Aus dem Institut für Mikrobiologie und Tierseuchen
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Comparative genotypic characterization of Methicillin-resistant and-susceptible *Staphylococcus pseudintermedius* of feline and canine origin in Germany

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
Yassmin Sayed Abou-Elnaga
Tierärztin aus Kairo, Ägypten

Berlin 2017
Journal-Nr.: 3917

mbvberlin | mensch und buch verlag

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Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Deskriptoren (nach CAB-Thesaurus):
cats; dogs; horses; *Staphylococcus pseudintermedius;* methicillin; drug susceptibility; drug resistance; genotypes; genetic variation; virulence factors; toxins; pulsed field electrophoresis; polymerase chain reaction; germany

Tag der Promotion: 31.03.2017

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-868-9
Zugl.: Berlin, Freie Univ., Diss., 2017
Dissertation, Freie Universität Berlin
D 188

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verlag@menschundbuch.de – www.menschundbuch.de

Für Meine Eltern

إلى أبي و أمي

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List of abbreviations

Abbreviation	Definition
°C	Degree Celsius
ABC- transporter	Group of ATP-binding and membrane spanning transport proteins
ack	Gene encoding acetate kinase
agr	Accessory gene regulator
AIP	Auto-inducing peptide
*bla*Z	beta-lactamase encoding gene
bp	Base pair
BW	Baden-Württemberg
ccr	Cassette chromosome recombinase
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase negative staphylococci
*cpn*60	Gene encoding Chaperonin 60
CPS	Coagulase positive staphylococci
DDW	Double-distilled water
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
dru	Direct-repeat unit
EF	Elongation factor
EMC	Extracellular matrix components
ETB	Exfoliative toxin type B (*S. aureus*)
exi	Gene encoding an exfoliative toxin (*S. pseudintermedius*)
ExpA	Exfoliative toxin type A (*S. pseudintermedius*)
ExpB	Exfoliative toxin type B in (*S. pseudintermedius*)
fdh	gene encoding formate dehydrogenase
G	Relative centrifugal force, gravity force
g	Gram
GC	Guanine-cytosine content (DNA)
h	Hour
ICD	Implantable cardioverter defibrillator
IgG	Immunoglobulin G
IMT	Institute of Microbiology and Epizootics
Kb	Kilobases
LS	Lower Saxony
Luk	Leukotoxin
m	Milli
*mec*A	Gene encoding methicillin resistance
MGEs	Mobile genetic elements
MIC	Minimum inhibitory concentrations
min	Minute
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
mM	milli-mol
mm	Millimeter

Abbreviation	Definition
MP	Mecklenburg Western Pomerania
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSP	Methicillin-resistant *Staphylococcus pseudintermedius*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
MSSP	Methicillin-susceptible *Staphylococcus pseudintermedius*
µ	Micro
ng	Nano gram
NRW	North-Rhine-Westphalia
ORF	Open reading frame
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pta	Gene encoding phosphate acetyltransferase
*pur*A	Gene encoding adenylosuccinate synthetase A
PVL	Panton-Valentine leukocidin factor from *S.°aureus*
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RP	Rhineland-Palatinate
sar	Gene encoding sodium sulfate symporter
SA	Saxony-Anhalt
SCC*mec*	Staphylococcal cassette chromosome *mec*
SE	Staphylococcal enterotoxins
Sec	Second
SH	Schleswig-Holstein
SIET	*Staphylococcus intermedius* exfoliative toxin
SIG	*Staphylococcus intermedius*-group
*sod*A	Gene encoding superoxide dismutase
spa	Gene encoding *Staphylococcus* protein A
sps	Gene encoding cell wall associated protein (*S. pseudintermedius*)
STs	Sequence types(STs)
Tn	Transposon
tuf	Gene encoding elongation factor
VNTR	Variable-number of tandem repeats
WGS	Whole genome sequencing
α	*alpha*
β	*beta*

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1 Introduction

The opportunistic and zoonotic pathogen *Staphylococcus pseudintermedius* is able to cause a wide range of infectious diseases in different companion animal species such as dogs, cats and horses. In the past, coagulase-positive staphylococci (CPS) isolated from animals that differed in their microbiological characteristics from *S. aureus*, namely *S. schleiferi* ssp. *coagulans* or *S. hyicus* were commonly (mis-)diagnosed as *S. intermedius* [1, 2]. Recent molecular taxonomic investigations revealed that *S. pseudintermedius* and not *S. intermedius* is the common causative agent of canine pyoderma [3]. These findings have led to the realization that most canine isolates previously identified as *S. intermedius* should have been classified as *S. pseudintermedius* [3].

S. pseudintermedius is widely distributed among companion animals. The relationship between companion animals and their owners has changed from working animals fulfilling a duty (e.g. herding sheep, watching the property, hunting rodents) to pets living in the household with similar levels of contact and attention provided only to human members of the family. This development was exemplarily shown by a recent study that showed that 69% of the 108 voluntarily participating dog owners allow their dogs to rest on the sofa, 40% allowed them on their beds and 53% let the dogs lick their face [4]. As a result of these changes, an elevated risk for transmission of microorganisms in terms of pathogenic and/ or drug resistant bacteria from pets to humans and vice-versa can be assumed, which provides new risks for public health in general. Consistent with this, first reports on human patients suffering from severe infections mediated by *S. pseudintermedius* were published [5-7], indicating at least a zoonotic potential for these bacteria.

Methicillin resistance is a prime example when it comes to describing the rising problem of antibiotic resistance factors accumulating in more or less pathogenic bacteria [1, 3, 8]. Methicillin resistance has been described as a “gateway” for antibiotic resistance [9], which is frequently associated with other resistance factors leading to a multidrug-resistant phenotype [1]. In the presence of ineffective antibiotic therapies, chances for mutational events and/or acquisition of mobile genetic elements increase, putatively leading to a general rise in multi-drug resistant bacterial phenotypes [9]. In the recent past the proportion of methicillin- resistant *S. pseudintermedius* (MRSP) in clinical samples of small animal and horses has increased, especially in specimens from wound infections and dermatitis [10]. Studies have shown that the *mec*A-encoded beta-lactam resistance in *S. pseudintermedius* (MRSP) is emerging in veterinary medicine worldwide [11, 12]. In addition, MRSP seems to be frequently associated with further resistance mechanisms towards different groups of antimicrobial agents, resulting in limited or even missing therapeutic options [13-15].

The successful treatment of MRSP-infected animals as well as the prevention of zoonotic spread is a major challenge to veterinarians worldwide [11]. As a result, animals are at risk for developing long-term suffering from infections or being euthanized for reasons of animal welfare [10, 14-16]. Therefore, the sudden emergence of a multidrug-resistant zoonotic pathogen frequently occurring in companion animals like MRSP requires more rapid detailed molecular and epidemiological studies [17].

The aim of this study was to determine the genetic relationship between methicillin-susceptible and –resistant *S. pseudintermedius* isolates (MSSP and MRSP) from specimens obtained from different body sites of companion animals and various regions in Germany isolated as part of an epidemiological study carried out by by Anja G. Brandenburg (IDEXX VetMed Lab, Ludwigsburg). In total, 220 *S. pseudintermedius* isolates were molecular-biologically characterized with respect to the three following main parameters:

A- Confirmation of methicillin-resistance by PCR, detection of important virulence factors including genes encoding leukotoxin (*luk*S, *luk*F) and exfoliative toxins (*exp*A, *exp*B)

B- Description of current MRSP and MSSP genotypes occurring in different regions in Germany

C- Determination of phylogenetic relationship of *S. pseudintermedius* circulating in Germany by pulsed-field gel electrophoresis (PFGE) and Multi-locus sequence typing (MLST)

2 Literature

2.1 Genus *Staphylococcus*

The genus *Staphylococcus* belongs to the Phylum Firmicutes, class Bacilli, order bacillales, family *Staphylococcaceae* [18], which currently contains 52 species and 28 subspecies, (http://www.bacterio.net/staphylococcus.html, 01/05/2016).

Staphylococci are Gram-positive, non-motile cocci (0.5-1.5 µm diameter), often clustering in grape-like structures [19, 20]. The name S*taphylococcus* is based on the grape-like appearance of the cells in the microscopic view (greek: Staphyle = grape) [19].

The cell wall structures of staphylococci include peptidoglycans and teichoic acid and a Guanine + Cytosine (GC) nucleotide content of deoxyribonucleic acid (DNA) of 30 – 40 mol% [21].

Staphylococci are mainly facultative anaerobes (with some exceptions as *S. aureus* ssp. *anaerobius*) [20, 22], do not form spores, and generally grow on simple and common culture media. Colonies are usually white to greyish-white or light –yellow, with regular edges [23]. These bacteria are characterized by a very high environmental tenacity, especially against dryness, which allows the individual cell to survive unfavorable conditions for several months [24].

Staphylococci are usually oxidase-negative and catalase-positive, the latter is the most important method for an easy-to-perform and first distinction between Staphylococci and Streptococci, which are not able to produce the enzyme catalase [19].

Staphylococci are common among the commensal bacteria residing on the skin and mucous membranes of humans and various animal species [20]. With respect to their medical importance, it is useful to differentiate between coagulase-positive (CPS) and coagulase-negative staphylococci (CNS). CPS include *S. aureus, S. pseudintermedius, S. intermedius, S. schleiferi subsp. coagulans, S. hyicus, S. lutrae* and *S. delphini*, and these bacteria were generally considered as being able to cause opportunistic infections in human and various animal species [19]. The bacterial coagulase produced by CPS is an important virulence-associated factor, since the enzyme enables the bacteria to coagulate blood plasma of mammals by inducing the conversion of fibrinogen to its activated form fibrin [19]. This activation is considered to be an important initial step towards adhesion and attachment of CPS within the invaded body, promoting the establishment of staphylococci during the first stages of infection. Later, the coagulum protects the staphylococci from the activities of defensive cells. Once established at the initial infection side, expression of a further enzyme (staphylokinase) enables the bacteria to escape the self-induced fibrin-mediated wall. Later, other virulence-associated factors such as hyaluronidase (spreading factor), lipase, collagenase, protease, nuclease and urease, all may play additional roles in the pathogenesis of staphylococcal infections [23].

2.2 Staphylococci of the intermedius-group (SIG)

Until 2005, most CPS originating from companion animals was diagnosed as *S. intermedius*. However, in recent years, phylogenetic analysis revealed that staphylococci regarded as *S. intermedius* include at least three distinct species, the so-called *Staphylococcus intermedius*-group (SIG). The members of this group include *S. intermedius*, *S. delphini* and *S. pseudintermedius* [25, 26]. Sequence analysis of the respective 16S rRNA genes revealed a 99% sequence similarity of the species within this group [22]. At present, a distinct differentiation between *S. intermedius*, *S. delphini* and *S. pseudintermedius* based on phenotypical properties including use of commercial identification tools is difficult and may lead to unreliable results [22, 26]. SIG are the most common coagulase-positive staphylococcal species (CPS) isolated from companion animals like dogs, cats and horses [27, 28]. More detailed information on the different species composing the SIG is given below.

In the following chapters, the literature review includes studies in the past when the members of the SIG were not known or not distinctly respected. Thus, to avoid confusion of the reader, these references have been reported as referring to the "*Staphylococcus intermedius*"- group (SIG).

For decades, most CPS were identified as *S. aureus*. In 1976, Hajek described a novel CPS species denominated as *Staphylococcus intermedius* isolated from specimens obtained from pigeons, dogs, mink and horses, previously described as *S. aureus* biotype E and F [29]. The original name "intermedius" derived from the biochemical properties of this species, which were somehow regarded as being "between" *S. aureus* and *S. epidermidis* [29, 30]. These bacterial species were described as being facultative anaerobes growing as slightly convex, round, smooth, shiny, translucent, white-grey or un-pigmented colonies [29].

In the past, staphylococcal isolates diagnosed as *S. intermedius* were regarded as common commensal bacterial species among the microbiota of dogs and a prototype of an opportunistic pathogen. Strains considered as *S. intermedius* were also isolated from diagnostic materials obtained from further animal species including cats and goats [25, 29, 31, 32].

S. intermedius was regarded as a major cause associated with the broad symptom complex of canine pyoderma, a topic of utmost importance in the field of small animal dermatology [3]. Similarly, *S. intermedius* was assumed to play an important role in staphylococcal-induced cases of otitis externa in dogs [33].

In addition, there had been some reports about human infections due to *S. intermedius*, including infected dog bite wounds [34-36], bacteraemia, sinusitis, otitis media and septic arthritis [35, 37].

However, as indicated in the introduction of this section, discrimination among the distinct members of the *S. intermedius* group (SIG) has not been performed in the past, and *S. pseudintermedius* was presumptively frequently misdiagnosed as *S. intermedius* [25, 26, 38]. At present, *S. pseudintermedius* seems to be common and widely distributed in microbiological samples of canine origin, while *S. intermedius* is only very rarely isolated and seems to be more or less exclusively associated with samples originating from pigeons [25, 26].

Varaldo et al. 1988 first isolated the bacterial species *S. delphini* from purulent skin lesions of dolphins, and it has rarely been reported since [39, 40]. This bacterium can also be isolated from wound infections in horses, cattle, mink, pigeons in addition to dolphins [26]. Usually, the colonies were described as having a diameter of five to seven mm, with a round and shiny form, a smooth and slightly convex surface and a colour ranging from opaque to translucent. This species was described as coagulase- and catalase-positive but negative for the clumping factor test. Knowledge about the prevalence and clinical significance of *S. delphini* is still scarce, and differentiation from *S. intermedius* and *S. pseudintermedius* is challenging due to their phenotypic similarities [22, 25, 26]. Study results from Sasaki et al. (2007) and Youn et al. (2011) showed that *S. delphini* seems to be uncommon among CPS originating from dogs or cats [26, 41]. In contrast, all SIG strains obtained from horses belonged to this particular species (Sasaki et al. [26]).

2.3 *Staphylococcus pseudintermedius*

Based on growth characteristics, biochemical features and DNA-DNA hybridizations, Devries et al. described a novel *Staphylococcus* species denominated as *Staphylococcus pseudintermedius* in 2005 [22]. 16S rRNA sequencing results of this species revealed that the closest phylogenetic relatives are *Staphylococcus intermedius* and *Staphylococcus delphini*.

As a consequence, isolates formerly regarded as *S. intermedius* were divided into three distinct species composing the *S. intermedius* group (SIG): *S. intermedius*, *S. pseudintermedius* and *S. delphini* [25, 26]. In addition, this re-classification revealed that *S. pseudintermedius* and not *S. intermedius* is the major pathogen associated with the symptom complex of canine pyoderma [25] and represents a major opportunistic pathogen in dogs and cats [14].

Of late, much more knowledge on *S. pseudintermedius* as a common inhabitant of the skin and mucosa being frequently isolated from nares, mouth, forehead, groin and the anal region of healthy dogs and cats is available [11, 15, 42]. SIG isolates originating from equine samples were mostly identified as *S. delphini,* and *S. pseudintermedius* seems to be not widely distributed among horses in general [43]. However, sporadic cases were reported [44]. Since *S. pseudintermedius* is considered a typical opportunistic pathogen, it is able to cause infectious diseases of the skin, the ears as well as of many other body tissues, including soft tissues, and represents an important cause of (post–operative) wound infection in small animals like dogs and cats [45, 46].

2.3.1 Virulence factors of *S. pseudintermedius*

Recently, the first two complete genome sequences of *S. pseudintermedius* (ED99, HKU 10-03) were published contributing to the analysis of the virulence potential and special host adaptation of this versatile opportunistic pathogen [47, 48]. Sequence analysis of ED99 and HKU10-03 yielded numerous putative virulence factors including leukocidins, exotoxins, haemolysins, exoenzymes, predicted superantigens and adherence factors [47, 48].

2.3.1.1 Excreted virulence-associated factors

S. pseudintermedius is a CPS species and possesses various virulence factors including some which are similar to those described for *S. aureus* [49, 50] such as thermonuclease and proteases as well as surface associated proteins like clumping factor A and B and protein A.

Different toxins including haemolysins, enterotoxins as well as exfoliative toxins can be produced by *S. pseudintermedius*, resulting in significant damage of affected host tissues [49, 51]. *S. pseudintermedius* commonly produce alpha- (pore-forming toxin) and beta-haemolysins (phospholipase C; specific for sphingomyelin) causing haemolysis of erythrocytes (e.g. of rabbit origin) and also the characteristic hot-cold haemolysis on plates containing sheep erythrocytes [51].

While production of exfoliative toxins and their destructive effect on human skin was firstly described for *S. aureus* strains by Dajani in 1972 [52], proteins inducing similar negative effects on the structural integrity of the canine skin produced by SIG were reported by Hesselbarth et al. in 1994 [53]. Thus, exfoliative toxins are important virulence factors known to contribute significantly to the symptom complex referred to as canine pyoderma in veterinary dermatology [54, 55].

In recent years, a novel toxin designated as “EXI” (encoding an exfoliative toxin in *S. pseudintermedius*), which seems to be closely related to the exfoliative toxin B (ETB) of *S. aureus,* was detected in 23.3% of 43 *S. pseudintermedius* isolates from cases of canine pyoderma [56]. Furthermore, exfoliative skin lesions in neonatal mice were artificially induced by subcutaneous injections of the recombinant EXI protein [56].

A further exfoliative toxin (*Exp*B) was recently described for *S. pseudintermedius*, possessing the ability to disrupt single cell–cell adhesion molecules (desmoglein-1) within the canine epidermis, causing the typical histological pictures of intra-epidermal splitting [55].The authors recommended that the toxin formerly designated as “EXI” in *S. pseudintermedius* should be renamed as *Exp*A in accordance with the denomination of the second exfoliative toxin *Exp*B [55].

