (1/37) to ciprofloxacin) (Figure 22). Seven isolates were multi-resistant in the case of *S. pseudintermedius* (172).

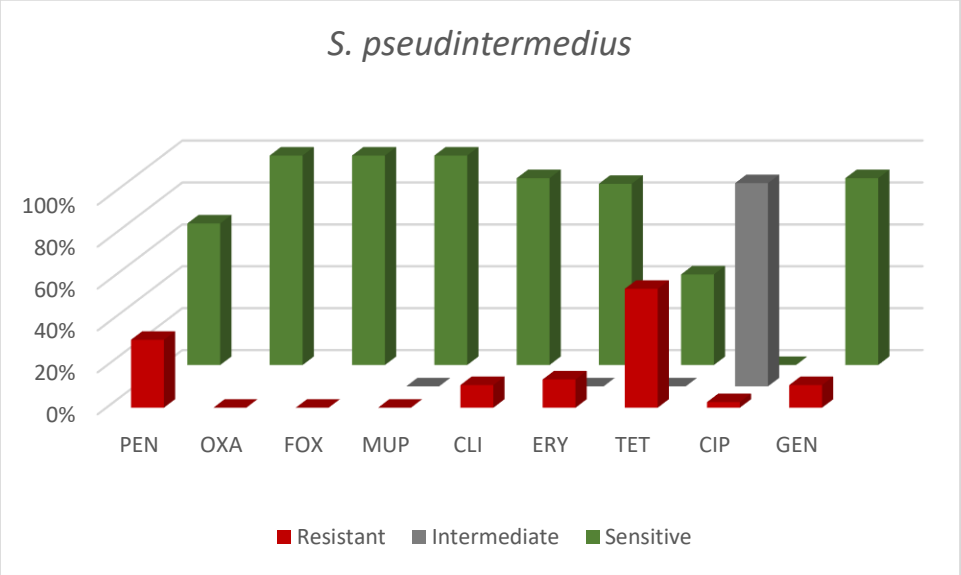

Figure 22. Antibiotic susceptibility in the *S. pseudinteremdius* strains isolated from humans and dogs from dog parks.
PEN -penicillin, OXA-oxacillin, FOX-cefoxitin, MUP-mupirocin, CLI-clindamycin, ERY-erythromycin, TET-tetracycline, CIP-ciprofloxacin, GEN-gentamicin

4.2.2. Antibiotic sensitivity in the veterinary hospital samples

S. aureus strains were mainly sensitive to the tested antibiotics, higher resistance rates were observed to penicillin (57.4% - 27/47) and to the macrolide-lincosamide group (MLSB) (clindamycin 31.9% (15/47) and erythromycin 23.4% (11/47)) and to tetracycline 8.5% (4/47). The D-test was positive in 11 strains, these had inducible clindamycin resistance. Five isolates were multidrug resistant. Only one strain taken from the nose of a dog (CS20/1) proved to be methicillin resistant (MRSA) by the disc diffusion method with cefoxitin (Figure 23). We established the presence of the *mecA* gene and categorized it as a *SCCmec* type IV isolate with PCR. Another strain (ÁO3O) showed resistance to oxacillin (MIC=8mg/l) but was susceptible to cefoxitin (inhibiton

zone size=24mm) without the presence of any *mec* gene. This is called a borderline oxacillin resistant *S. aureus* (BORSA).

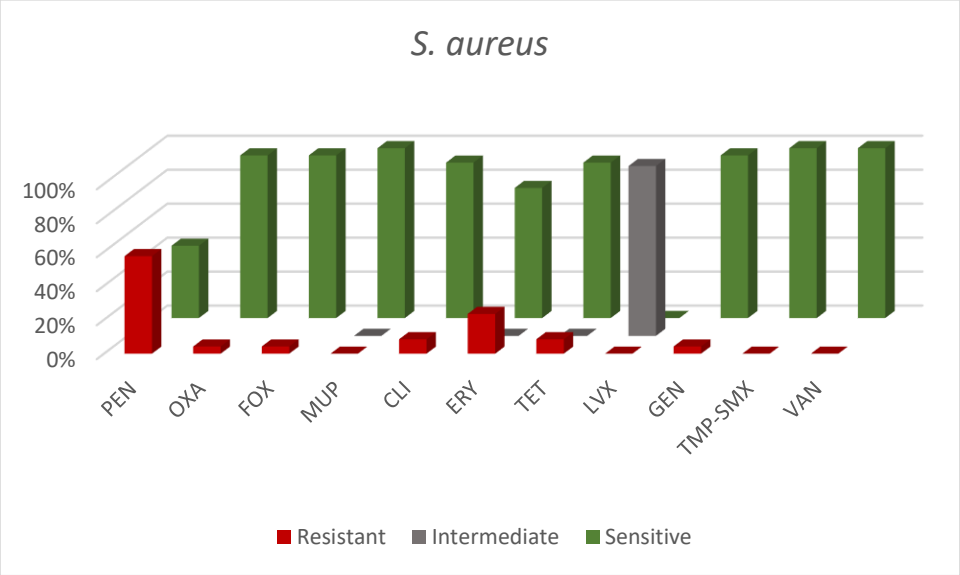

Figure 23. Antibiotic susceptibility in the *S. aureus* strains isolated from humans and dogs from veterinary hospitals.
PEN -penicillin, OXA-oxacillin, FOX-cefoxitin, MUP-mupirocin, CLI-clindamycin, ERY- erythromycin, TET-tetracycline, LVX-levofloxacin, GEN-gentamicin, TMP-SMX-trimetophrim-sulfamethoxazole, VAN-vancomycin

PCR examinations were carried out to search for resistance genes. These tests identified *ermA* (n=4) and *ermB* (n=1) genes as possible reasons behind the MLSB resistance.

Regarding the antibiotic susceptibility of *S. pseudintermedius* isolates, they showed the same trend as *S. aureus* strains. Elevated MICs were detected to penicillin 41.7% (28/67), erythromcyin 34.3% (23/67), clindamycin 31.3% (21/67) and tetracycline 29,8% (20/67) and 9,0% levofloxacin (6/67) (Figure 24). In this study group, inducible clindamycin resistance were not found in any of the isolates. Six samples proved to be methicillin resistant (MRSP) both with cefoxitin disc diffusion and *mecA* PCR methods. 14 strains were multi-resistant.

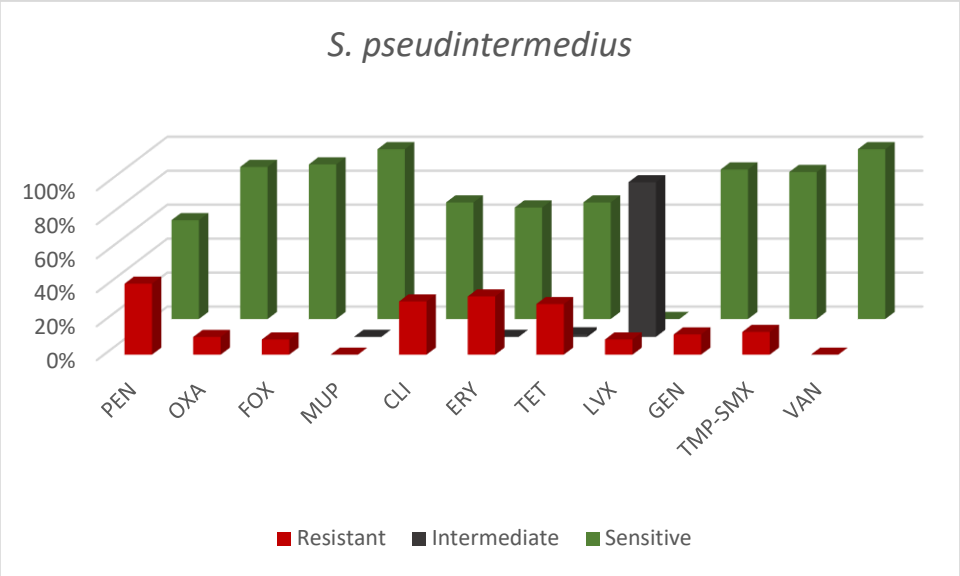

Figure 24. Antibiotic susceptibility in the *S. pseudintermedius* strains isolated from humans and dogs from veterinary hospitals.
PEN -penicillin, OXA-oxacillin, FOX-cefoxitin, MUP-mupirocin, CLI-clindamycin, ERY- erythromycin, TET-tetracycline, LVX-levofloxacin, GEN-gentamicin, TMP-SMX-trimetophrim-sulfamethoxazole, VAN-vancomycin

PCR examinations were carried out to identify resistance genes. The molecular tests identified *ermB* gene as responsible for the MLSB resistance, *tetK* (n=1) and *tetM* (n=15) behind tetracycline resistance.

S. pseudintermedius strains showed higher resistance rates to tetracycline compared to *S. aureus* strains (dog park samples: 57% and 8% respectively, p = 0.0001) (veterinary hospital samples: 31% and .8%, p=0.005).

4.2.3. Antibiotic sensitivity in the hedgehog isolates

Multi-resistant isolates were not found, and the isolated *S. aureus* strains were sensitive to most of the antibiotics used for susceptibility testing. Apart from penicillin, to which 6/13 (46.1%) of the *S. aureus* samples showed resistance. Reduced sensitivity were observed to ciprofloxacin (13/13 – 100%) and elevated MICs to erythromycin (1/13 – 7.6%) (Figure 25). Phenotypically two isolates were identified as cefoxitin resistant and it was verified by PCR. Eventually these two strains proved to be MRSAs: one contained the *mecA* gene, the other the novel homologue *mecC* (173).

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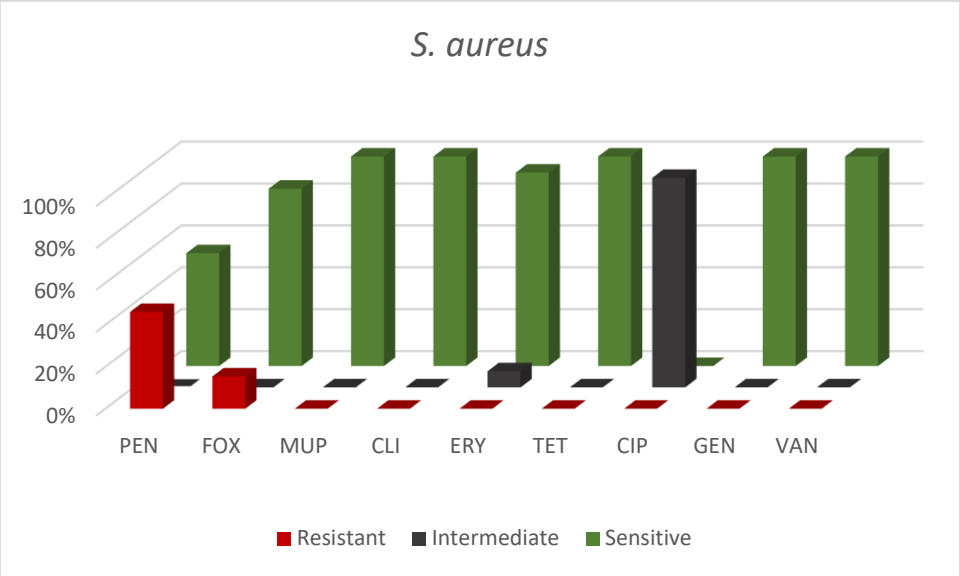

Figure 25. Antibiotic susceptibility in the *S. aureus* strains isolated from wild hedgehogs. PEN -penicillin, FOX-cefoxitin, MUP-mupirocin, CLI-clindamycin, ERY- erythromycin, TET-tetracycline, CIP-ciprofloxacin, GEN-gentamicin, VAN-vancomycin

4.3. Virulence factors and toxin genes

4.3.1. Virulence factors and toxin genes in the dog park isolates

Only the minority of the *S. aureus* strains possessed enterotoxin genes: *sea* (3/25), *seb* (1/25), *sec* (3/25). One isolate co-carried the staphylococcal enterotoxin type A and C. The *tsst* gene was present only in one *S. aureus*. Toxin genes were only found in *S. aureus* isolates from human origin. All tested *S. pseudintermedius* strains (both from animal and human samples) carried the *siet* gene (37/37) (172).

4.3.2. Virulence factors and toxin genes in the veterinary hospital samples

In the case of the isolates from the veterinary hospital study several genes responsible for biofilm and toxin production were detected in the *S. aureus* strains both from human and animal origin. A huge number of them carried at least one kind of hemolysin gene: *hla* (23/47), *hlg* or *hlg-v* (17/47), *hlb* (5/47). *Tsst* gene was found in a surprisingly high proportion of the samples (6/47) mainly in human strains (5/6), while the staphylococcal enterotoxin type C had almost the same prevalence (5/47). Two strains were detected containing the *seb* gene. and one isolate each carried *sea* and *eta*. From the genes

responsible for biofilm production and adhesion, we detected *icaD* (33/47), *cna* (11/47), *fnbA* (39/47), and *fnbB* (19/47).

In the *S. pseudintermedius* isolates both from human and animal origin the following toxin genes were detected*: siet* (48/67), *lukF-I* (58/67), *lukS-I* (58/67), *hld* (64/67), *psm* (64/67) moreover we identified *icaD* as part of the *ica*-operon in 64 of the 67 strains.

The MRSA isolate only carried one toxin gene: the enterotoxin type A, meanwhile the MRSP isolates contained several toxin genes: *siet* (4/6), *lukF*, *lukS* (5/6), *hld* (6/6), *psm* (6/6).

4.3.3. Virulence factors and toxin genes in the hedgehog isolates

In the isolated *S. aureus* strains there were no toxin genes found with PCR, whereas the majority of the, had genes responsible for biofilm production or adhesion to host tissue (*icaD* 13/13, *cna* 1/13, *fnbA* 13/13, *fnbB* 11/13). Only the SS2 strain possessed the collagen-binding adhesin (*cna*) gene. Apart from their *mec* genes, the two MRSAs differed only one characteristic, which is the absence of the *fnbB* gene in the *mecC*-MRSA (173).

4.4. Clonality

4.4.1. Clonality of the CPS isolates from the dog park study

According to the interpretation guidelines of the PFGE method the isolates can belong into four main categories: (1) ‘‘indistinguishable’’: isolates with identical banding patterns (same number and size of bands); if they are epidemiologically related these will be considered the same strain (i.e., isolates from an owner-pet pair) (2) ‘‘probably related’’: isolates with banding patterns differing from one and other in one band, as a result of one genetic event; these can be considered subtypes if the epidemiologic data suggest connection between the samples; (3) ‘‘possibly related’’: isolates differing from each other with two bands as a result of two genetic events; these will be considered subtypes if the epidemiologic information suggests linkage; and (4) ‘‘different’’: isolates with banding patterns differing from each other as a result of three or more genetic events (141)**.**

The PFGE analysis of the CPS isolates (*S. aureus* n = 25, *S. pseudintermedius* n = 37) showed similar banding patterns in specimens from different geographical areas and between unrelated human-dog isolates.

From the two cases where the owner and his/her dog co-carried *S. aureus* isolates, only one had indistinguishable PFGE pattern (Q37-Q38). The other co-carried pair (Q84-Q85) showed diverse appearance. However, the owner's sample (Q84) was identical with the previous Q37-Q38 pair, while the dog's sample (Q85) slightly differed from another human *S. aureus* isolate (Q47) from a different geographical location (Figure 26). When we look at the *S. pseudintermedius* isolates, indistinguishable banding patterns were recognized in samples from different towns, and from unconnected humans and dogs and the only human-animal co-carriage (Q81-Q82) had an identical restriction pattern with the PFGE analysis (Figure 27) (172).

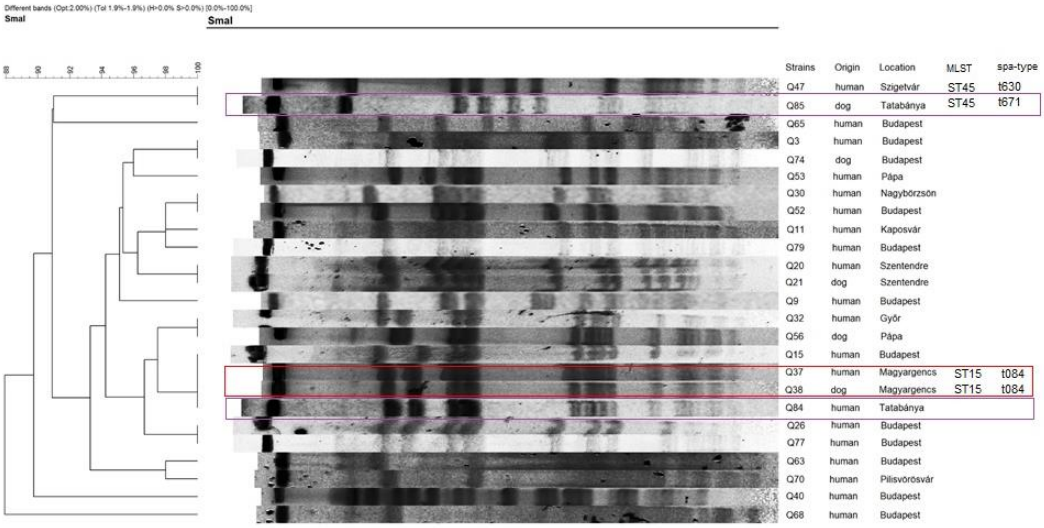

Figure 26. PFGE dendogram of *S. areus* isolates from the dog park screening study.
The co-carried isolates are encircled with the same colour.

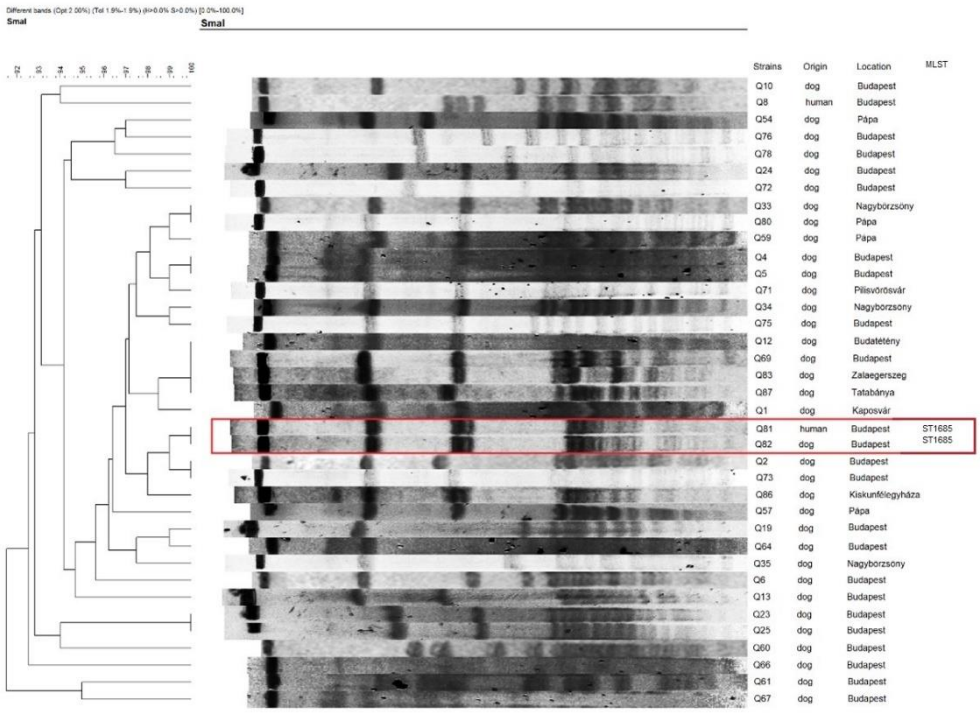

Figure 27. PFGE dendogram of *S. pseudintermedius* isolates from the dog park screening study. The co-carried isolates are encircled.