In the past, a further factor excreted by SIG, referred to as *Staphylococcus intermedius* exfoliative toxin (SIET), has been regarded as an important exfoliative toxin [57]. The authors reported that the typical clinical signs of canine pyoderma (crusts, erythema and exfoliation) were developed after the injection of SIET in canine skin [57]. However, a further study reported that after intradermal injection of recombinant SIET in canine skin no typical histological or clinical lesions were observed [58]. Thus, the biological importance of SIET is still uncertain [57, 58].

S. pseudintermedius also produces a beta-pore-forming, bi-component cytotoxin known as LukI which is very similar to the Panton-Valentine leukocidin (PVL)-factor described for *S. aureus.* This factor is encoded by two co-transcribed genes (*luk*S and *luk*F) [31]. LukI exhibits a strong leukotoxicity on various polymorphonuclear cells of canine origin. The leukotoxicity titer of LukI was found to be 129-fold higher in canine polymorphonuclear cells compared to those of pigeon origin [31]. Furthermore, the haemolytic activity of LukI on rabbit erythrocytes is very strong [59]. At present, knowledge is scarce on the prevalence and distribution of LukI within the *S. pseudintermedius* population and its potential contributing role in the pathogenesis of important diseases such as canine pyoderma [39].

S. pseudintermedius is able to express different staphylococcal enterotoxins (SE) such as SEA, SEB and SEC and the toxic shock syndrome toxin [60]. In canine pyoderma, SEC is frequently detected either individually or in combination among SIG isolates [60]. In general, enterotoxins produced by staphylococci are known as strong immune-activating super antigens that induce a non-specific T cell-proliferation and a typical cytokine storm inducing undirected damage within different host tissues [61].

In 1991, an enterotoxigenic SIG strain producing type A enterotoxin was identified as the etiological agent of a food-associated disease outbreak in the western United States by PFGE, comprising 265 cases in total [62]. However, the enterotoxigenic potential of *S.* (*pseud*)*intermedius* has not been fully clarified yet.

2.3.1.2 Surface-associated factors

Initial adherence of pathogenic bacteria to host tissues is a common requirement for colonization and infection, and the ability of *S. pseudintermedius* to adhere to canine, feline and human corneocytes were investigated in various studies [63-65]. In addition, *S. pseudintermedius* is known as a strong biofilm former [51].

S. pseudintermedius can also adhere to several different host extracellular matrix components (EMC) [47, 66, 67], which are targeted by microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [68]. For instance, a putative protein A homologue (encoded by *spa*) was identified in *S. pseudintermedius*. Protein A has been described as an important virulence factor in *S. aureus*, possessing four to five immunoglobulin-binding domains able to bind the FC-part of immunoglobulin G (IgG), interfering with opsonisation and phagocytosis of the bacteria by immune cells of the host immune system [69]. However, further investigation is warranted to unravel the distinct role of protein A as a virulence factor in *S. pseudintermedius* [69].

Analysis of strain ED99 revealed 18 open reading frames (ORFs) encoding putative surface proteins, which were named as \`Sps\` (*Staphylococcus pseudintermedius* surface proteins), followed by a capital letter (SpsA to SpsR) [70]. These predicted proteins have hallmark features of MSCRAMM's [70-72]. At the cell surface of ED99, three Sps (SpD, SpsL and SpsO) were expressed. SpsD and SpsL mediate adherence to proteins of the host extracellular matrix and presented a certain affinity to canine fibrinogen [70]. Both SpsD and SpsO also confer adherence to canine corneocytes [67].

At present, knowledge about the structure and function of *S. pseudintermedius* adhesins is limited. Some studies reported that SIG were able to adhere to fibrinogen, fibronectin, cytokeratin 10, elastin, collagen type I, vitronectin and laminin [66, 73].

2.3.1.3 Global regulation of gene expression in SIG

The accessory gene regulator (*agr*), firstly described for *S. aureus* in 2002, is part of a staphylococcal quorum-sensing system and acts as a global regulatory system, optimizing the expression of different bacterial virulence factors under certain conditions in order to enhance colonization and infection properties [74, 75]. DNA sequence analysis of the *S. pseudintermedius-agr* locus revealed an operon comprising of five open reading frames, *agr*B, *agr*D, *agr*C, *agr*A and *hld*, representing the classical two component regulatory system, including *agr*D encoding a short auto-inducer peptide (AIP) [76]. In 2006, Sung et al. identified three *agr*D alleles, which are similar to *agr* specificity groups, encoding different AIP variants in canine SIG isolates. The biological activity was elucidated for two of them [33].

A further broad study among SIG verified these three predicted AIPs as well as a further AIP variant (I to IV). However, AIP I-III were found for each of the three closely related members of the SIG, indicating that a common quorum-sensing capacity has been conserved in spite of species differentiation in distinct ecological niches [25].

2.3.2 Phenotypic identification of *S. pseudintermedius*

Colonies of *Staphylococcus pseudintermedius* are of medium size (3-4 mm), convex and have a translucent greyish-white appearance, which is typical for staphylococci [22]. On agar plates containing red blood cells of sheep or bovine origin, this staphylococcal species usually exhibits a large incomplete haemolysis zone, and a smaller "margin" of complete haemolysis in the proximity of the colony, a phenomenon known as "double-zone" haemolysis [39]. Horse blood agar is not recommended for primary isolation of *S. pseudintermedius*, because no haemolytic activity was observed on this medium [39]. The characteristically appearance on sheep blood agar helps to distinguish between the two CPS species *S. pseudintermedius* and *S. aureus*. The latter exhibits a variable colour (greyish-white to light-yellow) and, by comparison, a smaller margin of incomplete haemolysis after 18h of incubation (**Figure** 1).

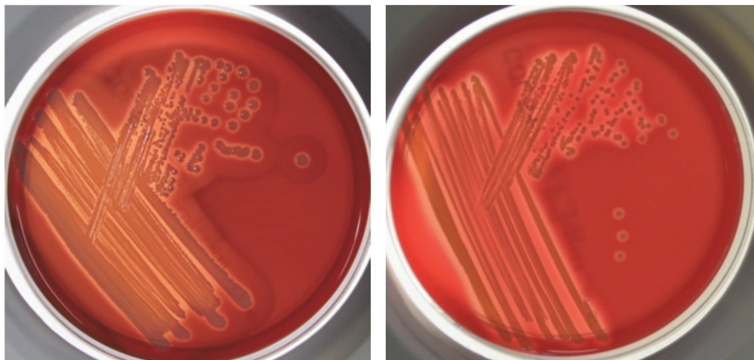


Figure 1: Characteristic "double-zone" hemolysis of *S. pseudintermedius* (left) on sheep blood agar. In contrast, hemolytic *S. aureus* usually lacks a zone of incomplete hemolysis (right). These pictures provided by Yassmin Abou-Elnaga and Andrea Schmidt.

Since *S. pseudintermedius* is, by comparison, a relatively new described species, possibilities for fast and accurate identification depend on the establishment of a reliable SIG reference collection, which is not available to date [11]. *S. pseudintermedius* isolates were commonly negative in both the rapid slide clumping factor test and the latex agglutination test identifying protein A in *S. aureus* [77]. This may lead to misidentification of *S. pseudintermedius* as CNS, particularly in human diagnostic laboratories [78].

Consequently, sound phenotypical identification of *S. pseudintermedius* is often challenging and isolates might be misidentified as *S. aureus* or *S. intermedius* [5, 79] or, in case of *S. pseudintermedius,* erroneously identified as coagulase–negative staphylococci [78]. The following example illustrates this problem: A methicillin-resistant *S. (pseud)intermedius* was initially misidentified as MRSA because the identification as *S. aureus* was solely based on a positive tube coagulase test [2]. The isolate was then re-identified as *S. intermedius*, but it was probably a *S. pseudintermedius* isolate since the source of this isolate was a dog [11]. Consequently, molecular-based methods are recommended for species identification of the members of the SIG at present [11].

2.3.3 Molecular identification techniques for *S. pseudintermedius*

After re-classification of the SIG in 2005, a study from Japan reported the usefulness of partial *sodA*- and *hsp*60 gene sequencing for distinct discrimination between *S. intermedius, S. pseudintermedius* and *S. delphini* [2, 26].

A multiplex PCR approach was published for species differentiation of CPS, targeting the thermonuclease (*nuc*) gene locus. To evaluate sensitivity and specificity, this PCR method used staphylococcal strains that had been previously identified at the species level by *hsp*60 sequencing, and rates of 99.8% (sensitivity) and 100% (specificity) were reported [80]. However, this method may have some limitations, since this very simple approach has not established itself to date as a “key tool” for CPS differentiation in the field of veterinary microbiological diagnostics.

A further simple but effective approach to identify *S. pseudintermedius* utilizes polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP). This method is based on the fact that the *pta*-gene of *S. pseudintermedius* harbors a single *Mbo*I restriction site, while a comparable site is absent in *S. intermedius* and *S. delphini* [3].

However, a heterogeneity of the *Mbo*I restriction site was reported for a small fraction of the *S. pseudintermedius* population (approximately 1%), resulting in either non-cutting or an additional band [81].

2.3.4 Genotypic typing techniques of *S. pseudintermedius*

Typing methods used for identification of different bacterial isolates are important epidemiological tools in the field of infection prevention and control. The development of different molecular methods in the second half of the last century has provided new tools applicable for surveillance, outbreak investigation and enhanced dramatically our general understanding of pathogenesis and transmission of bacteria. Nowadays, a broad range of molecular typing methods are available for the molecular characterization of methicillin-resistant staphylococci, which basically differ in their applicability and discriminatory validity.
Typing methods developed for *S. pseudintermedius* include pulsed-field gel electrophoresis (PFGE) analysis [69, 82], *Staphylococcus* protein A (*spa*) typing [69], staphylococcal cassette chromosome *mec* (SCC*mec*) typing and Multi-locus sequence typing (MLST) [25]. However, none of these techniques is suitable for all forms of investigation and scientific questions.

2.3.4.1 Pulsed-Field Gel Electrophoresis (PFGE)

(PFGE) was regarded as the ‘gold standard’ among molecular typing methods for a variety of clinically important bacteria for decades [83]. In the 1990s, this technique was adopted for most bacterial species as a sufficient epidemiological tool [84, 85]. To date, it has been considered as one of the most discriminatory typing methods and in particular indicated for surveillance and outbreak investigations [39].
To perform PFGE, a purified bacterial DNA is cleaved with a restriction endonuclease (e. g. SmaI, ApaI) that recognizes infrequently distributed chromosomal restriction sites among the genome of the bacteria. The resulting high molecular-weight restriction fragments can be separated on an agarose gel by pulsed-field gel electrophoresis [86, 87]. For each bacterial isolate, a certain PFGE pattern can be obtained (“molecular fingerprint”), and further comparative analysis is possible with suitable software [87].
At present, a standardized protocol for *S. pseudintermedius* does not exist. Since a PFGE protocol was developed for *S. aureus* [88], this protocol has been used as a “basic concept” for the development of PFGE protocols for *S. pseudintermedius* in different studies [14, 27]. However, differences between individual PFGE protocols and interpretation rules led to difficulties with respect to comparison of results between studies and communication of PFGE data between laboratories [39]. As a consequence, PFGE is generally not suitable for long term epidemiological surveillance [11].

The major drawbacks associated with PFGE are the time-consuming and technical demands of the procedure, the costs of the reagents and the equipment. However, the largest challenge is interpretation of results, as with any band-based technology. It is complicated and requires experience, although published guidelines for the interpretation of bands has made it simpler to associate the PFGE results with available epidemiological data in order to conclude on the potential epidemiological relationship between strains [89, 90].
In general, interpretation of DNA fragment patterns obtained by PFGE analysis is possible with respect to two clearly different points of view: a) an assumed genetic relationship of isolates possessing similar restriction sites among their genome resulting in similar PFGE patterns and b), a potential epidemiological relationship of individual isolates, if further information with respect to time, place and patient are available. In the latter case, PFGE pattern of isolates which are closely related might be indistinguishable (= similar) or differ in one to three bands, reflecting a very limited number (usually: one) of genetic changes affecting the genomic restriction sites. Genetic events such as point mutations, insertions and/or deletions of DNA might change the resulting PFGE pattern. Accordingly, a stratification system for PFGE results was published by Tenover et al. in 1995. Three categories were created to classify isolates according to their PFGE profile with respect to an outbreak situation: indistinguishable from the outbreak pattern (I) closely or possibly related to the outbreak pattern (II) and unrelated to the outbreak pattern (III). The robustness and discriminatory ability of the criteria will be unknown if PFGE resolves fewer than 10 distinct fragments [90].

2.3.4.2 Staphylococcus protein A (*spa*) typing

Spa typing was first described in 1996 as a typing method for *S. aureus* [91]. It is based on sequence analysis of the hyper-variable region (X region) of the *spa* locus, encoding protein A [91]. In general, *spa* typing is less time consuming and simplifies the communication between laboratories. Furthermore, *spa* typing data can be stored in a database, available at any time for further analysis or new scientific questions [39]. However, s*pa* typing does not have the resolving power of PFGE sub-typing [92].

In 2009, Moodley et al. published a species-specific *spa* typing protocol for *S. pseudintermedius* [69]. This approach was intended to realize the similar principles which were used for *spa* typing of *S. aureus* [93]. However, *spa* typing turned out to be an ineffective method when typing methicillin-susceptible *S. pseudintermedius*, because a significant number of isolates turned out to be “non-typeable”. Some *S. pseudintermedius* harbor more than one *spa* gene, resulting in multiple non-specific bands which inhibit sequencing [69].

2.3.4.3 Multi-Locus Sequence Typing (MLST)

The MLST method has been developed to overcome the poor portability of traditional molecular typing approaches. It is an extension of phenotypic Multi-locus enzyme electrophoresis (MLEE) [94], which depends on differences in the electrophoretic mobility of enzymes present in a bacterium [83]. The first MLST scheme was developed for *Neisseria meningitides* in 1998 [95]. Since then, MLST has become a very useful tool for global epidemiological studies, including molecular evolution of pathogens [96-100].

This technique is based on partial sequencing (usually: 450–500 bp) of generally seven to eight housekeeping genes. These genes (including their distribution within the genome) were specifically chosen to provide a robust structure of a 'population framework': Isolates exhibiting similar or identical genotypes are considered as very closely related and presumptive descendants from a recent common ancestor [101].

For each MLST locus, each unique sequence is assigned as a distinct allele (indicated by a number) and together these alleles provide an allelic profile, which defines the sequence type (ST) of each isolate [97, 102, 103].

The main advantage of MLST is that all data produced by this approach are unambiguous and electronically portable. Furthermore, the allele sequences and ST profiles are available in large central databases via the internet (f.e. www.mlst.net).

These databases allow investigation of the genetic relatedness of bacterial isolates within a species with high discriminatory power [83]. The application of MLST is huge, and provides a reliable tool for the scientific, public health, and veterinary communities. For instance, the MLST scheme developed for *S. aureus* is among the most frequently used typing method to investigate this opportunistic pathogen worldwide. The disadvantage of MLST is that its discriminatory power is insufficient for outbreak investigation and surveillance [83].

While methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolation rates increased in some areas of the world in the second half of the last decade, information about the genetic structure of the *S. pseudintermedius* population was very limited [82, 104, 105]. A first sequencing approach including three housekeeping genes, namely heat shock protein (*cpn60*), elongation factor (*tuf*) and phosphate acetyltransferase (*pta*) together with the 16S rRNA gene, was developed by Bannoehr et al. and was primarily intended to discriminate the distinct members of the SIG [25]. However, based on the sequencing results for *cpn*60, *tuf*, *pta* and 16SrRNA, an MLST scheme was utilized in the following years. First results showed that two different major genetic lineages seem to spread in Europa (ST71) and North America (ST68).

Since this first scheme was not originally intended to provide MLST data for large population analyses, a more refined MLST scheme based on seven housekeeping genes was recently developed. In addition to *pta*, *cpn*60 and *tuf*, four additional loci [adenylosuccinate synthetase (*pur*A), formate dehydrogenase (*fdh*), acetate kinase (*ack*), and sodium sulfate symporter (*sar*)] were selected to increase the discriminatory power of the original approach [106]. This method allowed detection of multiple STs within MRSP ST68 and revealed the occurrence of methicillin resistance in different genetic backgrounds [39, 106]. In addition, the expanded MLST approach suggests slow evolution among the lineages that have acquired methicillin resistance [39].

2.3.4.4 Staphylococcal Cassette Chromosome (SCC*mec*) typing

Like MRSA, MRSP can be investigated by PCR with respect to the structural components of the *mec*-harboring, potential mobile element Staphylococcal Cassette Chromosome *mec* (*SCCmec*). This element carries different variants of a site-specific recombinase system designated as cassette chromosome recombinases (*ccr*), certain insertion sequences (e.g. IS431), transposons and regulator genes (*mec*I, *mec*R1) [107, 108]. These main components were usually used to characterize SCC*mec* types.

SCCmec elements are by comparison large (21-67 kb) when compared to other classes of mobile genetic elements. These elements harbor the determinant for broad-spectrum β-lactam resistance (PBP2a) encoded by either *mec*A or *mec*C. The emergence of methicillin resistant staphylococcal lineages is a consequence of the acquisition and insertion of the SCC*mec* element into the chromosome of susceptible strains [109].

To date (2014), 11 *SCCmec* types (I-XI) have been identified based on a) allotype of recombinase genes and b) the class of *mec* gene complex, as well as a variety of subtypes depending on variation in the three joining regions (J1-J3), which constitute nonessential components of the cassette (IWG-SCC, 2009) [110].