4.4.2. Clonality of the CPS isolates from the veterinary hospital study

During the PFGE analysis of the CPS isolates (*S. aureus* n = 47, *S. pseudintermedius* n = 67) some of the strains (*S. aureus* n=4, *S. pseudintermedius* n=1) could not be digested with the *smaI* enzyme. The remaining CPS isolates showed similar banding patterns in specimens from different geographical areas and between unrelated human-dog isolates.

The clonality of the co-carried strains was examined here as well to determine the possibility of bacterial exchange between humans and animals just like with the dog park samples. The PFGE patterns of *S. aureus* showed the presence of identical isolates from pets and their owners in two cases of the six co-carriage cases (B13-B15, B9-B11) (Figure 28)., with *S. pseudintermedius* we detected similar banding pattern in seven cases (P11-P12, B28-B29, K5-K7, A34-A37, K37-K39, E26-E28, SL12-SL13) out of the ten co-carriages. (Figure 29).

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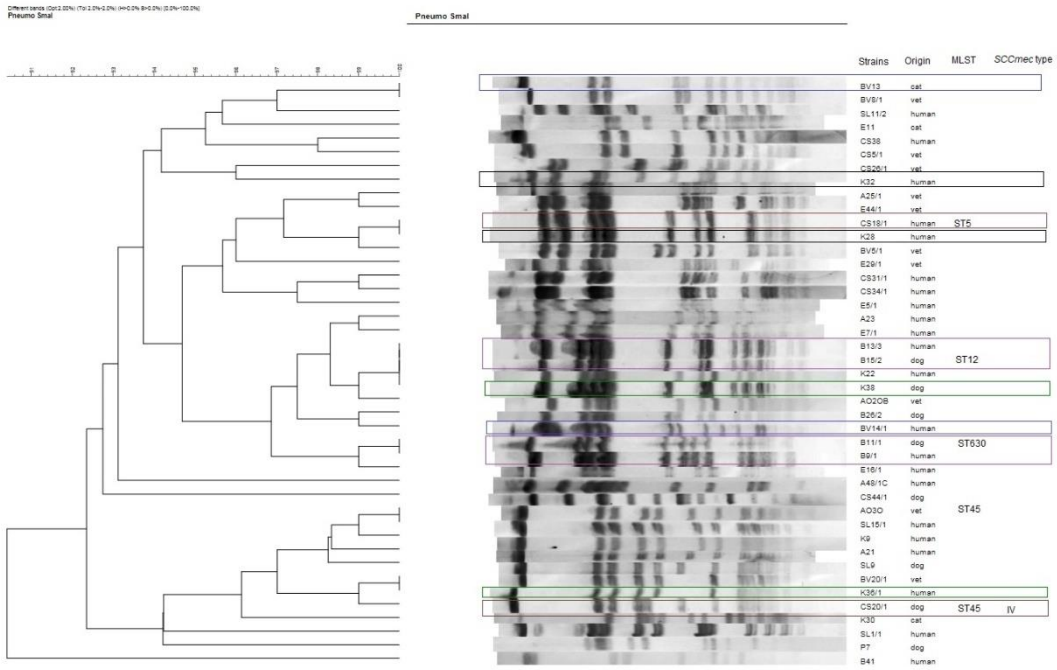

Figure 28. PFGE dendogram of *S. aureus* isolates from the veterinary hospital screening study. The co-carried isolates are encircled with the same colours.

DOI:10.14753/SE.2022.2741

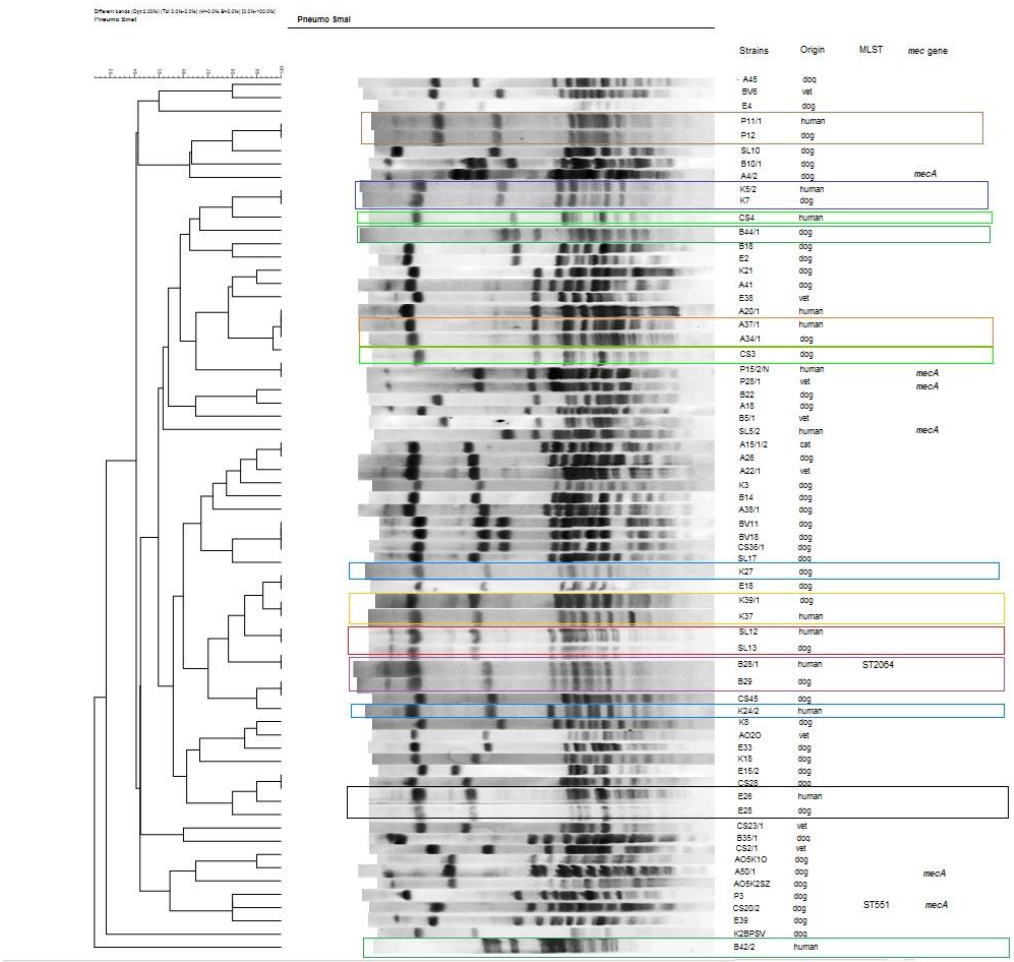

Figure 29. PFGE dendogram of *S. pseudintermedius* isolates from the veterinary hospital screening study.
The co-carried isolates are encircled with the same colours.

4.4.3. Clonality of the *S. aureus* isolates from hedgehogs

Two major clusters could be seen at 93% similarity level on the PFGE dendrogram of the 13 hedgehog *S. aureus* isolates , and only the *mecA*-MRSA had a very divergent banding pattern (Figure 30) (173).

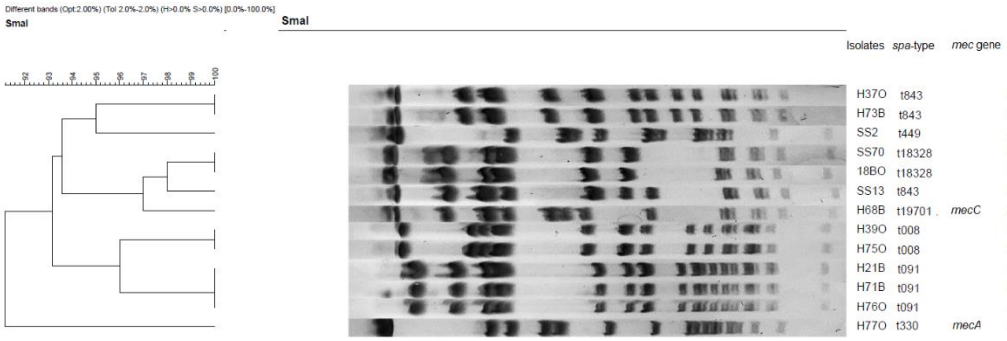

Figure 30. PFGE dendogram of *S. aureus* isolates from the wild hedgehog screening study.

4.5. MLST, spa, SCC*mec* typing

4.5.1. MLST and spa typing in selected dog park isolates

The Q37-Q38 isolates were part of ST15, while Q47-Q85 belonged into the ST45 MLST types. The ST15 isolates both had the t084 spa type, while the ST45 isolates identified as t630 and t671.

The sequenced *S. pseudintermedius* isolates both were assigned to a new sequence type, the ST1685 because they displayed a new allele combination (10-24-4-2-19-26-2) (172).

4.5.2. MLST and SCC*mec* typing in selected veterinary hospital isolates

The *mecA*-MRSA was *SCCmec* type IV and both this isolate and the BORSA strains belonged to ST45. One further human strain was ST5, and two more dog strains, were ST630 and ST12.

The examined MRSP isolate belonged into ST551 and the other analyzed MSSP had a novel allele combination (1-63-4-2-8-1-1) which was categorized as ST2064.

4.5.3. MLST, spa and SCC*mec* typing in the hedgehog isolates

All hedgehog related *S. aureus* isolates were typed with spa typing and the following types were defined: t091 (n=3), t843 (n=3), t18328 (n=2), t008 (n=2), t449 (n=1), t330 (n=1), and t19701 (n=1).

The *mecA*-MRSA isolate had the t330 *spa* type and was recognized as a CC45-ST3060 MLST type and belonged to the *SCCmec* type IV. The *mecC*-MRSA strain demonstrated a new spa repeat combination (04-82-17-25-17-25-25-110) which was assigned as a new *spa* type (t19701). This *mecC*-MRSA had a nucleotide difference in the *arcC* gene from the ST130. Therefore a new allele number (*arcC*-793) and a new sequence type (ST6736) were assigned to it by the MLST database (173).

4.6 WGS analysis results

4.6.1. The WGS analysis of selected CPS from the dog park study

All four WGS-analyzed *S. aureus* isolates carried the *blaZ* ß-lactamase gene as an acquired antibiotic resistance gene (199,200). Furthermore, the Q37-Q38 pair possessed the *tetK* gene and the *fosB* gene. In the Q47-Q85 pair the following amino acid changes were detected: the I45M in the topoisomerase GrlA, the E291D, T396N in the MurA transferase and A100V in the GlpT transporter. Resistance genes were not detected in the *S. pseudintermedius* isolates (Table 8).

Table 8. Typing data and resistance gene pattern in whole genome sequenced isolates.

Isolate	Species	Origin	Sampling location	ST - spa typing	Resistance phenotype	Resistance genotype
Q37	AUR	human nasal swab	Magyargencs	ST15-t084	PEN,TET	*blaZ, tet(K), lmrS, fosB*
Q38	AUR	dog nasal swab	Magyargencs	ST15-t084	PEN, TET	*blaZ, tet(K), lmrS*
Q47	AUR	human nasal swab	Szigetvár	ST45-t630	PEN	*blaZ, lmrS*
Q85	AUR	dog nasal swab	Tatabánya	ST45-t671	PEN	*blaZ, lmrS*
Q81	PSE	human nasal swab	Budapest	ST1685	-	-
Q82	PSE	dog oral swab	Budapest	ST1685	-	-
H68B	AUR	hedgehog skin swab	Budapest	CC130-ST6736-t19701	PEN	*blaZ, mecC, lmrS*

All four examined *S. aureus* contained the *ica* operon. Several other MSCRAMM producer genes were found in the isolates such as clumping factors (*clfA*, *clfB*), collagen

binding protein (*cna*), elastin binding protein (*ebp*), fibronectin binding proteins (*fnbA*, *fnbB*), extracellular adherence protein (*map*), or Ser-Asp rich proteins (*sdrE*, *sdrC*). Moreover, phenol-soluble modulins (*psmß1; psmß2*) were found.

All *S.aureus* isolates carried the serotype 8 capsule genes (*cap8H-cap8K*) responsible for capsule production and the staphylococcal immunoglobulin binding protein gene (*sbi*) responsible for immune evasion.

The *S. pseudintermedius* isolates carried the *ica* operon, phenol-soluble modulin proteins (*psmß*) and quorum sensing genes (*agrA-agrD*). Furthermore, an immunoglobulin binding protein gene (*sbi*) was detected which is similar to the sbi protein in *S. aureus*.

In the examined *S. aureus* isolates the following enzymes were detected: aureolysin (*aur*), hyaluronate lysate (*hysA*), lipase (either *geh* or *lip* or both), serine protease (*sspA*), staphopain (*sspB, sspC*), staphylocoagulase (*coa*), von Willebrand factor binding-protein (*vWbp*).

All the *S aureus* isolates carried hemolysin genes: *hla*, *hlb*, *hld*, *hlg-a*, *hlg-b; hlg-c*, but only the Q37-Q38 pair owned the *lukE* and *lukD* leucocidins. The other *S. aureus* pair Q47 and Q85 possessed several staphylococcal enterotoxin (SE) genes: *seg*, *sei*, *sem*, *sen*, *seo*, *seu*, in addition the Q47 strain also carried the *sec* and *sel* genes.

S. pseudintermedius samples contained the constitutive enzyme genes responsible for tissue invasion and spread like coagulase (*coa*) or thermonuclease (*nuc*). From the cytotoxin coding genes hemolysin (*hlb*, *hly-III*) and leucocidin (*lukS*) genes were carried by the strains. Both of Q81 and Q82 genome contained enterotoxin (*sec-int*) and exfoliative toxin (*siet*, *expB*) genes (Table 9 and 10) (172).

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Table 9. Virulence factors detected in *S. aureus* isolates with WGS.

***S. aureus* strains**		**Q37**	**Q38**	**Q47**	**Q85**	**H68B1**
		owner	**dog**	**owner**	**dog**	**hedgehog**
leucocidins	*lukD*					
	lukE					
	lukG					
	lukH					
	lukS-PV					
	lukF-PV					
hemolysins	*hla*					
	hlb					
	hld					
	hlg-a					
	hlg-b					
	hlg-c					
tss toxin	*tsst*					
exfoliative toxins	*eta*					
	etb					
	etd					
	ete					
	edinB					
enterotoxins	*sea*					
	seb					
	sec					
	sed					
	see					
	seg					
	seh					
	sei					
	sej					
	sek					
	sel					
	sem					
	sen					
	seo					
	seu					
ica operon	*icaA*					
	icaB					
	icaC					
	icaD					

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	icaR					
adherence	*clfA*					
	clfB					
	fnbA					
	fnbB					
	bap					
	cna					
	ebpS					
	sdrC					
	sdrD					
	sdrE					
	atl					
	map					
	psm-α					
	psm-ß1					
	psm-ß2					
enzymes	*sspA*					
	sspB					
	sspC					
	hysA					
	lip					
	geh					
	coa					
	sak					
	nuc					
	aur					
	vWbp					
immune evasion	*spa*					
	adsA					
	cap					
	chp					
	sbi					
	scn					

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Table 10. Virulence factors detected in *S. pseudintermedius* isolates with WGS.

***S. pseudintermedius* strains**		**Q81**	**Q82**
		owner	**dog**
leukocidins	*lukS-I*		
	lukF-I		
hemolysins	*hla*		
	hlb		
	hld		
	hlg-a		
	hlg-b		
	hlg-c		
	hly-III		
exfoliative toxins	*expA*		
	expB		
	siet		
enterotoxins	*sea*		
	seb		
	sec		
	sed		
	see		
	sec-int		
adherence	*icaA*		
	icaB		
	icaC		
	icaD		
	icaR		
	agrA		
	agrB		
	agrC		
	agrD		
	psm-α		
	psm-ß		
enzymes	*coa*		
	nuc		
immune evasion	*sbi*		

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The BLAST Ring Image Generator (BRIG) diagrams of the bacteria compared the sequenced genomes to designated reference genomes and they showed that the Q37-38 and Q81-82 isolates were nearly identical on a genomic level, however between the Q47 and Q85 samples small differences were detected (Figure 31 and 32).

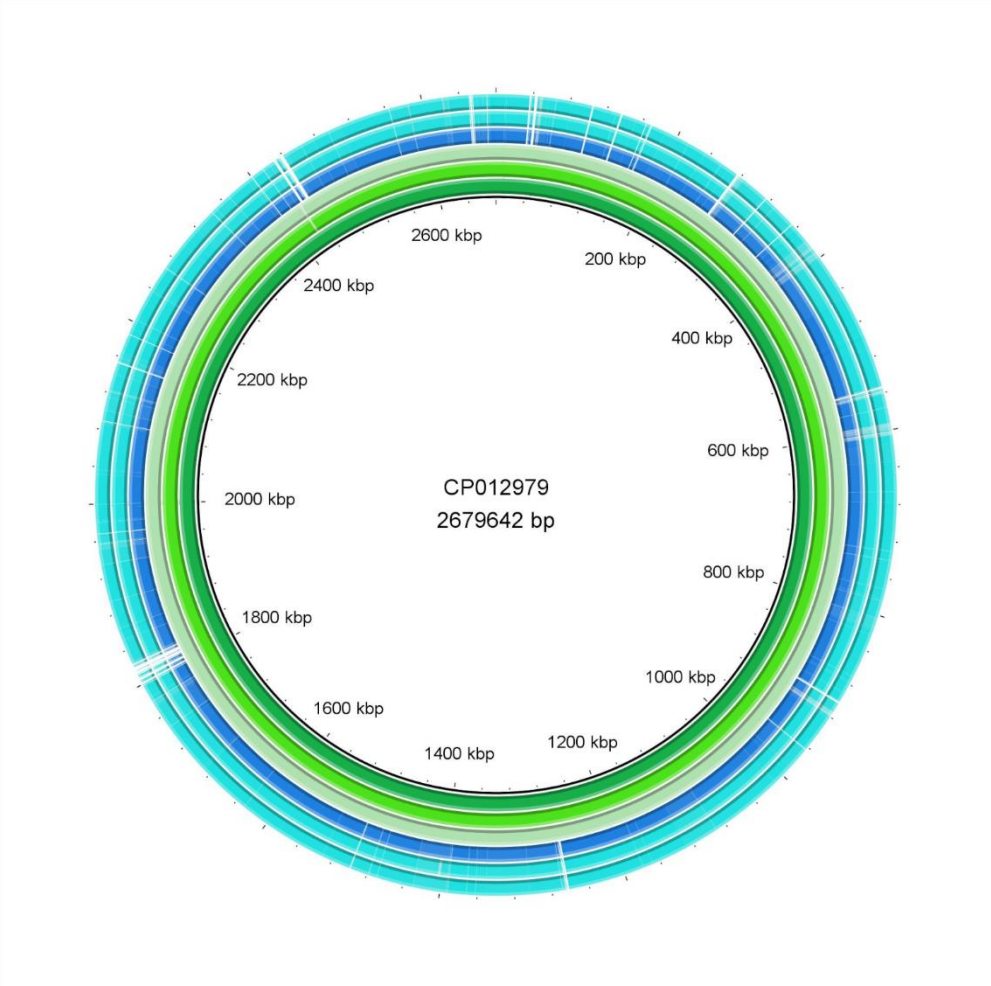

Figure 31. BRIG diagram of the S. *aureus* isolates.
The order of isolates from inside out is the following: CP012979 reference strain, Q47, Q85, CP014791 reference strain, Q37, Q38, (163).