Many multiplex PCR methods which had been developed for *SCCmec* typing of MRSA have been applied for MRSP characterization in the recent past [111-113]. However, while many *Staphylococcus pseudintermedius SCCmec* elements had been classified as *SCCmec*III in the past [26], other studies found non-typeable SCC*mec* elements as well as mosaic structured SCC*mec* elements. Such a bi-component element (*SCCmec* II-III) seems to be associated with the dominating European MRSP_ST71 lineage [112, 114]. For the major MRSP lineage spreading in North America (ST68), the element *SCCmec*V as well as non-typeable *SCCmec*'s were described [14, 27, 82]. Recently, *SCCmec*V was found to be the most prevalent element among MRSP isolates from South Korea [115].

2.3.4.5 Dru-typing

Variable-number tandem repeat (VNTR) sequence-typing was established as a method providing significant advantages for epidemiological typing purposes of bacterial pathogens. The direct-repeat unit (dru)-VNTR region adjacent to IS431 in *SCCmec* has shown to be useful in the epidemiological analysis of highly epidemic strains and in tracking the horizontal movement of *SCCmec*. Efficient use of dru-typing has been facilitated by a uniform system of nomenclature. However, optimum use of this typing approach requires a convenient interpretation tool for dru-typing results, which is now available online: http://dru-typing.org/site.

Recently, MRSP isolates were successfully dru-typed and confirmed two predominant dru type clusters. The largest was comprised predominantly by dt9a, a type that has been shown to correspond with sequence type ST71. The other predominant type, dt11a, is consistent with the other main MRSP clone, ST68 [116]. Consequently, dru-typing seems to be a further useful tool for MRSP typing and has become an objective, standardized, sequence-based method that is relatively cost-effective and easy to perform.

2.3.4.6 Whole genome sequence analyses (WGS)

At present, the emergence and easy accessibility of benchtop sequencers using next generation sequencing (NGS) technology promotes bacterial whole genome sequencing [83].
WGS is a powerful and attractive tool for epidemiological investigations as well as for routine clinical use with its ability for highly accurate characterization of bacterial isolates [117-120]. In addition to traditional epidemiological applications, WGS can also be used to identify factors associated with virulence and antibiotic resistance in a particular pathogen [121]. A definitive and accurate typing of different bacterial isolates can be done by WGS as this approach has the potential to detect a single base difference between two genomes. WGS has already been used to investigate and trace back in several large outbreak situations in Europa such as the outbreak of a multidrug resistant enterohaemorrhagic *E. coli* (EHEC) O104:H4 in Germany in the recent past [83, 86].
The first whole genome sequencing data on a methicillin-resistant *S. pseudintermedius* strain (E140) was only recently published by Moodley et al. in Denmark (2013) [122]. This strain represents the dominant clonal lineage associated with canine MRSP infections in Europa (ST71). The draft genome of MRSP-E140 has a size of 2,769,487bp, which is 150-200kb larger than the two published MSSP genomes of strain ED99 [47] and strain HKU10-03 [48].

Overall, the genome sequencing of MRSP will simplify future studies to understand the rapid spread and virulence of multidrug resistant *S. pseudintermedius*, including the dominating clonal lineages spreading in Europa, Asia and North America.

2.4 Antibiotic resistance in bacteria: General aspects

Microbes compete with each other for nutritional resources, and a broad range of strategies have evolved to shield the individual habitat from the microbial competitors, including antimicrobial agents. Since the original producers of antibiotics as well as the competitors were forced to develop methods to survive these antimicrobials or to die, resistance mechanisms towards antibiotics have evolved along the evolutionary process. Furthermore, exchange of genetic information harboring resistance mechanisms between bacteria is a common event in order to survive and to adapt to environmental changes in general. The sum of these strategies is commonly referred to as the “environmental resistome”.

Consequently, nearly each antimicrobial agent has a natural progenitor, which was discovered by researchers (e.g. A. Flemming 1928: Penicillin) and extracted from the original producing species (e.g. Florey and Chain 1939) or re-constructed according to a natural template.

After the introduction of antimicrobial drugs as therapeutic agents in the mid 1940´s of the last century, pathogenic as well as saprophytic and commensal bacteria such as staphylococci were found to accumulate and exchange genes encoding resistance factors towards these drugs from other bacteria. The excessive use (and abuse) of antibiotics in agriculture, and in both human and veterinary medicine, has played a critical causative role in the selection of antibiotic resistance in bacteria, which is now recognized as a global public health threat [123].

In general, resistance properties of the organisms can be differentiated in either an intrinsic (“inherent” or "natural" resistance) or an acquired antimicrobial resistance. Intrinsic resistance refers to the innate ability of a bacterial species to tolerate a particular drug or antimicrobial class due to inherent structural or functional characteristics [124]. In contrast, acquired resistance of bacteria is a specific property of certain bacterial populations and/or strains; it can be a result of either the mutation of one or more gene(s) (endogenous resistance) as in fluoroquinolone resistance and/or of the horizontal acquisition of resistance-conferring genes (exogenous resistance). Horizontal spread of antimicrobial resistance is possible, since resistance genes are often located on mobile genetic elements like plasmids and phages [124, 125].

Only a few general resistance mechanisms have been developed by bacteria to inhibit the effects of different antibiotics, preserving the organisms’ vital functions and reproduction abilities. These mechanisms include: enzymatic inhibition, target site alteration, changes in membrane permeability and efflux of the antibiotic [126].

Finally, sub-therapeutic applications of antimicrobial agents such as penicillin and tetracycline can enhance the acquisition of antibiotic resistance by gene transfer, which has led to regarding therapeutic failure as a hazard to animal and human health [124].

2.4.1 Antibiotic resistance in *Staphylococcus pseudintermedius*

Since 2006, the proportion of methicillin- and multi-drug-resistant *S. pseudintermedius* (MRSP) in clinical samples of small animal and horses has increased [10]. MRSP proved to be a notorious pathogen exhibiting multi-drug resistance towards almost all antimicrobial agents applicable in the field of veterinary medicine [127]. Consequently, MRSP has been recognized as a significant health problem in veterinary medicine [15].

2.4.2 Resistance towards beta-lactam antibiotics

Antibiotics based on the beta-lactam ring constitute the most important class used to treat staphylococcal infections during the last decades. By binding to the transpeptidase domain of the penicillin-binding protein (PBP) in the bacterial cell wall, beta-lactam antibiotics impede peptide cross-linking provoking instability in newly synthesized cell walls towards osmotic pressure [128]. The main mechanisms in staphylococci resulting in resistance towards this antibiotic of this class is the production (and overproduction) of beta-lactamases encoded by *blaZ* or acquisition of an additional penicillin-binding protein, PBP2` (or PBP2a). The latter confers resistance towards the whole class (with some exceptions) and it is called "methicillin resistance" according to the CLSI recommendations [127]. These antimicrobials have a low affinity to PBP2a [129], thus cell wall stabulity is no longer impaired by beta–lactams. PBP2a is encoded by either the *mec*A or *mec*C gene, which can be transferred among staphylococci due to its location on *SCCmec* (see also chapter 2.3.4.4) [8].

Resistance towards beta-lactamase sensitive penicillins, such as penicillin G, ampicillin or amoxicillin, seems to be frequently associated with *S. pseudintermedius*. A study published in 1992 including canine SIG isolates from Germany and the USA revealed 62.1% of 116 isolates as ampicillin resistant [130]. Also 62% of 50 isolates obtained from France were found to be beta-lactamase producers [131].

Moreover, microarray studies revealed that the gene encoding a narrow-spectrum beta-lactamase (*bla*Z) detected in most of the (investigated) canine and feline MRSP isolates from Europa and North America [13, 14]. Occurrence of *bla*Z was also confirmed by PCR in MRSP and MSSP isolates from dogs and MRSP isolates from cats [132].

During the last decades, an obvious increase of MRSP among clinical isolates of *S. pseudintermedius* has been identified. While the first case of a MRSP in Europe was published in 2007 [133], a retrospective study showed an increase of MRSP from 5 to 30% among isolates in the USA during the years 2001 to 2007 [1]. At present, MRSP isolation has been reported for microbiological samples taken from healthy and diseased dogs, cats, horses, birds and urban rats [15, 27].

2.4.3 Further antibiotic resistances described for *S. pseudintermedius*

Beyond the resistance associated with the presence of *mec*A and *bla*Z, MRSP can possess a wide range of additional antibiotic resistance genes, culminating in a phenotype expressing resistance to almost all classes of antimicrobial agents used in veterinary medicine, including fluoroquinolones, lincosamides, macrolides, tetracyclines, trimethoprim, rifampicin, aminoglycosides and fusidic acid [1, 13, 14, 27]. Antimicrobial resistance among *S. pseudintermedius* has been elaborately reviewed by Kadlec and Schwarz 2012 [127] and Wendlandt et al (2013) [134]. The following section intends to give a short overview on the relevant antibiotic resistances described for *S. pseudintermedius* so far.

Resistance to tetracyclines

Tetracycline is a bacteriostatic antibiotic agent targeting the bacterial process of protein synthesis; it binds to the 30S subunit of the bacterial ribosome, inhibiting further protein constitution. Three mechanisms are known to mediate tetracycline resistance: (i) enzymatic inactivation, (ii) efflux pumps and (iii) ribosomal protection. Genes encoding tetracycline resistance in staphylococci include *tet*(K) and *tet*(L) (encoding efflux pumps belonging to the major facilitator superfamily), while the genes *tet(*M) and *tet*(O) code for ribosome protective proteins [127, 135]. Tetracycline resistance seems to be common among *S. pseudintermedius*, with *tet*(M) being an important factor mediating this kind of resistance [127]. In some studies, also *tet*(K) was frequently detected among *S .pseudintermedius* isolates of obtained from different animal species [13, 14, 127, 136].

Resistance to aminoglycosides

Aminoglycosides (such as neomycin, kanamycin, streptomycin, tobramycin, gentamycin and amikacin) are broad spectrum antibiotics widely used in human and veterinary medicine. These agents bind to the 30S subunit of ribosomes, resulting in mRNA mistranslation and interfered peptide elongation during protein synthesis [137].

Susceptibility of bacteria towards this antimicrobial class can be lowered by (i). reduction of drug uptake, (ii.) chromosomal mutations, or (iii.) enzymatic modification of the antibiotic leading to decreased ribosomal affinity [137]. The latter is conferred by a variety of genes encoding for aminoglycoside-inactivating enzymes in *S. pseudintermedius* [127]. Especially genes conferring resistance to gentamycin, tobramycin and kanamycin (*aac*A-*aph*D) have been reported for canine MRSP isolates [14, 79, 127].

Moreover, streptomycin and kanamycin resistance coded by the *aadE* and *aph*A-3 genes appear to be predominant in *S. pseudintermedius*. [127, 138].

Resistance to phenicols

Similar to aminoglycosides, phenicols reduce bacterial growth by preventing protein synthesis. Antimicrobials of this class specifically bind to the 50S ribosomal subunit, inhibiting the activity of bacterial peptidyltransferases.

Three mechanisms are known to cause resistance against phenicols: (i) reduced bacterial cell membrane permeability for phenicols, (ii) efflux pumps that often act as multidrug extrusion transporters, thereby reducing the effective intracellular drug concentration [139, 140], and (iii) production of inactivating enzymes, mainly acetyltransferases (*cat*) or by chloramphenicol phosphotransferases [141, 142].

Three *cat* gene variants (*cat*p_{C194}, *cat*p_{C221}, *cat*p_{C223}), commonly located on plasmids, have been described for staphylococci [143] including *S. pseudintermedius* [127, 130, 144, 145].

Resistance to macrolides and lincosamides

Both macrolides and lincosamids bind to the 50S subunit of the bacterial ribosome, causing dissociation of the peptide chains during protein synthesis. Target-site modification mediated through methylation or mutation (i.), efflux of the antibiotic (ii.) and enzymatic inactivation (iii.) are the most common mechanisms leading to resistance against macrolides and lincosamids For *S. pseudintermedius* isolates, different genes encoding resistance mediating factors with respect to macrolides and lincosamides. These genes are: *inu*(A) (lincosamide nucleotidyl transferase), *msr*(A) (ABC-transporter) as well as *erm*(A), *erm*(B) and *erm*(C) encoding rRNA methylases [127]. Resistance to Macrolide-lincosamide encoded by the Tn917-associated *erm*(B) gene seems to be predominant among German canine and feline *S. pseudintermedius* isolates [127, 138, 146]. It can be also found in combination with *inu*(A) in canine MRSP isolates from USA and Canada [14] or with *msr*(A) in two German *S. pseudintermedius* isolates [146].

Resistance to fusidic acid

Fusidic acid acts as a bacterial inhibitor of protein synthesis by preventing the release of elongation factor G (EF-G) from the ribosome. Point mutations in the *fus*A gene, which encodes EF-G, are the most important resistance mechanism. Acquisition of the gene *fus*B, which encodes an EF-G-binding protein, promotes fusidic acid resistance. Furthermore, *fus*C and *fus*D, which are very similar to *fus*B, have also been reported for different staphylococcal species [147, 148].

Resistance to mupirocin

Mupirocin (pseudomonic acid) is a topical antimicrobial agent that binds to the bacterial enzyme isoleucyl-tRNA synthetase (*ileS*) and led to inhibition of protein synthesis [149]. Resistance occur either by a mutation in *iles* gene or acquisition of a separate gene, called *mup*A which also encodes an isoleucyl tRNA synthetase enzyme without affinity for mupirocin [149]. At present, resistance towards mupirocin is rarely found in *S. pseudintermedius* [127].

Resistance to quinolones

Quinolones inhibit the activity of two enzymes, topoisomerase II (or: DNA gyrase, encoded by *qyrA* and *qyr*B) and topoisomerase IV (encoded by *qrl*A and *qlr*B). Inhibition of these two topoisomerases leads to DNA fragmentation. Quinolone resistance is either mediated by efflux pumps, leading to decreased intracellular quinolone concentrations [150] and/or specific point mutations within the topoisomerases. Nucleotide substitution at some distinct loci in *gyr*A, *gyr*B, *qrl*A and *qrl*B results in amino acid substitution (f.i. Ser84Leu and Glu88Gly in *gyr*A or Ser80Ile and Asp84Asn in *grl*A) conferring different levels of quinolone resistance [151]. Further reports were published on those changes exists for *gyr*A and *grl*A genes of *S. pseudintermedius* isolates [127, 152-155].

Resistance to trimethoprim

Trimethoprim has a bacteriostatic activity, inhibiting the bacterial dihydrofolate reductase (DHFR), resulting in folate deficiency, an essential substrate for the process of bacterial DNA synthesis [156]. Resistance to trimethoprim is mediated by different mechanisms [157]: (i.) efflux pumps, (ii.) mutations in the DHFR-encoding gene *dfr,* and (iii.) production of different variants of the DHFR enzyme. The latter is reported for staphylococci mediated by DHFR enzyme variants encoded by *dfr*A, *dfr*D, *dfr*G and *dfr*K [158]. *dfr*G seems to be common among canine MRSP isolates [13, 14, 127].

2.5 Epidemiology of *S. pseudintermedius*

In February 2011, the Committee for Veterinary Medicinal Products (CVMP), an agency of the European Union, published definitions for the terms contamination, colonization and infection with MRSP. According to these definitions, colonization is the “presence, growth, and multiplication of MRSP in one or more body sites without observable clinical signs or immune reaction”. Furthermore, the term “carrier” (animal or human) refers to an individual colonized with MRSP. The most common site of MRSP colonization in dogs is the nose and the anus. In addition, “infection” is defined by CVMP as” a condition whereby MRSP has invaded a body site, is multiplying in tissue, and is causing clinical manifestations of disease.” Furthermore, these conditions should be distinguished from the condition “contamination”, defined as an individual state when bacteria can be washed off easily (http://www.ema.europa.eu/ema/).

In general, *S. pseudintermedius* seems to be a bacterial species adapted to the family of *canidae* and colonization of dogs appears to be common. In addition, colonization rates reported for *S. pseudintermedius* in dogs seem to be higher than those reported for human carriers’ with *S. aureus* [3, 39].

Nonetheless, a variability with respect to carriage rates reported in cross section studies is obvious: depending on the body site(s) sampled, the preferred screening method and the chosen isolation procedure (e.g. use of enrichment broth), identification rates range from 46 to 92% [46, 159, 160]. In addition, a study published by Fazakerley et al. reported a *S. pseudintermedius* carriage rate of 87% for atopic dogs (21 of 24) while only 37% of healthy individuals (16 of 43) were also colonized, indicating at least that affected skin is often positive for *S. pseudintermedius* [161].

S. pseudintermedius carriage rates reported for cats were considerably lower than those known for dogs [45, 160]. Furthermore, Hariahan et al. reported that *S. haemolyticus*, *S. felis* and *S. simulans* were the most common staphylococcal species isolated from feral cats [162], indicating that cats are not necessarily a natural host for *S. pseudintermedius* [39]. However, *S. pseudintermedius* can be isolated from clinical cases of feline pyoderma, especially from inflammatory skin lesions [45, 133].

Like every skin-colonizing staphylococcal species, vertical or horizontal transmission of *S. pseudintermedius* is possible [16, 39]. Puppies were reported to be colonized with *S. pseudintermedius* within eight hours’ *post-partum* [163, 164].

At present, data on the prevalence of MRSP colonization is limited. Furthermore, in most studies, a distinction between “contamination” and a real “colonization” is not possible due to the lack of “follow up” investigations (http://www.ema.europa.eu/ema/).

Reported carriage rates were 1.5-2% among healthy dogs in the community and upon admission to a veterinary hospital [15, 46], and 0-7% in dogs with skin disease [46, 165].

For healthy cats, one study reported a MRSP carriage rate of 4% [45, 166].

While *S. pseudintermedius* seems to be predominately associated with canine hosts, occasionally it can be found in human samples as well [4, 39] [167]. Again, distinction between contamination and colonization is not easy, but it seems to be reasonable that a certain degree of “microorganism exchange” occurs if a certain habitat (the flat, furniture, the bed *etc.*) is shared by dog owners and their animals. Furthermore, intensive, repeated and daily skin-to-skin contact is a common attribute indicating an intensive “friendship” of dog owners with their individual animals [4]. A study amongst owners of dogs which were suffering from deep pyoderma associated with *S. pseudintermedius* showed that 46% (6 of 13) of these owners carried the same particular strain which was identified in specimens from their dogs within their own nasal and oral cavities [168]. *S. pseudintermedius* carriage rates up to 4.1% were described for humans living in a household with pets [4, 160, 169, 170]. Since dog-to-human transmission of *S. pseudintermedius* in the household seems to be very easy, reports on transmission events (including MRSP) are available from all over the world [11, 16, 160, 168, 171-173].

Consequently, cases of human infection due to *S. pseudintermedius* (including MRSP) associated with e.g. dog bite wounds, bacteremia, wound infection, onycholysis, otitis externa, sinusitis, and a brain abscess have been reported recently [34, 36, 78, 174-180]. Since the distinct identification of *S. pseudintermedius* among CPS causing disease in humans is not easy to achieve (see: 2.3.2) and knowledge about this staphylococcal species is not widely distributed in microbiological laboratories with a focus on human samples, SIG members may have been misidentified as *S. aureus* in the past decades [15]. Since 2000, severe cases of *S. pseudintermedius* infection with *S. pseudintermedius* were reported in elderly patients (associated with Implantable cardioverter defibrillator (ICD) devices) and in a child with haemophilia [5-7].

In one of these reports, the source of infection remained unknown, but in the other cases close contact with a pet dog has been assumed as the pivotal predisposing factor. However, the isolation of MRSP has also been described from patients with gastric adenocarcinoma and pneumonia without exposure to animals [78, 181].

2.5.1 Molecular epidemiology of *S. pseudintermedius*

Due to the lack of knowledge with regard to *S. pseudintermedius* being a distinct staphylococcal species before 2005 and the development of sequence-based typing schemes in 2007 and 2013 [22, 25, 106], gene-based phylogenetic analysis is a young scientific research area in comparison with data available on the genetic background of *S. aureus*.

At present (date: February 2016), 626 distinct STs were present in the MLST database (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_spseudintermedius_isolates).

The information on the genetic diversity among the *S. pseudintermedius* population is still limited [39]. Since re-definition of the MLST scheme by Solyman et al. in 2013, isolates formerly designated as ST68, a lineage often reported for MRSP originating in North-America, was subdivided in three different ST (ST29, ST30 and ST68). Nonetheless, many reports about ST71 showed the worldwide emergence of this ST since its first description [13, 14, 27, 69, 106, 172, 182].

In Norway, the first case of MRSP infection in a dog was recorded in 2008. From that time until June 2011, an additional 22 cases were observed [183]. Surprisingly, MLST analysis revealed ST106 and not ST71 being the predominant ST among that strain collection representing various geographic origins (8 of 23), while ST71 constituted the clonal background of a further 6 MRSP isolates [183]. Another report published by these Norwegian Authors revealed an additional 9 STs associated with MRSP isolates in 2011 [183].

This diversification of MRSP seems to be a recent development, since 245 distinct MRSP STs were recorded in the MLST database. A minimum spanning tree (MST) of 626 data base entries (23.02.2016) reveals a remarkable distribution of MRSP within the whole tree (**Figure** 2).

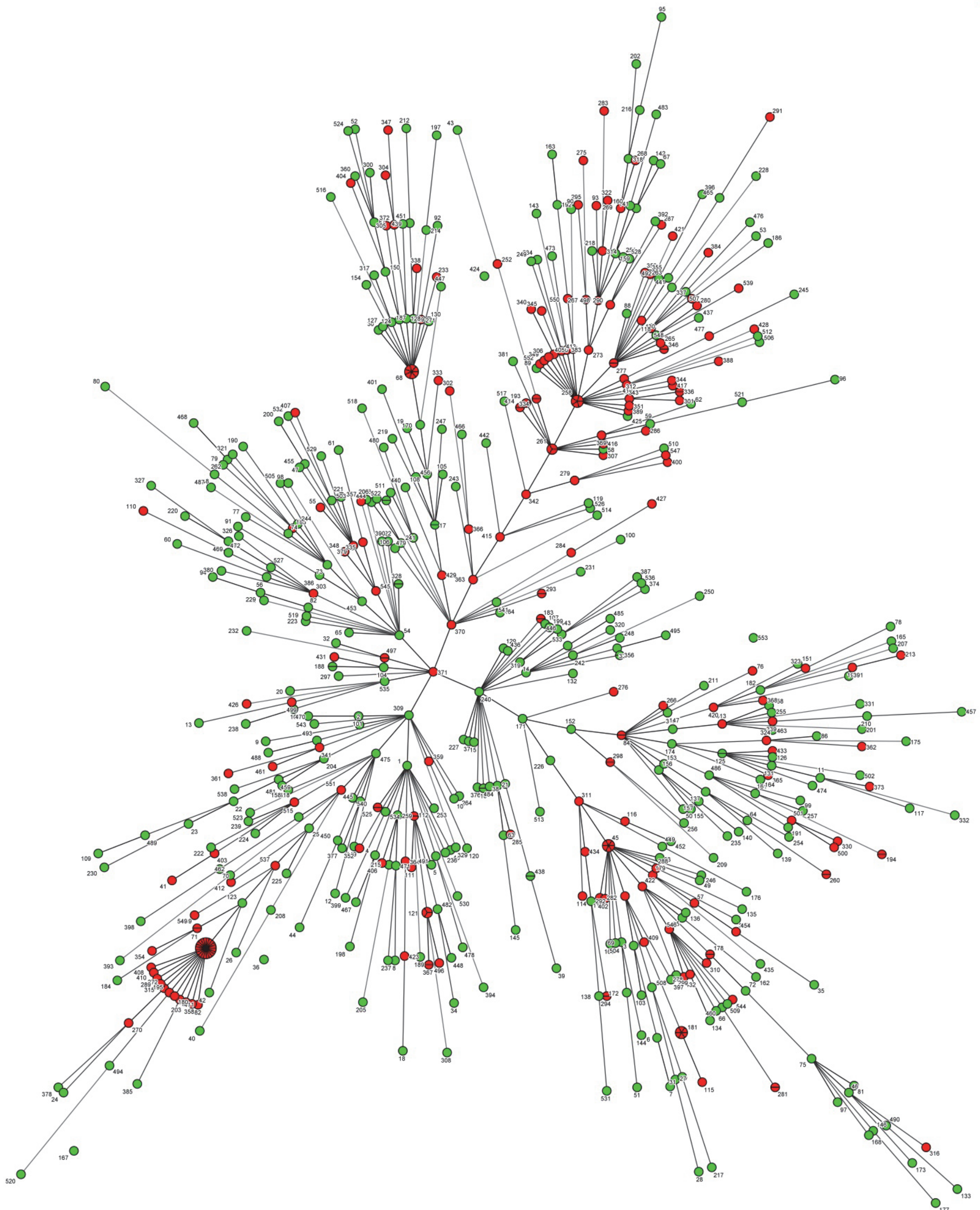


Figure 2: Minimum spanning tree based on MLST data of MRSP and MSSP.
Minimum spanning tree (generated by Dr. B. Walther) of *S. pseudintermedius* based on 626 data base entries (www.mlst.net) database entries (23.02.2016) with bionumerics 7.5 (AppliedMath, Belgium).
Each circle represents a unique sequence type (ST), the size of the circles corresponds to the number of database entries for distinct STs (red: MRSP, green: MSSP).

3 Materials

3.1 Bacterial isolates, reference-strains, Polymerase chain reaction components

A total of 220 (n= 110 classified as methicillin-susceptible and n= 110 as methicillin-resistant) staphylococcal isolates used in this study were obtained from IDEXX Vet-Med Lab (Ludwigsburg) during a risk factor assessment study by A. G. Brandenburg. **Figure** 3 showing the distribution of all *S. pseudintermedius* isolates in different federal states of Germany.

Information on all isolates including date of isolation, host species, body site affected and geographic origins were provided in **Appendix 1**

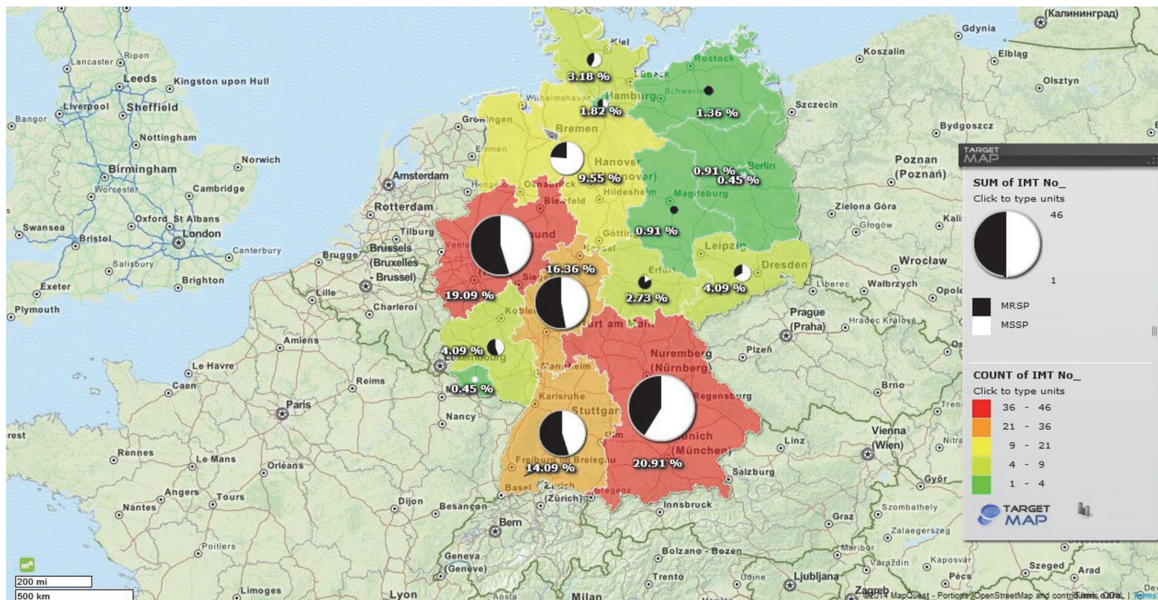


Figure 3: Geographic origin of *S. pseudintermedius* isolates reported in this study.
This map was created by PhD. Szilvia Vincze by use of targetmap (www.targetmap.com).

Table 1: List of reference- and control-strains used in this study

Strain	Species	Strain properties
NCTC 8325	*S. aureus*	PFGE reference strain, *mecA*- negative
IMT6637	*S. aureus*	*mec*A-positive
IMT6689	*Staphylococcus sp.* of the *intermedius*-group	*mec*A-negative
ATCC29663	*S. intermedius*	*pta* gene lacking an MboI restriction site
CCUG30107	*S. delphini*	*pta* gene lacking an MboI restriction site
IMT21470	*S. pseudintermedius*	*pta* gene harboring MboI restriction site
IMT21652(JN604832)	*S. pseudintermedius*	*exp*A-positive
IMT24468	*S. pseudintermedius*	*exp*B-positive

Oligonucleotide primers for PCR

Oligonucleotide primers used in this study are given in **table** 2. All primers were obtained from Eurofins MWG Operon, Ebersberg (Germany) in a lyophilized form. Primers were re-suspended in sterile, deionized (Millipore) water to obtain a stock solution of 100 pmol/ml as recommended by the manufacturer. Stocks were stored at -20°C.

Table 2: Oligonucleotide primers used in this study

Target gene	Species	D	Primer Sequence (5'-3')	Size	Location	Reference
nuc	*S. aureus*	F R	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGACGAACTAAAGC	367 bp	Thermonuclease	Merlino et al. 2002 [8]
16S rDNA	*S. intermedius*	F R	CCGTATTAGCTAGTTGGTGG GAATGATGGCAACTAAGTTC	901 bp	16S rDNA	Wakita et al.2002 [184]
*mec*A	*S. intermedius*	F R	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533 bp	SCCmec	Merlino et al. 2002 [8]
*exp*A	*S. pseudintermedius*	F R	GCGCGTCCTTCTGATCCAGAACT AACGTCCCCCTTTACCTACGTGAAT	574 bp	Exfoliative toxin	Walther et al., 2012 [4]
expB	*S. pseudintermedius*	F R	GCGGCATGCCTAAAACATATGATGAAGCCGAA TCTGGATCCATCTTCTGATTCAGCTCTTTTTTTCAAA	843 bp	Exfoliative toxin	Iyori et al., 2010 [55]
pta	*S. pseudintermedius*	F R	AAAGACAAACTTTCAGGTAA GCATAAACAAGCATTGTACCG	320 bp	Phosphoacetyltransferase	Bannoehr et al., 2009 [3]
*luk*S	*S. pseudintermedius*	F R	TGTAAGC AGCAGAAAATGGGG GCCCGATAGGACTTCTTACAA	503 bp	Leukotoxin	Futagawa-Saito et al., 2004 [31]
*luk*F	*S. pseudintermedius*	F R	CCTG TCTATGCCGCTAATCAA AGGTCATGGAAGCTATCTCGA	572 bp	Leukotoxin	Futagawa-Saito et al., 2004 [31]
*pur*A	*S. pseudintermedius*	F R	GATTACTTCCAAGGTATGTTT TCGATAGAGTTAATAGATAAGTC	490 bp	Housekeeping gene	Solyman et al.,2013 [106]
fdh	*S. pseudintermedius*	F R	TGCGATAACAGGATGTGCTT CTTCTCATGATTCACCGGC	408 bp	Housekeeping gene	Solyman et al.,2013 [106]

Target gene	Species	D	Primer Sequence (5'-3')	Size	Location	Reference
sar	*S. pseudintermedius*	F	GGATTTAGTCCAGTTCAAAATTT	521 bp	Housekeeping gene	Solyman et al.,2013 [106]
		R	GAACCATTCGCCCCATGAA			
tuf	*S. pseudintermedius*	F	CAATGCCACAAACTCG	500 bp	Housekeeping gene	Bannoehr et al., 2007 [25]
		R	GCTTCAGCGTAGTCTA			
cpn60	*S. pseudintermedius*	F	GCGACTGTACTTGCACAAGCA	550 bp	Housekeeping gene	Bannoehr et al., 2007 [25]
		R	AACTGCAACCGCTGTAAATG			
pta	*S. pseudintermedius*	F	GTGCGTATCGTATTACCAGAAGG	570 bp	Housekeeping gene	Bannoehr et al., 2007 [25]
		R	GCAGAACCTTTT GTTGAGAAGC			
ack	*S. pseudintermedius*	F	CACCACTTCACAACCCAGCAAACT	680 bp	Housekeeping gene	Solyman et al.,2013 [106]
		R	AACCTTCTAATACACGCGCACGCA			

Abbreviations: D: direction; F: forward, R: reverse

DNA ladder and commercial kits

1 kb plus DNA ladder	Fermentas, St. Leon-Rot
QIA amp DNA Mini Kit	Qiagen, Hilden
QIA quick PCR Purification Kit	Qiagen, Hilden
100 bp DNA ladder	Fermentas, St. Leon-Rot
Lambda DNA marker	New England Bio Labs Inc., Schwalbach/Taunus

Enzymes

DNA taq polymerase	Fermentas, St. Leon-Rot
Proteinase K	Carl Roth, Karlsruhe
Lysostaphin	Sigma-Aldrich, Steinheim
Restriction endonuclease *Sma*I and tango buffer with BSA	Fermentas, St. Leon-Rot

Media

Brain heart infusion (BHI)	Oxoid, Wesel
Columbia agar with sheep Blood Plus	Oxoid, Wesel
Mueller-Hinton-Bouillon (MHB)	Sifin (Berlin)
Müller-Hinton Agar	Oxoid, Wesel
Müller-Hinton solution	Carl Roth, Karlsruhe

Solutions and Buffers

a. Phosphate buffered saline (PBS), 1 L

	Sodium chloride	8.0 g
	Potassium chloride	0.20 g
	Sodium phosphate dibasic	1.44 g

b. 0.9 NaCl, 1L

	NaCl	9.0 g
	DDW	add 1 L

c. DNA extraction solutions

TE buffer	EDTA	1mM
	Tris Ultra Quality	10mM
	DDW	add 10 ml
	Adjust pH to 7.0 using HCl	

d. Solutions for agarose gel electrophoresis (DNA)

1. Tris Borate EDTA (TBE) 10x

Tris Ultra Quality	890 mM	107.82 g
Boric acid	890 mM	55.03 g
EDTA solution (pH 8.0)	500 mM	18.62 ml
NaOH		100 ml
DDW		Dilution 1:20

2. Loading dye

Glycerol	5.0 ml
Bromophenol Blue	10.0 mg
EDTA solution (0.5 mM)	2.0 ml
Tris pH 8.	0.1 ml
DDW	2.9 ml