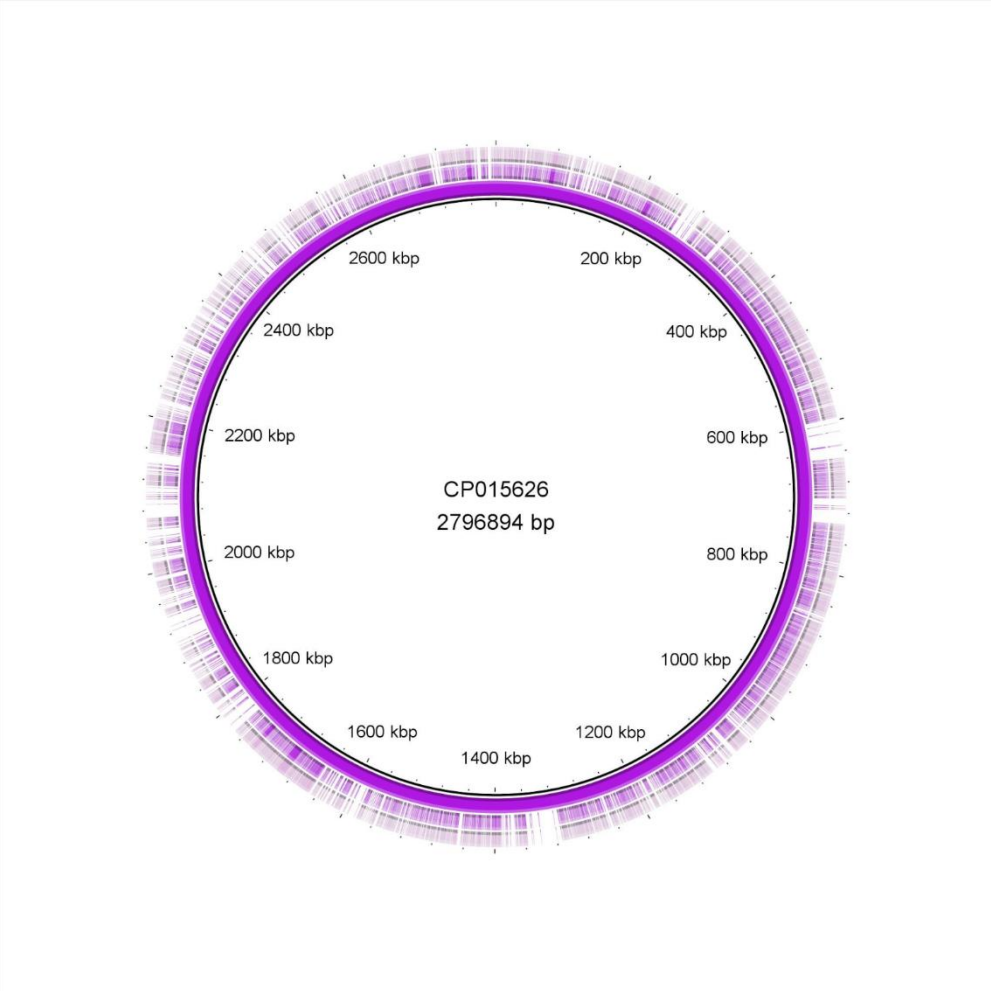

Figure 32. BRIG diagram of the *S. pseudintermedius* isolates.
The order of isolates from inside out is the following: CP015626 reference strain, Q81, Q82 (163).

4.6.2. The WGS analysis of the *mecC*-MRSA from the hedgehog isolates

Here only the *mecC*-MRSA isolate was further examined with WGS. During this analysis *blaZ*, *mecC*, and *lmrS* resistance genes were discovered in the genome of the bacteria.

Regarding toxin genes, the *hlg*-locus was identified in the bacterial genome, which contains the gamma hemolysin genes (*hlgA*, *hlgB*, *hlgC*) (79), and the following bicomponent leucocidins were found: *lukD-lukE*, *lukH-lukG* (74). The human MSSA associated Panton-Valentine leucocidin, the animal-associated leucocidin lukM/lukF-P83 (132) and the immune evasion cluster (IEC) (*sea, sep, chp, sak and scn)*, were missing (42,201). The epidermal cell differentiation inhibitor gene *edinB*, and the new

exfoliative toxin gene , *etE* (83) (previously called *etD2* (132)), were recognized close to each other. During our analysis, aureolysin (*aur*), coagulase (*coa*), lipase (*geh, lip*), protease (*sspA, sspB, sspC, splA, splB, splC, splD, splE splF*) and thermonuclease (*nuc*) enzyme coding genes were identified. Furthermore, this *mecC*-MRSA isolate possessed the *ica* operon and several other genes responsible for MSCRAMMs production, such as clumping factors (*clfA, clfB*), elastin binding protein (*ebp*), fibronectin binding protein A (*fnbA*), fibrinogen binding protein (*efb*), extracellular adherence protein (*map*), and Ser-Asp-rich proteins (*sdrC, sdrD, sdrE*). The *mecC*-MRSA had capsule genes (*cap8H-cap8K*) promoting serotype 8 capsule production. The immunoglobulin G-binding protein A (*spa*) and the staphylococcal immunoglobulin-binding protein gene (*sbi*) which belong to the immune evasion factors, were also detected.

The H68B1, *mecC*-MRSA incorporated the *ccrB3* and *ccrA1*, which were part of the *ccr* type 8. An arsenic-resistance operon could be recognized in the J1 region, consisting of three genes: a transcriptional repressor (*arsR)*; an arsenite efflux pump (*arsB*); and an arsenate reductase (*arsC*) (128). The SCCmecFinder typed the *SCCmec* cassette as *SCCmec* type XI (173).

5. DISCUSSION

5.1. Carriage

5.1.1. CPS carriage rates in pets

Given the close social interaction between humans and their pets, pathogens from human host can jump into animals, and this can lead to *S. aureus*/MRSA colonization and infection in a pet (38). However, scientific manuscripts rarely mention that humans also contribute to *S. aureus* infection as an emerging diseases in companion animals (202). Transmission of *S. aureus* among different host species in the community (i.e., between owners and their animals) and in veterinary practices (i.e., between veterinary personnel and pets) has been already reported. Intermittent bacterial carrier pets thought to be reservoirs of infection in humans with recurrent *S. aureus*/MRSA. Presumably humans were the original source of *S. aureus*, while the pets played only a part as reservoirs for reinfection (28,131,203,204). Carriage of *S. aureus* and MRSA by healthy animals and the transmission of these to humans is of great concern nowadays (38,205,206).

The transmission of *S. pseudintermedius* and MRSP was described between companion animals and humans or vice versa by several researchers (27,35). *S. pseudintermedius* is normally rare in humans, and they are only temporarily colonized, hence in this case the dog-to-human transmission route is the most probable direction (29,207). Animal infections or carriage of *S. pseudintermedius* might represent a potential hazard for people in contact with pets (29). In companion animals, *S. pseudintermedius* mostly responsible for skin and soft tissue infections, in humans it has been detected from various infections, such as prosthetic joint infections, endocarditis, ear, skin and wound infections (11,12,24,208).

The literature data about dogs with asymptomatic *S. aureus* colonization is minimal. According to several sources the prevalence rate varies between 2–8% (30,209,210). Fortunately, more data is accessible on asymptomatic carriage of *S. aureus* and MRSA prevalence in veterinary hospital environment. According to these screenings *S. aureus* prevalence is higher in veterinary environment and ranges from 5 to 36%

(27,38,209,211,212) while the MRSA carriage rate is between 0-11% (27,38,209,211,213). The skin, nasal and mucosal carriage of MRSA plays a critical role in the epidemiology and spread of *S. aureus* infections (214).

The documented carriage rates in the neighboring countries follow these trend: in Romania 12% *S. aureus* and 9% MRSA prevalence was documented in shelter dogs (214). In Austria, Vienna 0% MRSA was found in a study which screened healthy and veterinary visiting dogs (215). In Slovenia clinically healthy dogs were screened and 0% MRSA was detected (216).

Our results seem to mirror these numbers because the *S. aureus* prevalence in dogs in the community was 5%, and no MRSA was detected, furthermore the carriage rate of *S. aureus* was 15%, and 1% of the samples were MRSA in companion animals from veterinary environment. *S. aureus* was most frequently present in the nares and perineum of a companion animal. Therefore, these sites should be screened when a study is designed.

S. aureus nasal carriage in the human community is around 20–30%, while 0-4% is the MRSA prevalence (13,33,217–219). In veterinary personnel higher asymptomatic carriage rates were documented in the literature: *S. arueus* prevalence was around 20-39%, while MRSA was around 0-17% (19,38). In our previous *S. aureus* carriage studies in humans, the nasal colonization rates were 29.3% among the university students (174), 21.3% among children attending preschools (220) and 34.1% in preschool children (221). The MRSA carriage was 0.67% in university students and 0.8% in preschool children, there were no MRSA found in the day-care center study.

In this study, 24%-33% *S. aureus* carriage rate was observed in pet owners, however higher prevalence of *S. aureus* was detected in veterinary staff (38%). Moreover, no MRSA was detected from human samples. These results are in good correlation with the literature data.

The prevalence of S. pseudintermedius on the nasal mucosa has been rarely examined but according to the few data available it is somewhere between 0-12.5% (19,28,33) while for MRSP it is 0-3.9% (32,34). The literature data mainly focused on MRSP carriage in veterinary personnel, which is between 0-7% (19,32). In our study, we found

a lower percentage for *S. pseudintermedius* carriage (2%) in the dog park screening study, but do not forget we screened owners and not veterinary staff here. If we take a look at the veterinary hospital study, we detected 31% prevalence of *S. pseudintermedius* and 1% MRSP in veterinary personnel.

In dogs, 37% prevalence was documented in the UK in 2009 (then it was classified as *S. intermedius*) (212), more recently higher carriage rates were found in Denmark (69%) (222) and Australia (85%) (223) and according to Bannoehr et al it could vary between 46-92% (36). In this study the prevalence of *S. pseudintermedius* in dogs was between 34-58% in Hungary.

In the bordering countries the following prevalence rates documented: in Slovenia the MRSP carriage was 1.5% in dogs (216), in Austria it was also 1.5% in pets (215), in Romania the *S. pseudintermedius* carriage was 49%, and MRSP was 9% in Romanian shelter dogs (214). It seems like there is no major geographical difference in the prevalence of *S. pseudintermedius*.

5.1.2. *S. aureus* carriage rate in hedgehogs

Wildlife can serve as a reservoir for antibiotic resistant microorganisms such as MRSA, VRE or Enterobacterales (10,224,225). Although Northern European hedgehogs are recognized as a common reservoirs of *mecC* (39,43), during our screening we found a very low prevalence in hedgehogs in the Budapest region. The MRSA carriage rate was just 1% (2/200) in the sampled population.