3. 1.5 % Agarose Gel

Agarose	6.0 g
TBE 1x	400 ml
Midori Green, DNA dye	4 µl

e. Solution for Polymerase chain reaction (PCR)

10x Green Buffer	20 mM $MgCl_2$
dNTP's (PCR Nucleotide Mix: dNTP dATP, dCTP, dGTP, dTTP)	Each dNTP 10mM
Primer-Mix	10 pmol each
DreamTaqDNA Polymerase	5 U/µl
DNA-Template	2 µl

f. Solutions and buffers for pulsed-field gel electrophoresis (PFGE)

1. Cell suspensions buffer

EDTA	50 mM	1.861g
NaCl	20 mM	0.117g
Tris Ultra Quality	10 mM	1.211g
DDW (PH 7.2 with HCl)		100 ml

2. Lysis buffer

EDTA	20 mM	1.861g
NaCl	50 mM	0.292g
Tris Ultra Quality	10 mM	1.211g
Deoxycholat	0,20%	0.2g
Sarcosyl	0,50%	0.5g
DDW (PH 7.2 with HCl)		100 ml

3. Wash buffer

EDTA	0.1 mM	0.004g
Tris Ultra Quality	10 mM	1.211g
DDW (PH 7.2 with HCl)		100 ml

4. PK buffer

EDTA	250 mM	9.305g
Sarcosyl	1%	1g
DDW (PH 9 NaOH)		100 ml

List of chemicals

Agent	Company
Acetone	Carl Roth, Karlsruhe
Agarose	Biodeal, Markkleeberg
Agarose (peqGOLD Pulsed-field Agarose)	peqLAB
Boric acid	Carl Roth, Karlsruhe
Bromophenol Blue	Merck, Darmstadt
dNTP mix	TaKaRa
DreamTaq DNA Polymerase	Fermentas, St. Leon-Rot
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe
Ethanol	Carl Roth, Karlsruhe
Gel Red	Biotrend, Köln
Glycerol	Carl Roth, Karlsruhe
Green Buffer	Fermentas, St. Leon-Rot
Hydrochloric acid (HCl)	Carl Roth, Karlsruhe
Isopropanol	Carl Roth, Karlsruhe
Lauroylsarcosine	Sigma-Aldrich, Steinheim
Magnesium chloride (MgCl2)	Merck, Darmstadt
Methanol	Carl Roth, Karlsruhe
Midori Green, DNA dye	Biozym Scientific, Hessisch Oldendorf
Oxacillin	Sigma-Alderich (Deisenhofen)
Potassium chloride (KCl)	Carl Roth, Karlsruhe
Potassium Phosphate monobasic (KH2PO4)	Carl Roth, Karlsruhe
Sodium chloride (NaCl)	Carl Roth, Karlsruhe
Sodium hydroxide (NaOH)	Carl Roth, Karlsruhe
Sodium phosphate dibasic (Na2HPO4)	Carl Roth, Karlsruhe
Tris ultra-quality	Carl Roth, Karlsruhe

Equipments

Device	Company
Agitator	IKA Labortechnik, Staufen
Autoclave Systec, Wettenberg	Systec, Wettenberg
Balance	Sartorius, Göttingen
Centrifuge and angled rotor 19776-H	Sigma, Osterode am Harz
Chef-DR III Pulsed-field	Bio-Rad, München
Gel chambers for agarose electrophoresis	AGS, Heidelberg
Gel documentation system	herolab Laborgeräte, Wiesloch
Ice machine	Dr. Heinekamp, Karlsfeld
Incubator for bacteria plates	Binder, Tuttlingen
Laboratory centrifuge	Eppendorf, Wesseling-Berzdorf
Laminar flow cabinet	Nuaire, Plymouth, USA
Magnetic stirrer	IKA Labortechnik, Staufen
Microwave	MDA, Dresden
MiSeq Desktop Sequencer	Illumina
pH/ ion meter	Knick, Berlin
Pipettes	Eppendorf, Wesseling-Berzdorf
Pipettor	Brand, Wertheim
Power Supply for Electrophoresis	Bio-Rad, München
Scanner	Hewlett Packard, Böblingen
Spectrophotometer for DNA, Nanodrop	Thermo Scientific, Schwerte
Table centrifuge	Eppendorf, Wesseling-Berzdorf
Thermocycler, PCR	Biometra, Göttingen
Thermomixer	Eppendorf, Wesseling-Berzdorf
Vortex	IKA Labortechnik, Staufen
Water bath Theodor Karow, Berlin	Theodor Karow, Berlin
Water preparation	Merck Millipore, Darmstadt

Consumables

Component	Company
15 ml tubes, conical	Sarstedt, Nümbrecht
50 ml tubes, conical	Sarstedt, Nümbrecht
Cryotubes 1.5 ml	GreinerBio-One, Frickenhausen
Culture tubes, glass 10 ml, 20 ml	Carl Roth, Karlsruhe
Deep well plate, 96 well	Sarstedt, Nümbrecht
Disposal bags	Sarstedt, Nümbrecht
Filter pipette tips 10 µl, 200 µl, 1000 µl	Sigma-Aldrich, Steinheim
Glass ware (beakers, tubes, cylinders)	Schott, Mainz
Gloves Carl Roth, Karlsruhe	Carl Roth, Karlsruhe
Inoculation loops	Greiner Bio One, Frickenhausen
Light safe reaction tubes 1.5 ml	Carl Roth, Karlsruhe
Parafilm	Carl Roth, Karlsruhe
PCR reaction tubes 0.2 ml	Sarstedt, Nümbrecht
Petridishes	Sarstedt, Nümbrecht
Reaction tubes 1.5 ml, 2 ml	Sarstedt, Nümbrecht
Reaction tubes 0.5 ml, 2 ml ‘safe-lock‘	Sarstedt, Nümbrecht

Softwares

Software program	Application
BioNumerics version 7.5, AppliedMaths, Belgium	Phylogenetic analysis and dendrogram construction for *S. pseudintermedius* genome
Microsoft Office 2010, Microsoft, USA	Graphs, Tables, Images, Text
NanoDrop 1000 Version 3.6.0, Thermo Fisher, USA	DNA and RNA quantification, spectrophotometer

4 Methods

4.1 Bacterial cultivation, strain storage and disc diffusion antibiotic sensitivity testing

During the sampling period (2010-2012), isolates identified as *S. pseudintermedius* by use of Vitek 2 (bioMerieux, Germany) in our partner lab in Ludwigsburg were shipped on cotton swabs in Amies medium. On arrival, all swabs were streaked onto Columbia agar with sheep Blood Plus (Oxoid, Wesel). All strains were stored in stocks containing 15% glycerol at -80 °C in the IMT strain collection.

In cases of deviation of PCR results from the initial methicillin-resistant or -susceptible classification obtained by Vitek 2, the oxacillin (1µg) disk diffusion method was performed on Mueller-Hinton agar according to Clinical Laboratory Standards Institute (CLSI) guidelines [185].

4.2 Molecular genetic methods

4.2.1 DNA extraction

Three overnight grown colonies were suspended in 25µl Lysostaphin (0.1mg/ml; Sigma) and incubated for 10min at 37°C. Then, 25µl Proteinase K (0.1mg/ml, Sigma) and 75µl TE buffer (1mM EDTA, 10mM Tris pH 7.5) were added and the mixture was incubated for a further 20min at 37°C. In order to inactivate the enzymes and to denature proteins, the suspension underwent a boiling step at 95°C for 5min. When the DNA was to be used for whole genome sequencing (WGS), a QIAamp DNA Mini-Kit (Qiagen, Hilden) was used for DNA purification.

4.2.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a fast and inexpensive technique used to amplify well defined segments of DNA. Using this method allows to determine whether a certain DNA sequence is present in the sample of interest by evaluating the size (in base pair, bp) of the amplified sequence. The whole PCR process is based on the ability of a specialised enzyme (DNA polymerase) that synthesizes a complementary strand to a targeted segment of DNA by use of supplemented oligodeoxy-ribonucleotides. In addition, the mixture must also contain at least two further DNA fragments, each about 20 bases long, called primers. These primers are very short complementary sequences to each of the two DNA strands of interest, which mark the beginning of the target sequence in both directions (5`→3`and 3`→5`).

4.2.3 Gel electrophoresis

Agarose gel electrophoresis is the easiest and commonest method for size separation and visualization of DNA fragments such as PCR-amplified products. The DNA migrates through a semi-solid agarose matrix when placed under an electric current. All PCR products in this study were separated by horizontal gel electrophoresis. The running gel composed of 1.5 g agarose powder (Biodeal, Markkleeberg) in 100 ml 1 x TBE solution. After cooling down to 55°C, 4 µl of a Midori Green DNA dye (Biozym Scientific, Hessisch Oldendorf) (1ml) was added. The liquid agarose gel was poured into a gel chamber, and a plastic comb with 12 to 24 teeth for forming pockets was utilized. 5µl of each PCR product as well as a 100-bp DNA marker (Fermentas, St. Leon-Rot) have been pipetted in individual pockets. An electric field was applied to the electrophoresis chamber (45 min at 100 V). PCR fragments were photographed under UV light (transilluminator TI, Biometra, Wiesbaden; Photo Polaroid camera MP4 + instant camera system).

4.2.4 Triplex PCR

For verification of phenotypical methicillin resistance results gained by the Vitek 2 system in Ludwigsburg, all *S. pseudintermedius*-isolates were screened for *mec*A,16SRNA gene (*S. intermedius*-group) and *nuc* to rule out *S. aureus* (primer, references and controls: **table** 1 and 2).

The reaction mixture for the triplex PCR was composed as follows:

2.5 µl	10 x green buffer
0.6 µl	dNTPs (10mM each)
5 µl	Primer mix (10pM each)
0.1µl	DreamTaq DNA-polymerase 5 U/µl
2 µl	template DNA
add 14.8 µl	DDW*

* DDW: double distillated water

The reaction mixture was briefly centrifuged and immediately placed in the thermo cycler (Biometra, Göttingen). The PCR conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 25 cycles with 94 °C for 30 sec., 55 °C for 1 min and 72° C for 1 min, respectively. The final extension step was 7 min at 72 °C.

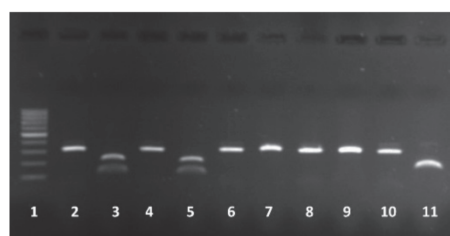
4.2.5 Verification of *S. pseudintermedius* among SIG isolates

Unambiguous identification of *S. pseudintermedius* was carried out by restriction fragment length polymorphism PCR (PCR-RFLP) analysis based on a single *Mbo*I restriction site in the *pta* gene of *S. pseudintermedius* according to a protocol published by Bannöhr et al., 2009 [3]. At first, PCR amplification of an internal 320-bp fragment of the *pta* gene was carried as follows:

5 µl	1 x green buffer
1.6 µl	dNTPs (0.2mM each)
3 µl	reaction primer (0.2µM each)
0.1µl	DreamTaq DNA-polymerase 5 U/µl
5 µl	template DNA
add 35.7 µl	DDW*

* DDW: double distillated water

The reaction mixture was briefly centrifuged and immediately placed in the thermo-cycler (Biometra, Göttingen). The PCR conditions were: 95 °C for 2 min (initial denaturation), 30 cycles starting with 95°C for 1 min. (denaturation), followed by 53 °C for 1 min (annealing) and 72° C for 1 min (elongation), respectively. Final elongation was done at 72 °C for 7min. 25 µl of the PCR mixture were then incubated with 5 U of *Mbo*I and 5 µl of 5X digestion buffer for 2 h at 37°C, and the digestion products were resolved in 1.5% agarose gel by electrophoresis. Since the *pta* gene of *S. pseudintermedius* harbors a distinct *Mbo*I restriction site, enzymatic digestion yielded two restriction fragments of 213 bp and 107 bp length.



row	content
1	100 bp marker
2	*S. pseudintermedius* PCR product (*pta*) (320bp)
3	*S. pseudintermedius* two fragments (213bp and 107bp) after digestion with endonuclease MboI
4	positive control *S. pseudintermedius* (IMT21470) PCR product (*pta*)
5	positive control *S. pseudintermedius* (IMT21470) two fragments (213bp and 107bp) after digestion
6	*S. intermedius* (ATCC29663) PCR product (*pta)* (320bp)
7	*S. intermedius* (ATCC29663): no restriction using endonuclease MboI
8	*S. delphini* (CCUG30107) PCR product (*pta)* (320bp)
9	*S. delphini* (CCUG30107): no digestion
10	*S. aureus* (NCTC 8325) PCR product (*pta*) 320bp
11	*S. aureus* (NCTC 8325): bands of 156 bp and 164 after digestion with endonuclease MboI

Figure 4: Restriction fragment length polymorphism PCR (RFLP-PCR) of *pta* (example) according to Bannöhr et al., 2009 [3].

4.2.6 Detection for virulence-associated genes in *S. pseudintermedius*

All *S. pseudintermedius* isolates were screened for the presence of virulence genes *lukS* and *lukF* encoding the bicomponent leukotoxin (LukI) using a PCR protocol described by Futagawa-Saito et al., (2004) [31]. The method based on amplification of a 503 bp fragment of the *luk*S gene and a 572 bp fragment of the *luk*F gene in a 25 µl reaction volume:

2.5 µl	1 x green buffer
0.2 µl	dNTPs (0.2mM each)
2.5 µl	reaction primer (0.2µM each)
0.07 µl	DreamTaq DNA-polymerase 5 U/µl
5 µl	template DNA (50 ng)
add 14.73 µl	DDW*

* DDW: double distillated water

The reaction mixture was briefly centrifuged and immediately placed in the thermo cycler (Biometra, Göttingen). DNA amplification was performed using 25 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and 10 min at 72°C.
Detection of the gene encoding *Exp*B was performed as described previously [55]. The presence of the exfoliative toxin *Exp*A was determined by PCR using a previously published protocol [4] at an annealing temperature of 58°C (25 cycles) including strain-JN604832 as a positive control.

The reaction mixture for both PCRs was composed as follows:

2.5µl	1 x green Puffer
0.5µl	dNTPs (0.2 mM each)
2µl	reaction primer (0.2 µM each)
0.1µl	DreamTaq DNA-Polymerase 5 U/µl
5µl	template DNA (50 ng)
add 19.75µl	DDW*

* DDW: double distillated water
The PCR conditions were as follows: initial activation of the Taq-DNA-Polymerase for 3 min at 94°C, followed by 25 cycles of 30 sec denaturation at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C. The program ended with a 10 min at 72°C.

4.2.7 Pulsed-field gelelectrophoresis (PFGE)

Macrorestriction of bacterial DNA using endonucleases (e.g. *SmaI*) followed by PFGE is a common tool of molecular epidemiologists and provides a “genetic fingerprint” which is uniquely associated with each individual isolate. Originally used to investigate outbreak situations like food poisoning or nosocomial infections, cluster analysis of the restriction patterns using specialized software (Bionumerics, AppliedMaths, Belgium) allows an initial grouping of *S. pseudintermedius* isolates [27].

At first, a single colony of *S. pseudintermedius* was suspended in 5 ml BHI and incubated for 16-18h at 37 °C incubator by gentle shaking (180-200 rpm). Then, 150 µl of the bacterial suspension was transferred to 1.5 ml reaction tubes and pelleted by centrifugation at 13.000 × g for 1 min. Pellets were suspended with 150 µl cell suspension buffer and kept at 37 °C in a thermomixer (Eppendorf, Wesseling- Berzdorf). 1.4 % peqGOLD Pulsed-field Agarose (peqLAB) was prepared according to the manufacturer's instructions with Millipore water and kept liquid at about 56 °C. 2 µl lysostaphin (1mg/ml) were added to the bacterial suspension and immediately mixed with 150 µl liquid agarose avoiding bubbles formation. 100 µl of the mixture was immediately filled into plug molds (2 plugs per sample). The plugs were allowed to cool down for 15 minutes at room temperature. Afterwards, the plugs were transferred into a new 1.5 ml reaction tubes with 500 µl of lysis buffer followed by incubation at 37 °C (gentle swirling) for 1 hour. After removing the lysis buffer, 500 µl PK-PK buffer was added (PK buffer supplemented with proteinase K 500 µg /ml, freshly added) and incubated for an additional 30 min at 55°C. Then the plugs were then washed repeatedly with wash buffer three times followed by an additional 45 min of equilibration. After the final wash, 1/3 of the plug was separated with a sharp scalpel and transferred into a new 0.5 ml reaction tube. The remaining “reserve plugs” were kept in the last wash buffer at 4°C.

The plugs were equilibrated on ice with 300 µl restriction buffer (Tango buffer with BSA) for 30 min. After removing the buffer, 100 µl of fresh Tango buffer with BSA with 2 µl *SmaI* were added to the plugs. The enzymatic digestion step was carried out at 37 °C by gentle shaking overnight.

A gel was prepared by dissolving 1.2 g peqGOLD Pulsed-field agarose (peqLAB) in boiling 120 ml TBE (0.5x) buffer and mixing until fully molten. The molten gel was then transferred to a water bath (Theodor Karow, Berlin) maintained at 55°C to equilibrate the agar to 55°C prior to casting of the gel. The Agarose was then poured into a 12 x 14 cm agarose gel form utilizing a 15-well comb. The macro-restriction treated small plugs were transferred with a spatula into the pre-formed pockets of the gel and sealed with gel residues. *S. aureus* PFGE

reference-strain NCTC8325 and a Lambda-DNS-Marker (New England Bio Labs Inc., Schwalbach/Taunus) were used in every run.