In Europe the *mecC*-MRSA seems to show significant geographical differences. The majority of the *mecC* isolate carrying animals and humans have been found in Western and Northern Europe (10,39,43,128,226,227). For instance, *mecC* seems to be extremely frequent among hedgehogs in Denmark (61%, 114/188) (39) and Sweden (64%, 35/55) (43). Whereas *mecC* has a low prevalence in the countries bordering Hungary. Only a few studies dealt with *mecC*-MRSA in animals in this region of Europe, but these seem to support our findings. 237 milk samples were examined from mastitis in Croatia, and only five *mecC*-MRSAs were detected from these cattle samples (2.1% prevalence) (228). Altogether almost two thousand wild animals and ruminants were screened for coagulase-negative *Staphylococcus* carriage in Austria, and only 15

mecC-CNS isolates were detected (229). Small mammals such as yellow-necked mice and striped field mice were evaluated for Staphylococcus carriage in Slovakia. From the 61 sampled rodents only seven *S. aureus* strains were identified - none of them carried the *mecC* gene (230). The first description of human *mecC*-MRSA strains in the countries bordering Hungary were reported in 2015. In Austria, 295 human MRSA strains, both from clinical settings and asymptomatic carriage, were investigated and only six *mecC*-MRSAs were detected (231). In Slovenia, from 395 CA-MRSA strains only six *mecC*-MRSAs were found (232).

In Hungary, between 2003 and 2018, Albert et al. screened hundreds of milk samples throughout the country, and found only 31 MRSA isolates - none of which carried the *mecC* gene (233). Based on this study the *mecC*-MRSA prevalence is presumably low in farm animals in the country. From human specimens in Hungary, *mecC*-MRSA has not been described so far.

5.2. Antibiotic susceptibility and resistance genes

5.2.1. The antibiotic susceptibility and resistance genes in pets

57 and 60% of *S. aureus* isolates of pets appeared to have higher penicillin MICs whilst only 32% and 42% of *S. pseudintermedius* were resistant in our two studies, respectively. Possibly ß-lactamase enzymes are in the background of these elevated MICs, as only seven isolates, one MRSA, and six MRSP were resistant to oxacillin and cefoxitin, and the *blaZ* gene was identified in the *S. aureus* genomes. Presumably, the ÁO3O isolate is borderline oxacillin resistant (BORSA) because of its high ß-lactamase production. Relatively low levels of penicillin resistance was found in the animal derived *S. pseudintermedius* isolates even if penicillins are one of the most widely used antibiotics in the veterinary field around the word (234–237). These results regarding the penicillin resistance are surprising since penicillins used frequently therefore usually higher resistance rates are documented. According to the annual reports of the National Health Care Institute of Hungary (238), the penicillin resistance of human *S. aureus* isolates from outpatients in the country is stagnating around 86–88% (between 2018-2020) furthermore in our preceding human carriage screening ~90% resistance was measured (220).

Tetracycline is one of the most often used antibiotic in livestock (239) and that is why the resistance caused by the *tet(K)* and *tet(M)* gene became the indicator of livestock associated strains and very frequent in animal adapted isolates (112,240,241). The *tet(K)* gene possession of Q37-Q38 *S. aureus* isolates was detected with WGS and *tet(K)* and *tet(M)* genes were found by PCR in the veterinary hospital CPS, as well. These genes code an efflux pump responsible for tetracycline resistance (136,242)

A possible cause of the elevated doxycycline usage in small animals could be the increased prevalence of *Dirofilaria immitis*, the so called heartworm, in Hungary (243,244). A month long doxycycline treatment is part of the management of this parasitic infection in dogs (245). Because of this reason there is a strong possibility that the *tet(K) and tet(M)* gene harboring S*taphylococcus* isolates in these studies evolved in animals. We found that *S. pseudintermedius* strains which are more frequent in animals are displayed significantly greater resistance to tetracycline than the predominantly human adapted *S. aureus* isolates.

Q37-Q38 isolates owned the *fosB* gene which isa thiol transferase that can inactivate fosfomycin therefore lead to resistance (199,246).

Because of the extensive use of macrolide, lincosamide, and streptogramin B antibiotics against Gram-positive bacteria, increasing cross-resistance to these antibiotics is a subsequent problem in staphylococcal isolates. According to literature data, in our geographical region the *ermC* gene is most commonly detected from human MSSA and MRSA strains (8). Interestingly in the CPS isolates in this study, *ermB* was the most frequently identified *erm* gene, followed by *ermA*. From humans the *ermB* gene is isolated more frequently in China and Egypt (8). On the other hand, in LA-MRSA/MSSA and *S. pseudintermedius* samples the *ermB* is the most frequent MLSB resistance gene which suggest that these genes very likely come from animal origin in our samples (100,247–256).

The Q47-Q85 *S. aureus* isolates had an amino acid change in the topoisomerase GrlA protein (I45M) which could cause higher ciprofloxacin MIC (167) The E291D, T396N amino acid changes in MurA transferase (257) and A100V in GlpT transporter protein all can lead to fosfomycin resistance either by triggering the overexpression of MurA or helping to reduce the import of fosfomycin into the microbes (126,127).

5.2.2. The antibiotic susceptibility and resistance genes in hedgehogs

In our present work, in the hedgehog associated *mecC*-MRSA the *blaZ* gene is the cause of the ß-lactamase enzyme production while *mecC* leads to a mutation in the penicillin binding protein resulting in resistance against ß-lactam antibiotics. Normally among *mecC*-MRSA isolates the MICs for oxacillin and cefoxitin are lower compared to *mecA*-MRSA strains (226), furthermore, in the *mecC*-MRSA resistance to non-*ß*-lactam antibiotics is very uncommon (39,132).

5.3. Virulence genes

5.3.1. Virulence genes in pet CPS isolates

During the WGS analysis of the dog park *S. aureus* isolates the *ica* operon was found in the genomes which will cause polysaccharide production (poly-n-succinyl-β-1,6 glucosamine (PNSG)) during infection allowing bacteria to stick to each other, adhere to human tissues, or inanimate plastic surfaces thus helping biofilm formation. Several genes were detected in the genomes responsible for the production of MSCRAMMs such as clumping factors (*clfA, clfB*), collagen binding protein (*cna*), elastin binding protein (*ebp*), extracellular adherence protein (*map*), fibronectin binding proteins (*fnbA, fnbB*) or Ser-Asp rich proteins (*sdrE, sdrC*). These genes also promote biofilm production, but in a different way than the *ica* operon. They can attach to specific, extracellular matrix molecules this way helping the production of bacterial films (49). Phenol-soluble modulin genes (*psmß1;psmß2*) were also detected which can trigger biofilm production, dissemination of infections in the body and can worsen inflammatory responses (57). The capsule production of the bacteria helps to escape opsonization and phagocytosis. The Q37-Q38 pair owns leucocidins which help the bacteria to lyse host cell membranes (259) (Table 9).

The majority of the *S. pseudintermedius* isolates had exfoliative toxin genes (*siet*, *expB*), which can lead to skin infection in dogs (93,104,111). Furthermore, an ecthyma-like, painful, lesion was documented in a husky dog owner caused by a toxin producer *S. pseudintermedius* (11), suggesting that these toxins could be pathogenic to humans as well (Table 10).

With WGS, we established that the *S. pseudintermedius* isolates (Q81-Q82) and one pair of the *S. aureus* isolates (Q47-Q85) possessed different kind of staphylococcal enterotoxins (SE). In the Q47-Q85 *S. aureus* isolates, we detected the animal associated enterotoxin gene cluster (*egc*) with the *seg*, *sei*, *sem*, *sen*, *seo*, *seu* genes (88,109,260,261). Amidst the *S. aureus* strains analyzed with WGS, the Q47 isolated from a human host contained enterotoxins and harbored the only classical SE (*sea-see*): the *sec* subtype 2. The *sec* gene was a common finding in cattle and human MRSA isolates, but it arose in bacteria with dog origin as well (109,110,260,262–266). In the Q47 isolate *sel* was also detected which has already been identified from uncooked meat samples (261). With PCR we found further *S. aureus* strains from the vet hospital and dog park isolates carrying one or more classical enterotoxins (*sea, seb, sec*). These heat-stable toxins are involved in staphylococcal food poisoning (259)

S. aureus food intoxications are well reported, whereas *S. pseudintermedius* food poisoning cases is scarcely discussed. However, *S. pseudintermedius* has been found in raw or processed food occasionally and it could be associated with human food-related outbreaks, as well (267). In this study, the *sec-int* gene was identified with WGS in the *S. pseudintermedius* strains.

The phage-encoded immune-evasion cluster (IEC) is recorded in the literature as a human adaptation specific element containing combinations of the following genes: the chemotaxis inhibitory protein (*chp*), the staphylococcal complement inhibitor (*scn*), the superantigen staphylococcal enterotoxin A and P (*sea, sep*) and the plasminogen activator staphylokinase (*sak*). Several reports described high IEC carriage rates among human *S. aureus* isolates, while the IEC was rarely identified in animal strains (268). The same connection with human origin can be established with the presence of the PVL gene (75). Our *S. aureus* isolates contained some of the genes belong into IEC which can mean that these strains originated from humans however over time they adapted to new hosts by losing some of their virulence factors from this IEC cluster.

5.3.2. Virulence genes in hedgehog *S. aureus* isolates

In the *mecC*-MRSA a novel exfoliative toxin was caught during WGS analysis, which was first reported in a paper by Harrison et al.(168), First, this toxin was mentioned as *etD2* and *etD*-like protein but its final name became *etE* in 2019 (83,132,269,270). This exfoliative toxin is thought to be a part of a pathogenicity island with the epidermal cell differentiation inhibitor gene (*edinB*) (269). The *mecC*-MRSA isolate in this study contained this *edinB* as well. In laboratory experiments, the *etE* hydrolyses the desmoglein 1 in human, swine, murine and ovine tissues (83) and has been detected in clinical or screening samples from ewe mastitis, humans and hedgehogs so far (83,132,168,226).

An association between *cna* ownership and MLST CC1, CC12, CC22, CC30, CC45, CC51 and CC239 has been noticed, so the presence of *cna* in *S. aureus* can be described as lineage-specific (85). Therefore, based on the presence of *cna*, specific clonal complexes can be differentiated within *spa* types (85). The *cna*-positive *S. aureus* isolate (SS2) found in this screening had the *spa* type t449 (26-23-13-23-31-05-05-17-25-17-25-16-28), which genomic structure is very similar with *spa* type t005 (26-23-13-23-31-05-17-25-17-25-16-28), and that type is associated with STs that belong to the CC22 (https://spa.ridom.de/). Based on these association, the SS2 isolate in this study probably belongs to MLST CC22, which is a frequent human epidemic lineage (271,272) and ST22 was already described as the major MLST clone among blood stream MRSA isolates in Hungary (154).

The lack of markers for human adaptation in the *mecC*-MRSA isolate, such as *pvl* and *iec*, (Table 6) firmly indicates that *mecC* originally appeared in animal-adapted CPS strains (132,273).

5.4. Clonal relatedness

Pets have been described more frequently as potential reservoirs of MRSA and MSSA lineages of human origin than livestock animals, indicating a spillover from people to their close contact animals (268,274). As a consequence, pet isolates mirror the local epidemiology of human MRSA and MSSA in a certain geographical area. Further

analysis revealed that there is no specific difference in the composition or the freqency of virulence and resistance genes between the canine and human isolates from these lineages. (268).

5.4.1. Clonal relatedness of CPS in pets

The PFGE pulsotypes looked rather diverse at first glance which can be typical in case of asymptomatic carriers, but taking a closer look it can be seen that all of the isolates had a >90% similarity level (Figures 26-30). Furthermore, high resemblances could be noticed between samples collected from hosts at different geographical locations. These results can imply that major genetic types circulate in the community. Nonetheless the number of strains in this study is very limited to draw firm consequences. However, owners and their pets shared identical PFGE patterns, suggesting transmission via direct contact from animals to humans or the other way round.

Clonal complex 15 and 45 were found in our dog park study. These CCs were primarily described from human isolates in the literature(16). However, the MLST database also contains ST15 and ST45 strains isolated from animal hosts from the last few years. ST15 was reported in China and Greece from companion animals (38,275) from wildlife in Spain, Germany, Denmark, Nigeria, and Zambia (10) from cows in Iran (276), while the ST45 was also found in cows from Iran (276), in wildlife from Italy (10) and in companion animals from Austria (277), Spain (28,112) and Serbia (255).

Both the Q37 (human) and Q38 (canine) *S. aureus* strains from the dog park study belonged to ST15-t084. This genomic type is commonly described from animals. ST15-t084 was described in Iran from cows (276) and in a few wildlife studies from banded mongoose, Egyptian fruit bat, naked mole rat, wild boar in Germany, Spain and Denmark (10). The t084 spa type was detected in dogs and a pig from Poland with a predicated ST15 (278). The t084 was documented in poultry carcasses and slaughter house workers in Nigeria (279). A very similar genomic type, ST15-t085 was identified from an environmental veterinary hospital sample. (275). This t084 spa type was the most commonly detected from human blood stream infections (BSI) in a Norwegian retrospective study in the early 2000’s (280), furthermore ST15-t084 strains were also documented amongst clindamycin resistant clinical MRSA isolates in Iran (281).

The other *S. aureus* sample pair from the dog park samples (Q47-Q85) were members of the ST45 clonal type. These isolates had distinct but related spa types: t630 and t671. Presumably, small genetic alterations developed in the two different hosts. According to the Ridom spa server t630 was detected in Sweden, Norway, Germany, Austria, the Netherlands, the UK, Switzerland, Spain and the t671 was documented in Germany, Norway, the Netherlands, USA, Argentina. From the veterinary hospital study, we also identified *S. aureus* strains belonging to ST45. This sequence type has been predominant among Belgian human MRSA isolates (282), and it was present also in Hungarian MRSAs—with low prevalence—in the early 2000s (153). However in a recent study it was not isolated anymore among Hungarian BSI MRSA samples (154). The so-called USA600 MRSA also associated with this clonal complex. Members of ST45 were formerly identified from human carriers in Germany, Philippines and from dog carriers in the USA (283). ST45 was also described in our earlier studies (represented by both MRSAs and MSSAs), from children and adults (82,155), so it seems to be present in the Hungarian community for some time now. This belief is further supported by the fact that Q47 and Q85 were detected from two different towns in Hungary.

In addition to ST15 and ST45, ST5 from a human, ST12 and ST630 from dogs were also identified. ST5 was documented as an important human clinical clone in Japan (284) and a major CA-MRSA clone in Argentina(219), In Europe it was also found in polish and British clinical samples (285,286), and it became a significant Hungarian human MRSA clone in the early 2000 (153) and it is still a frequent and important BSI pathogen(154).However it was detected from clinical samples of companion animals in Portugal (156), Spain (112), USA (287) and from asymptomatic carriage in Australia (288) and meat samples in the USA (289) and was the leading clone in dog samples in Serbia (255) and was found in a cat isolate in Austria (290).

ST12 is a less frequent type. In Europe it was detected in companion animal samples in Germany (268), in raw meat products in USA (289). From humans it was isolated from clinical samples in the UK (142,291) and Poland (292). It was detected in dairy water buffalo milk samples in the Philippines (293) and SEB producing isolates were found on shopping cart handles in Japan (294).

ST630 belongs to CC5 and it was found mainly in China and other Asian countries. ST630 were detected in human clinical samples in China (52,295–298), in nasal carriage samples in Hong Kong and China (299,300), in retail foods in China (301), in retail meat in Korea (302), in milk samples in clinical bovine mastitis cases in China (303).

In the case of the MRSP isolate typed in this study, the ST551 was identified in Switzerland in environmental samples from veterinary clinics and from a veterinarian (304). This ST was documented in a clinical sample from otitis externa from a dog in Italy (305) and from clinical samples in Slovenia (165). It was also cultivated from healthy and sick companion animals in Poland and it was one of the most frequent ST type there (306,307).

5.4.2. Clonal relatedness of *S. aureus* in hedgehogs

In hedgehogs, the most frequently described MLST clonal complex is CC130 in the *mecC*-positive MRSA isolates. Within this CC130, the several *spa* types were frequent isolated: t528, t843, t1048, t3256, t3570, t5771, t6220, t10755, t10893, t11015, t17133 (39,43,132,159). Moreover, CC1943-t978, t2345, t3391, t8835, t16868 and CC2361-t978, t3391, t9111, t15312 have been carried by hedgehogs (39).

The ST6736 *mecC*-MRSA strain described in this study is a single-locus variant (in the *arcC* gene) of ST130. Furthermore, this Hungarian *mecC*-MRSA strain has a new *spa* type, t19701 (repeats: 04-82-17-25-17-25-25-110), as well. The t19701 is very close genetically to t843 (repeats: 04-82-17-25-17-25-25-16-17), which has already been detected in hedgehogs in Sweden and Austria (43,132).

ST130 has also been isolated from other wild animals, not just hedgehogs and livestock: such as the European rabbit (ST130-t843), red deer (ST130-tNT) (10,160), European brown hare (ST130-t843, ST130-t10513) (10,42,159), common chaffinch (ST130-t6293) (175), yellow-necked mouse and domestic mouse (ST130-t843) (308), wild boar (ST130-t6220), Iberian ibex (ST130-t1736) (309), cattle (ST130-t843) (228,273,310) and sheep (ST130-t843) (175).

Isolates associated with the clonal complex (CC) 130 (which is a broader category than ST130) have additionally been described in brown rat, European fallow deer, red fox

(42), common seal, domestic dog (175), European otter (159), white stork (311), magpies and cinereous vultures (312).

The other MRSA strain identified from the hedgehogs - which contained the more common *mecA* gene - belongs to ST3060-t330. The ST3060 is part of the CC45, which we previously detected in humans in Hungary (221). Only another ST3060 isolate has been documented so far, and that was obtained in 2014 in Spain from a white stork (311), this isolate had the t015 spa type. Interestingly, spa type t330 and t015 are very similar to one another (t330: 09-02-16-34-34-17-34-16-34 and t015: 08-16-02-16-34-13-17-34-16-34) (https://spaserver.ridom.de/) (173).

6. CONCLUSIONS

In this work we report the first documentation of asymptomatic staphylococcal carriage among companion animals, owners, veterinary staff, wildlife and furthermore we report the first *mecC*-MRSA strains in Hungary isolated from a hedgehog.

We can assume based on the prevalence data and the antibiotic sensitivity results — e.g., high tetraycline resistance —that the human *S. pseudintermedius* strains isolated in the companion animal study are dominantly animal adapted.

On the other hand, the analysis of the *S. aureus* strains pointed into the direction that these isolates evolved from human sources, and only went through genetic changes when they colonized a new host species – e.g., presence or absence of *pvl* and *iec*.

In the veterinary hospital and dog park screening study we could verify the direct transmission of *S. aureus* and *S. pseudintermedius* between humans and their companion animals based on the PFGE results and the virulence and resistance gene patterns.

The majority of the strains possessed genes responsible for adhesion and biofilm production. These could be the cause of serious, medical device associated, nosocomial infections. A few of the isolates possessed enterotoxin genes so under certain circumstances, these could be involved in food poisoning cases. The *tsst* positive strains mostly were of human origin and have the potential to lead to severe infections. *S. pseudintermedius* also had exfoliative toxins which could damage the skin.

Fortunately, the MRSA and MRSP prevalence was low among these carried CPS isolates. One MRSA of animal origin and one BORSA from a veterinarian were detected. Furthermore, three MRSPs from humans and pets each were isolated.

In the case of the dog park study, some of the co-carried isolates were more deeply examined with WGS. The *S. aureus* samples, Q37-Q38 proved to be ST15-t084. ST15 is a widely distributed type in Europe both in humans and animals (283). The genetic resemblance of Q37-Q38 is also mirrored in their virulence and resistance genes, not just in their genetic typing results. The Q47-Q85 *S. aureus* samples were highly similar,

but not completely identical. As these were isolated in different towns, from unrelated hosts, they could have acquired various genes and mutations there. Both were ST45 which is a frequent ST in Europe (283). We also identified a *S. pseudintermedius* co-carriage in an owner and her dog (Q81-Q82). These bacteria had identical genome which supports the literature data that also humans can be colonized by *S. pseudintermedius* asymptomatically.