Electrophoretic separation of the restricted DNA fragments was performed in a contour-clamped homogeneous electric field chamber (CHEF DR III; Bio-Rad, München) in 0.5 × TBE as described by Ruscher et al. 2010 [27] with minor modifications regarding the running conditions: Initial switching time 8 h; temperature: 14°C; angle: 120°; voltage: 6 V; pulse time: 5 – 15 seconds followed by switches from : 15 - 60 sec ; 10 h; temperature: 14°C; angle: 120°; voltage: 6V.

The staining of the DNA fragments was performed using a Gel Red (Biotrend, Köln) concentration 30 µl/300 ml H_2O for 20 minutes in TBE buffer (0.5 %). The gel was then washed with 0.5X strength TBE and documented by a gel documentation system (Herolab Lab Systems, Wiesloch).

4.2.8 Analysis of PFGE pattern using Bionumerics

Analysis of macrorestriction pattern includes differences in size and total number of resolved fragments. In this study, the software Bionumerics 7.5 (AppliedMaths, Belgium) was employed to store and normalize PFGE restriction pattern for 200 *S. pseudintermedius* isolates. Generation of the final comparison was performed using dice coefficient of similarity with unweighed pair group method using arithmetic averages (UPGMA). Optimization was set at 0.5% with 1.5% position tolerances [27].

The published article “Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing” by Tenover et al. published in 1995 [90] was the source for definitions which were used whenever suitable in this thesis:

> **“Isolate**: is a general term for a pure culture of bacteria obtained by subculture of a single colony from a primary isolation plate, presumed to be derived from a single organism, for which no information is available aside from its genus and species.
>
> **Epidemiologically related isolates:** Epidemiologically related isolates are isolates cultured from specimens collected from patients, fomites, or the environment during a discrete time frame or from a well-defined area as part of an epidemiologic investigation that suggests that the isolates may be derived from a common source.

Genetically related isolates (clones): Genetically related isolates (clones) are isolates that are indistinguishable from each other by a variety of genetic tests (e.g., PFGE, multilocus enzyme electrophoresis, or ribotyping) or that are so similar that they are presumed to be derived from a common parent.

Strain: A strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic characteristics or genotypic characteristics or both. A strain is a descriptive subdivision of a species."
Original citation from: Tenover et al. 1995 [90].

Since outbreak events were not investigated in this work, identification of putative genetically related isolates was the primary focus of the PFGE analysis.

4.2.9 Multi-locus sequence typing (MLST)

Subsequent to PFGE analysis, individual strains (n=108/220) were selected for MLST analysis in order to keep the costs of sequencing manageable. In order to achieve a representative selection, isolates of each clade were selected. In Figures 8a-8c, selected isolates for MLST can easily be identified by a displayed ST.
In general, MLST bases on PCR amplification of internal fragments of housekeeping genes distributed in the bacterial genome. The genes chosen are essential for the cell viability and commonly associated with a low mutation rates. Analysis of allelic composition and changes allows determination of phylogenetic relationships among a given set of isolates. In this study, MLST analysis of representative *S. pseudintermedius* isolates (selected after PFGE analysis) was performed according to a recently developed scheme published by Solyman et al., 2013 [106].

The amplifications of the seven housekeeping genes (*purA, fdh, sar, tuf, cpn60, pta* and *ack*) required for the MLST analysis were prepared by the following reaction mixture:

5.4µl	10 x green Puffer
1.3µl	dNTPs (10mM each)
2.5µl (1.25µl F /1.25µl R)	reaction primer (10µM each)
0.1µl	DreamTaq DNA-Polymerase 5 U/µl
5µl	template DNA (50 ng)
add 35.7µl	DDW*

* DDW: double distillated water

The PCR conditions were 95°C for 90s, followed by 35 cycles of 30s at 52°C, 1min at 72°C, 30s at 94°C and a final extension at 72°C for 5min. The resulting amplicons were purified by using QIA quick PCR Purification Kit. Subsequent sequencing was carried out by LGC Genomics GmbH (Berlin). Allele sequences were analysed using the Bionumerics software (AppliedMaths, Belgium). Allele numbers were assigned according to the MLST scheme provided by Prof. Dr. V. Perreten (curator of the *S. pseudintermedius* MLST database: www.mlst.net).

4.2.10 MLST data analysis and generation of a minimum spanning tree

A minimum spanning tree was generated to display a ST-based model on the current *S. pseudintermedius* population including current data of available MSSP and MRSP isolates (as of 23.02.2016; **Figure** 2). An additional tree was calculated including all STs identified during this thesis (MST generation: Dr. B. Walther).

4.3 Statistical Analysis

Statistical significance testing regarding presence of exfoliative toxins in MSSP isolates obtained from wound and other body sites was performed using cross-tabulation followed by Fisher's exact test.

5 Results

5.1 Characterisation of *S. pseudintermedius* isolates with respect to origin, methicillin resistance and virulence factors

A total of 220 *S. pseudintermedius* classified as either methicillin-susceptible (MSSP n= 110) or -resistant (MRSP n= 110) were isolated between 2010 to 2012 from clinical specimens sampled during a study on risk factors associated with MRSP infections in companion animals conducted by Anja G. Brandenburg and Dr. Ivonne Stamm at the IDEXX MedVet Lab, Ludwigsburg, and the microbiological unit at IDEXX VetMed Lab Ludwigsburg, respectively. The majority of isolates were obtained from specimens of dogs (n=195, 88.6%), followed by those from cats (n= 23, 10.5%) and horses (n= 2, 0.9%). On arrival at the IMT, all *S. pseudintermedius* isolates underwent a PCR-based screening procedure for methicillin-resistance directed at the gene *mec*A. Three isolates initially classified as methicillin-resistant (IMT21472, IMT24467, IMT25853) yielded a negative PCR result for *mec*A, while none of the MSSP isolates were positive for *mec*A. Re-testing for methicillin-resistance by use of the agar diffusion method recommended by the CLSI revealed a susceptible phenotype for all of them (see methods 4.1). Consequently, 113 MSSP and 107 MRSP isolates (re-classification: 1.5% of 220 isolates) underwent further molecular-biological characterization procedures. The geographic distribution of all MRSP and MSSP isolates is presented in **table** 3 and **Figure** 3. Some clinics / practices provided more than one specimen considered in the original study: 15 veterinary settings were associated with two isolates each, further 15 with three isolates, three with four and one clinic provided six isolates.

Table 3: Overview on geographical distribution of MRSP and MSSP

	total		MRSP		MSSP	
Federal State	**n= 220**	**%**	**n= 107**	**%**	**n= 113**	**%**
Bavaria	46	20.9	19	17.8	27	23.9
North-Rhine-Westphalia	43	19.5	23	21.5	19	17.7
Hessia	36	16.4	19	17.8	17	15
Baden-Württemberg	31	14.1	17	15.9	14	12.4
Lower Saxony	20	9.1	5	4.7	16	14.2
Rhineland-Palatinate	9	4.1	5	4.7	4	3.5
Saxony	9	4.1	3	2.8	6	5.3
Schleswig-Holstein	7	3.2	3	2.8	4	3.5
Thuringia	6	2.7	5	4.7	1	0.9
Hamburg	4	1.8	2	1.9	2	1.8
Mecklenburg Western-Pomerania	3	1.4	3	2.8	0	0
Brandenburg	2	0.9	1	0.9	1	0.9
Saxony-Anhalt	2	0.9	2	1.9	0	0
Berlin	1	0.5	0	0	1	0.9
Saarland	1	0.5	0	0	1	0.9

Abbreviation: n: number of isolates, MRSP: Methicillin-resistant *Staphylococcus pseudintermedius*, MSSP: Methicillin-susceptible *Staphylococcus pseudintermedius*

The majority of isolates (n=60; 27.3%) were detected in cultures of ear specimens, followed by those from surgical site infections (n=38; 17.3 %), skin and accessory structures (include hair, nails, dermoid cyst and teats; n=33; 15%) and wounds (n=25; 11.4%). Furthermore, other body sites of specimen origin were derived from the respiratory system (n=20; 9.1%); uro-genital tract (n=31; 14.1%) and eye (n=13; 5.9%), see also **Figure** 5.Furthermore, regional differences between German Federal States regarding occurrence of either MRSP or MSSP strains were not compared and interpreted statistically due to the convenience sample nature of the included biological samples. Consequently, a representative association with an overlying population could not be assumed.

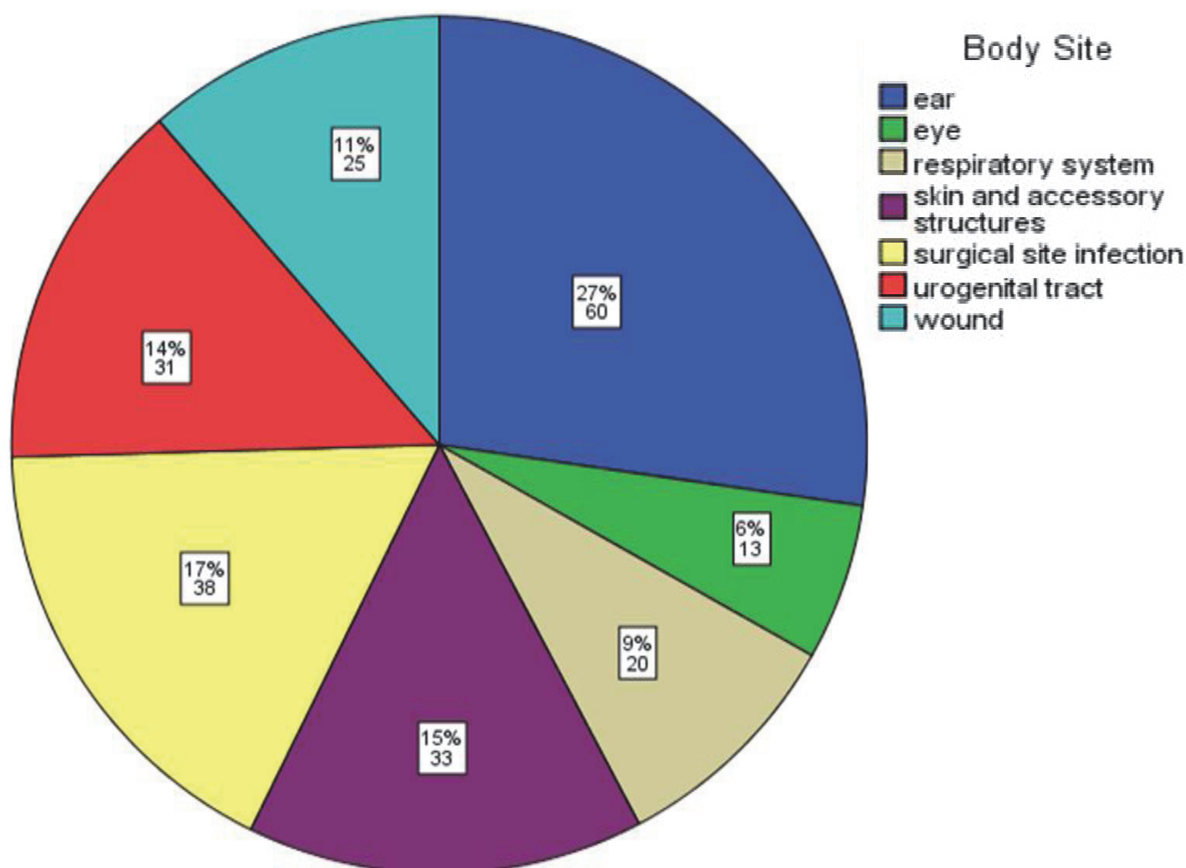


Figure 5: *S. pseudintermedius* isolates in relation to body sampling sites (n= 220), the majority of isolates were obtained from dogs (n=195, 88.6%), followed by cats (n= 23, 10.5%) and horses (n= 2, 0.9%).

5.2 Virulence-associated factors among *S. pseudintermedius* isolates

All 220 *S. pseudintermedius* isolates showed a positive PCR result for both constituents of the *Luk*I-operon (*Luk*F and *Luk*S). Screening for genes encoding exfoliative toxins (*Exp*A and *Exp*B) by PCR revealed 18 *exp*A-positive MSSP and 24 *exp*B-positive isolates, including one MRSP (0.93% of all MRSP). Since the majority of MRSP isolates in this study was assigned to a single genetic lineage (MRSP_ST71; see below), comparative analysis of MRSP with MSSP with respect to the presence of exfoliative toxins was not performed. All data concerning the presence/absence of exfoliative toxins within the isolates investigated here are given in **table** 4. However, within the group of MSSP, the total *exp*A proportion was 18/113 (15.9%) and for *exp*B 23/113 (20.4%). The higher rate of *exp*B in MSSP isolates obtained from wound specimens was statistically significant (p=0.024) compared with other body sites (95% confidence interval). There was no statistical significance for the presence of *exp*A.

Four isolates were found positive for both genes 4/113 (3.5%). In additon, the distribution of exfoliative toxins with respect to distinct sequence types are shown in **Figure** 10 .

Table 4: Distribution of exfoliative toxins among MRSP and MSSP isolates with respect to distinct body sites

				Body site of isolation															
				Ear		SAS		Wound		SSI		UTI		Eye		RS		**All**	
				No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
***mec*A**	n	*exp*A	n	31	79.5	21	84.0	9	69.2	4	80.0	12	100	9	100	9	90.0	95	84,1
			p	**8**	**20.5**	4	16.0	**4**	**30.8**	1	20.0	0	0	0	0	1	10.0	**18**	**15,9**
		*exp*B	n	31	79.5	22	88.0	7	53.8	4	80.0	10	83.3	7	77.8	9	90.0	90	79,6
			p	8	20.5	3	12.0	**6**	**46.2**	1	20.0	2	16.7	2	22.2	1	10.0	**23**	**20,4**
	p	*exp*A	n	21	100	8	100	12	100	33	100	19	100	4	100	10	100	107	100
			p	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		*exp*B	n	21	100	8	100	12	100	32	97.0	19	100	4	100	10	100	106	99,1
			p	0	0	0	0%	0	0	1	0	0	0	0	0	0	0	1	0.9

Abbreviations: n: negative, p: positive; No: number of isolates; *mec*A: gene encoding methicillin resistance; SAS: skin and accessory structures; SSI: surgical site infections; UT: uro-genital tract; RS: respiratory system.

5.3 Results of multi-locus sequence typing

MLST results for the 108 selected representatives of each *S. pseudintermedius* clade (see: **Figure** 8a-8c for detailed information on each selected isolate) were converted into the Bionumerics database of the IMT (File: Yassmin 2) and further analysed with respect to their phylogenetic relationship, geographic origin of certain STs and distribution among PFGE clades (see: **Figures** 6, 9). MLST analysis of 108 representative *S. pseudintermedius* isolates (n= 53 MSSP and n= 55 MRSP) which were isolated between 2010 and 2012 were assigned to 57 different sequence types (STs).

In this study, 51 of 53 MSSP isolates investigated by MLST belonged to novel STs, resulting in 50 new data base entries (STs) including four new allelic variants for *sar* (IMT27350), *cpn*60 (IMT27449), *pur*A (IMT30128) and *fdh* (IMT24470). Only two of the novel STs occurred twice, namely ST186 and ST438. The new STs were represented by unique isolates while four isolates belonged to ST186 (n=2) and ST438 (n=2). Interestingly, one ST (ST370) was already present in the MLST database, associated with an MRSP from Korea. Here, ST370 is represented by an MSSP.

Six distinct (STs) were detected for the 55 MRSP investigated in this study. The majority of them belonged to ST71 (n=48), while ST258 (n=2), ST335 (n=2), ST341 (n=1), and the novel types ST461 (n=1) and ST525 (n=1) were represented only rarely. A detailed overview on all isolates associated with novel STs with respect to geographic and host origin and presence of *exp*A and *exp*B genes is presented in **appendix 1**.

MLST analysis was carried out using the advanced cluster analysis tool choosing the predefined template with default settings for creating minimum spanning trees. This analysis was carried out by Dr. B. Walther. Among the MST tree, all MRSP and MSSP isolates were clustered according to the sequence types and the Federal States of Germany (**Figure** 6). Further phylogenetic tree was performed showing the clonal distribution of our sequence types within all *S. pseudintermedius* MLST database regardless of any clusters (**Figure** 7).

Figure 6: Phylogenetic relationship of 108 *S. pseudintermedius* isolates and their sequence types as well as geographic origin.

Abbreviations: LS: Lower Saxony, SH: Schleswig-Holstein, MP: Mecklenburg Western Pomerania, SA: Saxony-Anhalt, RP: Rhineland-Palatinate, BW: Baden-Württemberg, NRW: North-Rhine-Westphalia.Clustering of 56 STs using minimum spanning tree: Each circle represents a unique sequence type (red= MRSP, green= MSSP). The size of the circles is related to the number of isolates. Sample origin is indicated to Federal States of Germany.