These findings also support the theory that pets can be the source of human infections because they can act as reservoirs of CPS. Furthermore, it seems like that some major clonal types (like ST45) are circulating in the community in Hungary. Although this study has its limitations, it certainly draws attention to the fact that the bacterial flora of pets can be an infection risk for certain patient groups (172).

The *mecC*-MRSA isolate found here was very similar genetically to those isolated in Northern-European countries, however when it comes to comparing the carriage rates it was much lower in Hungary (39,43). The majority of *mecC*-MRSA samples from livestock have also been identified in Northern Europe (264,313,314). This finding seems to further support the idea that *mecC*-MRSA strains have clonal spread. The *mecC*-MRSA strain cultivated from the hedgehog screening, carried a novel exfoliative toxin (*etE)*. This hypothetically can also bind to human desmoglein (83), and lead to toxin-mediated skin diseases in humans. Although there has not been any clinical evidence to support this theory so far.

This *mecC*-MRSA strain – just like the majority of the *mecC* isolates - was sensitive to all examined antibiotics except the ß-lactams. Even so, monitoring the antibiotic susceptibility in *mecC*-MRSA isolates is necessary as they may acquire resistance genes along the way.

The main limitation of our study is the limited source of hedgehogs; therefore, no strong conclusions can be drawn on the prevalence and geographic distribution of *S. aureus*/MRSA in hedgehogs for the whole country. Furthermore, other potential hosts, including companion animals and livestock, should be investigated in the future.

Based on our results and the data already available in the literature (233), the risk of contracting the *mecC*-MRSA from an animal source is currently highly unlikely in

Hungary. Nevertheless, awareness is needed as *mecC*-MRSA could be overlooked or misidentified in the clinical microbiology laboratories and ultimately that could help the spread of this resistance gene and *mecC*-MRSA could potentially emerge as a novel human pathogen (173).

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6.1. The novel findings based on this thesis are the following:

1. This is the first study in Hungary about the prevalence of the two major important coagulase-positive staphylococci: *S. aureus* and *S. pseudintermedius* among pets and their owners as well as veterinary personnel.

2. Based on the high genomic similarities, resistance and virulence patterns, we have provided evidence for potential transmission of staphylococci between humans and dogs.

3. Methicillin resistance was very rare among both investigated staphylococcal species. Only one MRSA was found during the study (in a dog), one BORSA isolated from a veterinarian, and six MRSPs in total from humans and dogs.

4. Higher antibiotic susceptibility rates were detected among the carried isolates compared to the contemporary human clinical isolates.

5. Virulence factors (toxin genes, enzymes, adhesion factors) were found in both human and animal-originated staphylococcal isolates, and a novel exfoliative toxin gene (*etE*) was detected in hedgehogs.

6. Based on PFGE, MLST and *spa*-typing, several well-known international clones (both from carriage and clinical origin) could be detected also in this study.

7. Some of the *S. aureus* clones (e.g., ST45) were described several years ago in our human carriage studies, indicating the long-term circulation of these clones in Hungary.

8. This is the first survey of *Staphylococcus spp*. carriage among wild animals in Hungary.

9. This is the first report on the occurrence of *mecC*-MRSA in Hungary.

10. Prevalence of *mecC*-MRSA seems to be much lower in Hungarian hedgehogs compared to Northern European observations. However, due to the relative frequent direct contact between these animals and people, coupled with the fact that *mecC*-MRSA will very likely be described in domestic animals sooner or later, this bacterium might emergence as a potential novel human pathogen as well.

7. SUMMARY

Staphylococcus aureus and *S. pseudintermedius* are both coagulase positive staphylococci (CPS), and both can cause life-threatening diseases in humans and animals. Especially MRSA strains are hard to treat. This type of resistance linked to the *mecA* gene, but in 2011, a new homologue, *mecC* was described. Very high prevalence of *mecC*-MRSA has been found among hedgehogs in Sweden and Denmark.

In this study, we aimed to survey pets, and their owners for CPS colonization and the hedgehog population for *mecC*-MRSA in Hungary. We have screened 184 pets and 187 humans, as well as 200 hedgehogs. Resistance and virulence genes were detected by PCR and the antibiotic susceptibility was tested. To determine the clonality of isolates, PFGE, MLST, *spa* typing and WGS was used.

Carriage rate of *S. aureus* was between 24%-35% in humans and 5%-15% in dogs, while that of *S. pseudintermedius* was inversely 2%-20% versus 34%-58%. Co-carriages were also detected. In total, 99 *S. aureus* and 104 *S. pseudintermedius* isolates were found. Only one isolate proved to be MRSA, one BORSA, and six MRSPs. High tetracycline resistance was noted in *S. pseudintermedius*, otherwise the strains were mainly sensitive to the tested drugs. Various toxins were detected. The isolates belonged to several different clones, including well known international ones. Some of these were already found in Hungary, indicating their long-term persistence. Of the 200 screened hedgehogs, 13 were carriers of *S. aureus* (6.5%). Among these, one isolate was *mecA*- and one was *mecC*-MRSA. The isolates were sensitive to non-beta-lactam antibiotics. The majority carried genes accountable for adhesion and biofilm production. The *mecC*-MRSA carried a novel exfoliative toxin (*etE*) and was a single-locus variant of ST130. Based on the genomic data, antibiotic susceptibility and virulence genes, dog and owner co-carried strains displayed only insignificant differences, hence provided evidence for potential interspecies transmission. Our findings seem to strengthen the theory that pets can serve as reservoirs for human CPS infections.

Finally, this is the first report of *mecC*-MRSA and the first survey of Staphylococcus carriage among wild animals in Hungary. The *mecC* prevalence was much lower than in Northern European countries and more like countries in our region. *MecC*-MRSA could emerge as a novel human pathogen in the future, especially where close contact occurs between humans and animals.

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8. ÖSSZEFOGLALÁS

A *Staphylococcus aureus* és *S. pseudintermedius* a két leggyakoribb koaguláz-pozitív Staphylococcus (KPS), minkettő képes életveszélyes fertőzést is okozni emberekben és állatokban is, melyek sokszor nehezen kezelhetők a széleskörű rezisztencia miatt. Leginkább az MRSA törzsek jelentenek problémát. Ennek genetikai alapja a *mecA* gén, de 2011-ben leírták egy új változatát, a *mecC*-t. Feltűnően magas volt a *mecC*-MRSA-k előfordulása svéd és dán sünök között.

Jelen kutatás célja elsősorban a KPS hordozás felmérése volt állatokban és gazdáikban, illetve sünök szűrése *mecC*-MRSA-ra Magyarországon. 184 társállatot, 187 embert és 200 keleti sünt szűrtünk. A rezisztencia és virulencia géneket PCR-rel mutattuk ki, az antibiotikum érzékenység meghatározására is sor került. A genetikai rokonság megállapítására PFGE-t, MLST-t, spa tipizálást és WGS-t alkalmaztunk.

A *S. aureus* hordozás 24%-35% volt emberekben és 5%-15% kutyákban, míg a *S. pseudintermedius* hordozás éppen fordítva: 2%-20% illetve 34%-58% között alakult. Állat-gazda közös hordozásokat is találtunk. Összesen 99 *S. aureus* és 104 *S. pseudintermedius* törzset izoláltunk. Ezek közül csupán egy volt MRSA, egy BORSA, és hat MRSP. A S. *pseudintermedius* izolátumok magas tetracyclin rezisztenciát mutattak, egyébiránt jellemzően érzékenyek voltak az antibiotikumokra. Többféle toxint is ki tudtunk mutatni PCR-rel. A törzsek genetikai diverzitást mutattak, több ismert, illetve hazánkban már leírt klónt is detektáltunk. A 200 sün közül 13 volt *S. aureus* hordozó (6.5%). Ezekből egy törzs volt *mecA* és egy *mecC*- MRSA. A törzsekre jellemző volt az adhéziós faktorok és biofilm gének jelenléte. A talált *mecC*-MRSA törzs a leggyakoribb ST130-tól egy nukleotidban különbözött, továbbá egy nemrég azonosított exfoliatív toxin gént (*etE*) is hordozott. A kutyák-gazdák által közösen hordozott KPS törzsek klonalitása, antibiotikum érzékenysége és hasonló virulencia gén mintázata bizonyítékul szolgálhat a gazdafajok közötti baktérium átvitelre, tehát a társállatok humán KPS fertőzések forrásai is lehetnek.

A vizsgálatok során elsőként mértük föl a KPS hordozást kutyákban, gazdáikban, és vadon élő állatokban Magyarországon, valamint elsőként írtuk le a *mecC*-MRSA előfordulását. Ez utóbbi jóval alacsonyabbnak bizonyult az északi országokénál. A *mecC*-MRSA potenciális humán patogénként is szerepet játszhat a jövőben, különösen ahol emberek és állatok szoros közelségben élnek.

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10. BIBLIOGRAPHY OF THE CANDIDATES'S PUBLICATIONS

10.1. Publications related to the topic of the thesis

Judit Sahin-Tóth, Ervin Albert, Alexandra Juhász, Ágoston Ghidán, János Juhász, Andrea Horváth, Martin C Steward, Orsolya Dobay (2022):
Prevalence of *Staphylococcus aureus* in wild hedgehogs (*Erinaceus europaeus*) and first report of *mecC*-MRSA in Hungary.
Science of The Total Environment, 815:152858. **IF(2020)=7.963**

Judit Sahin-Tóth, Eszter Kovács, Adrienn Tóthpál, János Juhász, Barbara Forró, Krisztián Bányai, Orsolya Dobay (2021).
Whole genome sequencing of coagulase positive staphylococci from a dog-and-owner screening survey.
PLOS One, 16(1):0245351. **IF(2020)=3.240**

10.2. Publications not related to the topic of the thesis

Andrea Horváth, Orsolya Dobay, **Judit Sahin-Tóth**, Emese Juhász, Júlia Pongrácz, Miklós Iván, Kristóf Kristóf (2020).
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