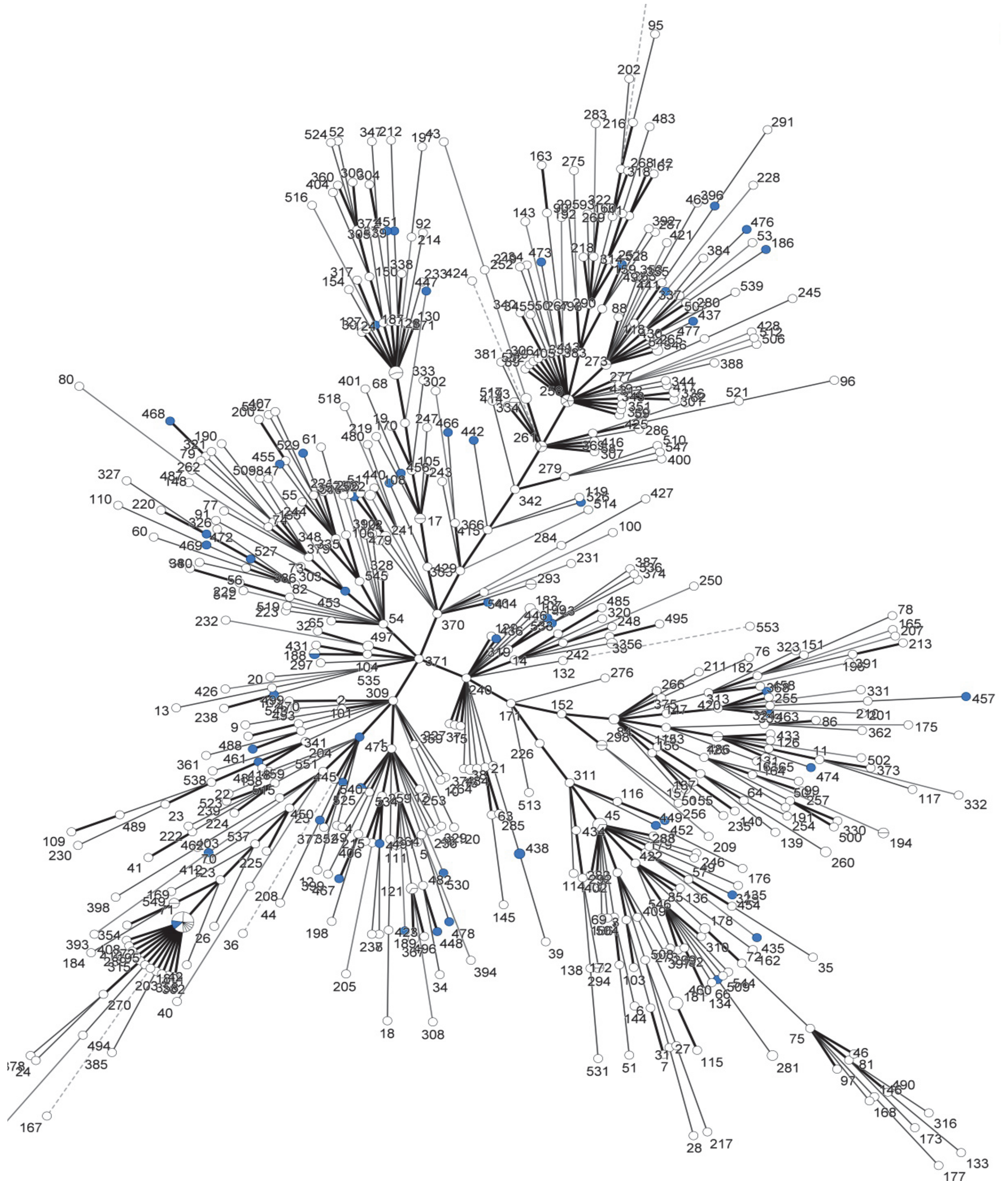


Figure 7: MST based on MLST database entries of *S. pseudintermedius.*

MST based on more than 600 *S. pseudintermedius* MLST database entries from all continents represented by white circles with sequence types identified in this work (blue circles): Lack of any association of STs representing *S. pseudintermedius* obtained from diseased animals of different federal states with respect to branches or putative clusters Thus, a certain ST-subgroup associated with strains from Germany is missing so far.

5.4 Results of PFGE analysis

Analysis of the pulsed-field gel electrophoresis (PFGE) was performed by use of Bionumerics 7.5 (AppliedMaths, Belgium). Based on this analysis, 219/220 *S. pseudintermedius* isolates *Sma*I cleavage patterns yielded 20 distinct clusters using 80% similarity as a cut-off criterion [27, 186, 187]. A further three isolates were assigned as "singletons" since their PFGE-patterns seemed to be unrelated to the other clusters. **Figure** 8a and 8c.

Altogether, five PFGE clades (1, 2, 8, 12 and 16) comprising 163 isolates (74.4%) represented the majority of the *S. pseudintermedius* investigated here. Each of these clades incudes at least ten isolates. PFGE pattern associated with MSSP isolates were distributed among 19 of the 20 clades and were represented by two of the three singeltons.

5.4.1 Detailed description of five major PFGE clades

In the following section PFGE clades comprising more than ten isolates were analysed with respect to commonalities and differences in sample origin, host species, patient history and molecular characteristics such as genomic background (sequence type assigned for representatives of each clade) and presence of genes encoding exfoliative toxins. Detailed information about all isolates is provided in **Figure** 8a-8c.

5.4.1.1 Description of major PFGE clade 1

The first clade comprises 10 MSSP, including the novel STs ST462, ST463, ST466 and ST475 representing each sub-group of the clade, and one MRSP (ST341) isolate which originated from canine and feline samples of six different federal states, including veterinary settings located in northern, western, middle and eastern regions of Germany (Dendrogram Part 1; **Figure** 8a). Within this clade, three of 11 (27.3%) isolates were derived from samples of skin and accessory structures and 3/11 from the respiratory system. A further five isolates were from specimens of the ear, eye, uro-genital system, wound and surgical site infection, respectively. Two MSSP were positive for *exp*A.

5.4.1.2 Description of major PFGE clade 2

This clade comprises 14 canine and one feline isolate, the majority of which are MSSP (n=13). Six of the MSSP were isolated from ear samples, three from the uro-genital tract, two from eyes, one from the respiratory system and one from a surgical site infection, respectively. Three MSSP were selected for MLST and subsequently assigned to the novel sequence types ST438, ST443, and ST450, originating from Bavaria, Baden-Württemberg and the Saarland. Six strains yielded a positive PCR signal for *exp*A and five for *exp*B (including one isolate positive for both genes). (Dendrogram Part 1; Figure 8a).

Two canine MRSP (ST71) from skin infection and uro-genital system specimens were included also in this PFGE clade originated from Hessen and Baden-Württemberg, respectively.

5.4.1.3 Description of major PFGE clade 8

Clade eight, the largest group of this study comprises 101 isolates, including 89 MRSP and 12 MSSP which originated from a wide range of federal states, hosts and body sites. 39 representatives of the 89 MRSP were selected for MLST and all belonged to ST71. MRSP were isolated from specimens of canine origin (n=77), eleven from cats and one from a horse. Most were associated with surgical site infections (n= 31), followed by ear samples (n=15) and specimens from the uro-genital tract (n=14). Samples originated predominately from North Rhine-Westphalia (n=18) and Bavaria (n=17), followed by Hessia (n=16) and Baden-Württemberg (n=14), (see also dendrogram part 1 and 2; Figure 8a and 8b).

Three canine MRSP originating from Baden-Württemberg (IMT25838), North Rhine-Westphalia (IMT25843) and Hessia (IMT25849), showed indistinguishable PFGE patterns. A further four “pairs” of canine MRSP with a similar pattern were identified within this clade. The first pair (IMT25807 and IMT29752) were both isolated from specimens of the uro-genital tract (UT), one sent from a praxis located in Thuringia, and one from Hessia. The second, third, and fourth pairs were isolated from different body sites (SSI; UT; ear and wound) and different Federal States. Moreover, two MRSP (from specimens of a dog and a cat) from Hessia and Bavaria showed 100% similarity and had been collected from ear and respiratory system samples, respectively. Likewise, three MRSP isolated from specimens of uro-genital tract (IMT25798, cat), a wound (IMT25810, dog) and skin (IMT25813, cat) showed similar PFGE patterns.

On the other hand, 12 MSSP (dog n=11, cat n=1) clustering within this clade showed heterogeneous PFGE patterns and most of them had been isolated from skin and accessory structures (5 of 12). Furthermore, ten representative MSSP were characterized by MLST and subsequently assigned to sequence types ST186, ST444, ST446, ST447, ST448, ST449, ST454, ST464, ST465 and ST527. Five MSSP and one MRSP yielded a positive PCR result for *exp*B and none for *exp*A.

5.4.1.4 Description of major PFGE clade 12

A set of 24 canine isolates from various federal states in Germany clustered within this clade, including 23 MSSP (n=11 isolated from ear samples) followed by those from skin specimens (n=5), the uro-genital tract (n=4) as well as singletons from wound, respiratory system and infected skin samples. Two isolates harbored *exp*A, one *exp*B and one was positive for both genes. Sequence types were assigned for 12 isolates as ST187, ST189, ST435, ST437, ST438, ST457, ST458, ST469, and ST528. (Dendrogram Part 3; Figure 8c).

Interestingly, only one MRSP (IMT25802, ST71) was associated with this clade and whole genome sequencing performed by T. Semmler and B. Walther exhibited significant differences with respect to other MRSP_ST71 of this study in the high resolution phylogeny analysis (unpublished data, personal communication).

5.4.1.5 Description of major PFGE clade 16

This clade comprises 12 (10 MSSP and 2 MRSP) canine isolates which derived mostly from Bavaria (n= 4), Baden-Württemberg (n= 3) and Lower Saxony (n=2). Isolates were from ear samples (6/12), wound samples (2/12), the respiratory system (2/12), the uro-genital system and skin. Three MSSP isolates were positive for the presence of exfoliative toxins (one for *exp*A and two for *exp*B). In addition, four MSSP isolates belonged to ST441, ST451, ST476, ST530 respectively, while a single MRSP was assigned as ST258. (Dendrogram Part 3; Figure 8c).

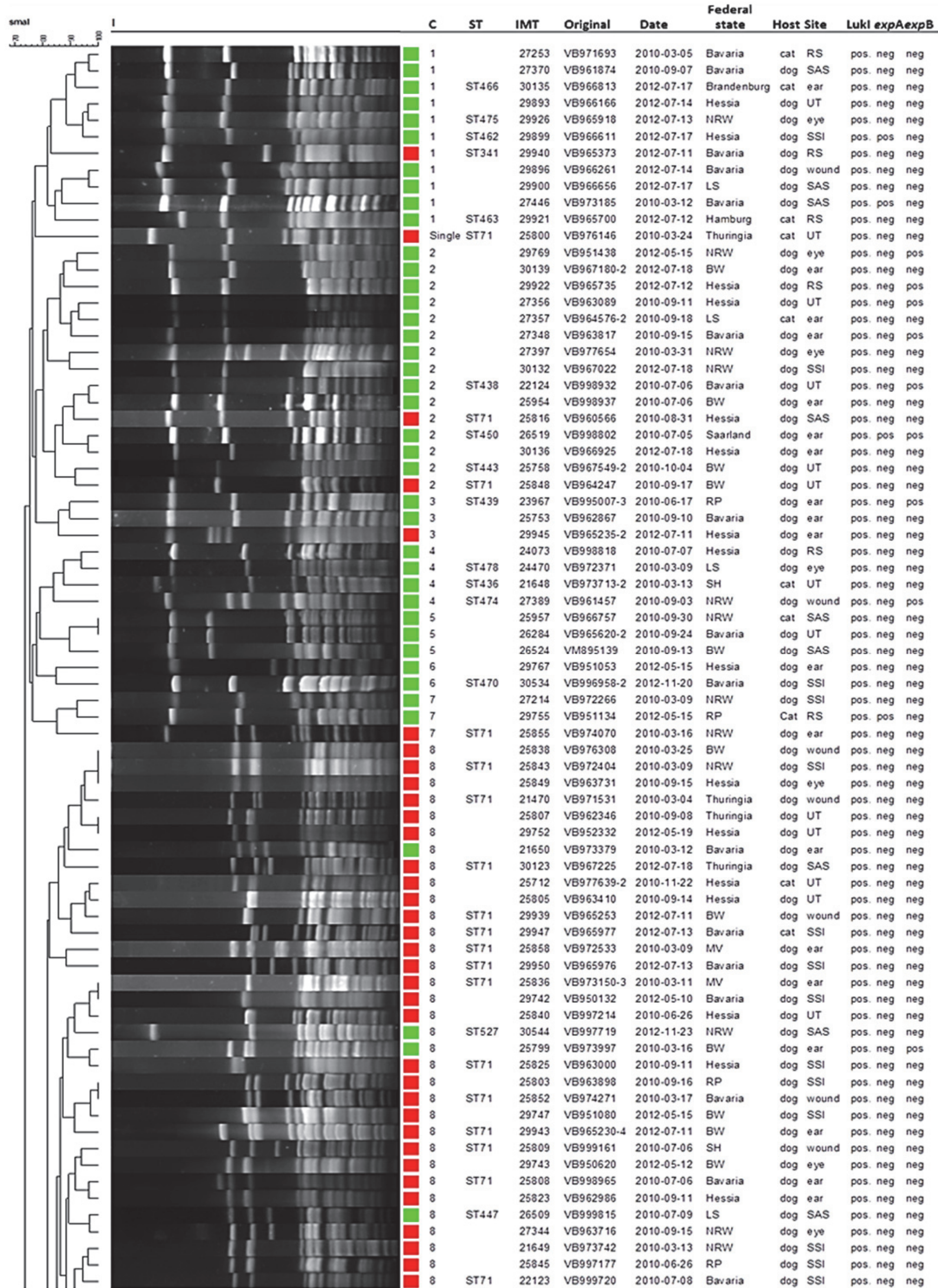


Figure 8a: Dendrogramm based on *Sma*I-restriction pattern of 219/220 *S. pseudintermedius* isolates (part 1).

Abbreviations: red squares: MRSP; green squares: MSSP, ST: sequence type; C: Clade; IMT: IMT strain collection number, Original: Isolate number by IDEXX VetMed Lab Ludwigsburg; UT: genitourethral system; SAS: Skin and accessory structures; RS: Respiratory system; SSI: surgical site infection; LS: Lower Saxony; NRW: North Rhine Westphalia, BW: Baden-Wuerttemberg, RF: Rhineland Palatine, Brandenb.: Brandenburg, SA: Saxony-Anhalt, MV: Mecklenburg-Hither Pomerania; SH: Schleswig-Holstein; LukI: *luk*S and *luk*F encoding leukotoxin I; *exp*A: gene encoding exfolitive toxin A; *exp*B: gene encoding Exfolitative toxin B

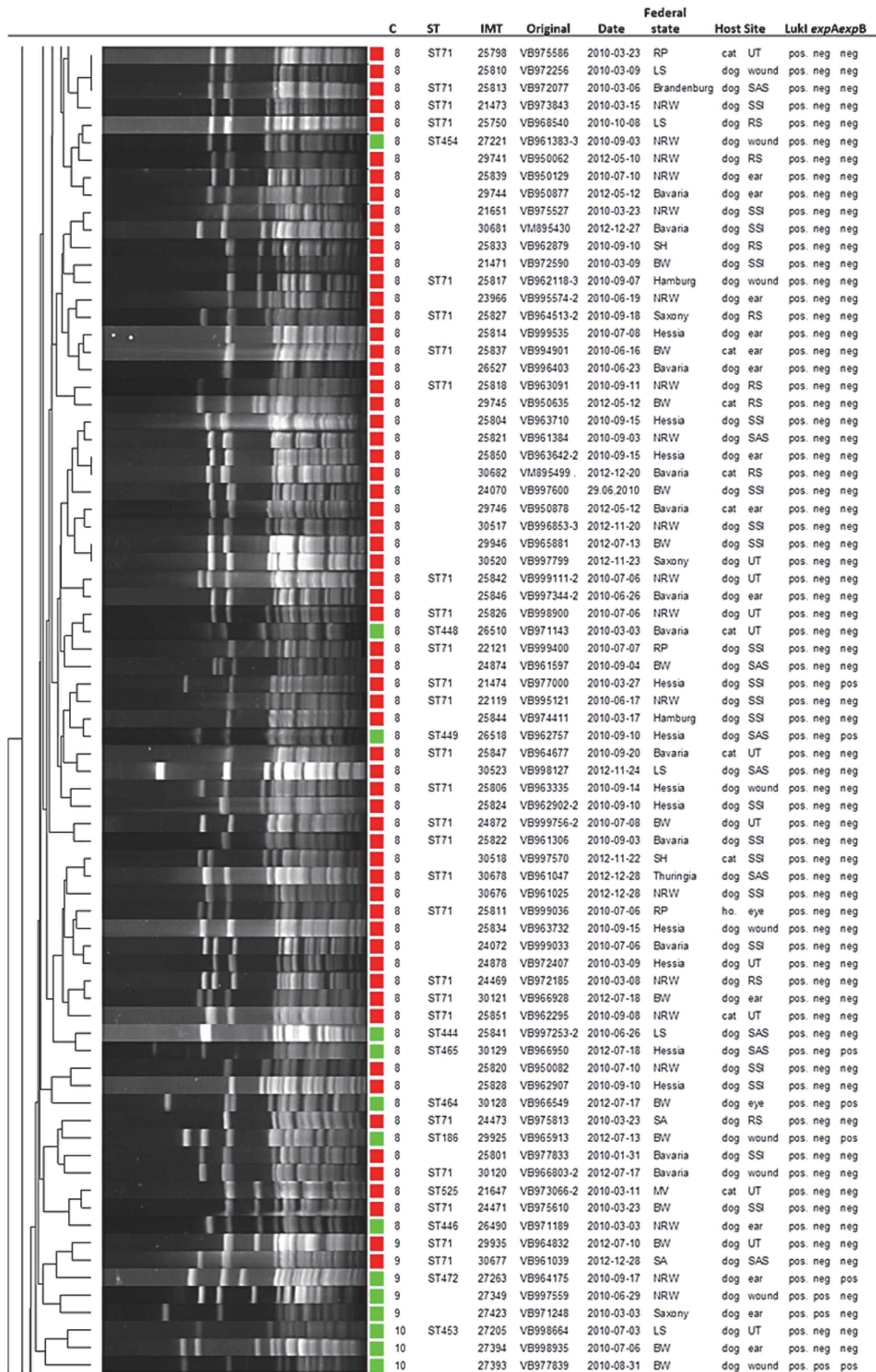
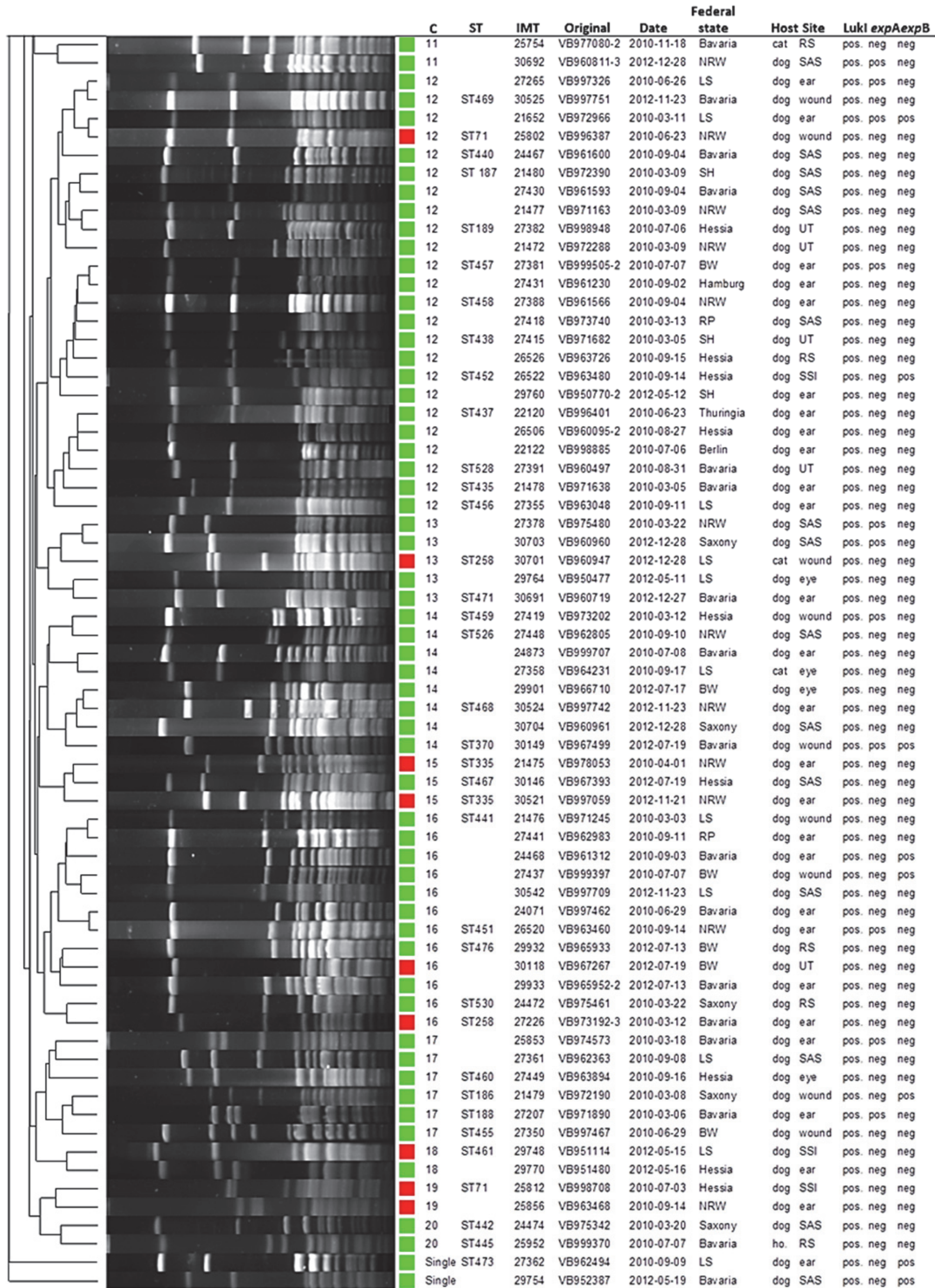


Figure 8b: Dendrogramm based on *Sma*I-restriction pattern of 219/220 *S. pseudintermedius* isolates (part 2).

Abbreviations: red squares: MRSP; green squares: MSSP, ST: sequence type; C: Clade; IMT: IMT strain collection number, Original: Isolate number by IDEXX VetMed Lab Ludwigsburg; UT: genitourethral system; SAS: Skin and accessory structures; RS: Respiratory system; SSI: surgical site infection; LS: Lower Saxony; NRW: North Rhine Westphalia, BW: Baden-Wuerttemberg, RF: Rhineland Palatine, Brandenb.: Brandenburg, SA: Saxony-Anhalt, MV: Mecklenburg-Hither Pomerania; SH: Schleswig-Holstein; LukI: *luk*S and *luk*F encoding leukotoxin I; *exp*A: gene encoding exfolitive toxin A; *exp*B: gene encoding Exfolitative toxin B



C	ST	IMT	Original	Date	Federal state	Host	Site	LukI	*exp*A	*exp*B
11		25754	VB977080-2	2010-11-18	Bavaria	cat	RS	pos.	neg	neg
11		30692	VB960811-3	2012-12-28	NRW	dog	SAS	pos.	pos	neg
12		27265	VB997326	2010-06-26	LS	dog	ear	pos.	pos	neg
12	ST469	30525	VB997751	2012-11-23	Bavaria	dog	wound	pos.	neg	neg
12		21652	VB972966	2010-03-11	LS	dog	ear	pos.	pos	pos
12	ST71	25802	VB996387	2010-06-23	NRW	dog	wound	pos.	neg	neg
12	ST440	24467	VB961600	2010-09-04	Bavaria	dog	SAS	pos.	neg	neg
12	ST 187	21480	VB972390	2010-03-09	SH	dog	SAS	pos.	neg	neg
12		27430	VB961593	2010-09-04	Bavaria	dog	SAS	pos.	neg	neg
12		21477	VB971163	2010-03-09	NRW	dog	SAS	pos.	neg	neg
12	ST189	27382	VB998948	2010-07-06	Hessia	dog	UT	pos.	neg	neg
12		21472	VB972288	2010-03-09	NRW	dog	UT	pos.	neg	neg
12	ST457	27381	VB999505-2	2010-07-07	BW	dog	ear	pos.	pos	neg
12		27431	VB961230	2010-09-02	Hamburg	dog	ear	pos.	neg	neg
12	ST458	27388	VB961566	2010-09-04	NRW	dog	ear	pos.	neg	neg
12		27418	VB973740	2010-03-13	RP	dog	SAS	pos.	neg	neg
12	ST438	27415	VB971682	2010-03-05	SH	dog	UT	pos.	neg	neg
12		26526	VB963726	2010-09-15	Hessia	dog	RS	pos.	neg	neg
12	ST452	26522	VB963480	2010-09-14	Hessia	dog	SSI	pos.	neg	pos
12		29760	VB950770-2	2012-05-12	SH	dog	ear	pos.	neg	neg
12	ST437	22120	VB996401	2010-06-23	Thuringia	dog	ear	pos.	neg	neg
12		26506	VB960095-2	2010-08-27	Hessia	dog	ear	pos.	neg	neg
12		22122	VB998885	2010-07-06	Berlin	dog	ear	pos.	neg	neg
12	ST528	27391	VB960497	2010-08-31	Bavaria	dog	UT	pos.	neg	neg
12	ST435	21478	VB971638	2010-03-05	Bavaria	dog	ear	pos.	neg	neg
12	ST456	27355	VB963048	2010-09-11	LS	dog	ear	pos.	neg	neg
13		27378	VB975480	2010-03-22	NRW	dog	SAS	pos.	pos	neg
13		30703	VB960960	2012-12-28	Saxony	dog	SAS	pos.	pos	neg
13	ST258	30701	VB960947	2012-12-28	LS	cat	wound	pos.	neg	neg
13		29764	VB950477	2012-05-11	LS	dog	eye	pos.	neg	neg
13	ST471	30691	VB960719	2012-12-27	Bavaria	dog	ear	pos.	neg	neg
14	ST459	27419	VB973202	2010-03-12	Hessia	dog	wound	pos.	pos	neg
14	ST526	27448	VB962805	2010-09-10	NRW	dog	SAS	pos.	neg	neg
14		24873	VB999707	2010-07-08	Bavaria	dog	ear	pos.	neg	neg
14		27358	VB964231	2010-09-17	LS	cat	eye	pos.	neg	neg
14		29901	VB966710	2012-07-17	BW	dog	eye	pos.	neg	neg
14	ST468	30524	VB997742	2012-11-23	NRW	dog	ear	pos.	neg	neg
14		30704	VB960961	2012-12-28	Saxony	dog	SAS	pos.	neg	neg
14	ST370	30149	VB967499	2012-07-19	Bavaria	dog	wound	pos.	pos	pos
15	ST335	21475	VB978053	2010-04-01	NRW	dog	ear	pos.	neg	neg
15	ST467	30146	VB967393	2012-07-19	Hessia	dog	SAS	pos.	neg	neg
15	ST335	30521	VB997059	2012-11-21	NRW	dog	ear	pos.	neg	neg
16	ST441	21476	VB971245	2010-03-03	LS	dog	wound	pos.	neg	neg
16		27441	VB962983	2010-09-11	RP	dog	ear	pos.	neg	neg
16		24468	VB961312	2010-09-03	Bavaria	dog	ear	pos.	neg	pos
16		27437	VB999397	2010-07-07	BW	dog	wound	pos.	neg	pos
16		30542	VB997709	2012-11-23	LS	dog	SAS	pos.	neg	neg
16		24071	VB997462	2010-06-29	Bavaria	dog	ear	pos.	neg	neg
16	ST451	26520	VB963460	2010-09-14	NRW	dog	ear	pos.	pos	neg
16	ST476	29932	VB965933	2012-07-13	BW	dog	RS	pos.	neg	neg
16		30118	VB967267	2012-07-19	BW	dog	UT	pos.	neg	neg
16		29933	VB965952-2	2012-07-13	Bavaria	dog	ear	pos.	neg	neg
16	ST530	24472	VB975461	2010-03-22	Saxony	dog	RS	pos.	neg	neg
16	ST258	27226	VB973192-3	2010-03-12	Bavaria	dog	ear	pos.	neg	neg
17		25853	VB974573	2010-03-18	Bavaria	dog	ear	pos.	pos	neg
17		27361	VB962363	2010-09-08	LS	dog	SAS	pos.	neg	neg
17	ST460	27449	VB963894	2010-09-16	Hessia	dog	eye	pos.	neg	neg
17	ST186	21479	VB972190	2010-03-08	Saxony	dog	wound	pos.	neg	pos
17	ST188	27207	VB971890	2010-03-06	Bavaria	dog	ear	pos.	pos	neg
17	ST455	27350	VB997467	2010-06-29	BW	dog	wound	pos.	neg	neg
18	ST461	29748	VB951114	2012-05-15	LS	dog	SSI	pos.	neg	neg
18		29770	VB951480	2012-05-16	Hessia	dog	ear	pos.	neg	neg
19	ST71	25812	VB998708	2010-07-03	Hessia	dog	SSI	pos.	neg	neg
19		25856	VB963468	2010-09-14	NRW	dog	ear	pos.	neg	neg
20	ST442	24474	VB975342	2010-03-20	Saxony	dog	SAS	pos.	neg	neg
20	ST445	25952	VB999370	2010-07-07	Bavaria	ho.	RS	pos.	neg	neg
Single	ST473	27362	VB962494	2010-09-09	LS	dog	ear	pos.	neg	pos
Single		29754	VB952387	2012-05-19	Bavaria	dog	SAS	pos.	neg	pos

Figure 8c: Dendrogramm based on *Sma*I-restriction pattern of 219/220 *S. pseudintermedius* isolates (part 3).

Abbreviations: red squares: MRSP; green squares: MSSP, ST: sequence type; C: Clade; IMT: IMT strain collection number, Original: Isolate number by IDEXX VetMed Lab Ludwigsburg; UT: genitourethral system; SAS: Skin and accessory structures; RS: Respiratory system; SSI: surgical site infection; LS: Lower Saxony; NRW: North Rhine Westphalia, BW: Baden-Wuerttemberg, RF: Rhineland Palatine, Brandenb.: Brandenburg, SA: Saxony-Anhalt, MV: Mecklenburg-Hither Pomerania; SH: Schleswig-Holstein; LukI: *luk*S and *luk*F encoding leukotoxin I; *exp*A: gene encoding exfolitive toxin A; *exp*B: gene encoding Exfolitative toxin B

5.4.2 Comparison of PFGE clades with genomic background of MRSP and MSSP isolates

The following **Figure** 9 shows a minimum spanning tree (generated by Dr. B. Walther) calculated on MLST data base entries (24.02.16) for *S. pseudintermedius*. The distribution of PFGE clades (isolates clustering within the same clade were assigned to a distinct colour) are displayed for all 108 representatives together with their sequence types. *S. pseudintermedius* isolates clustering within one PFGE clade (exemplarily highlighted with black squares for clade 8) were widely distributed on the MST, indicating a lack of congruence between PFGE pattern and MLST data.

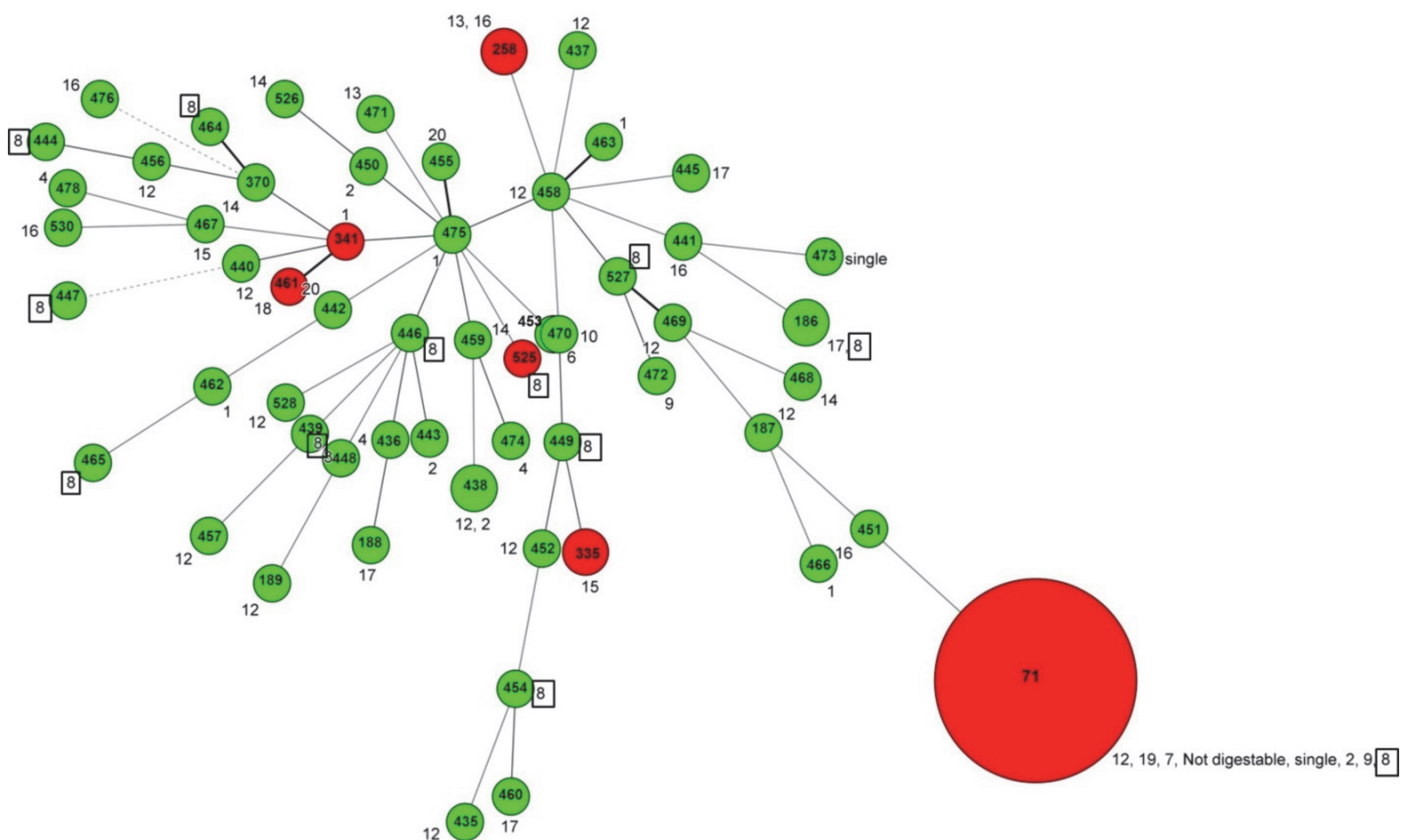


Figure 9: MST displaying PFGE cluster assignment and corresponding STs distribution of PFGE clades (isolates of the same clade share a single colour) together with their sequence types: Some isolates clustering within one PFGE clade (exemplarily highlighted with black squares for clade eight) were widely distributed on the MST. Each circle (red= MRSP, green= MSSP) represents one of each sequence types. Size of circles represents number of isolates.

Figure 10: MST displaying STs and distribution of detected exfoliative toxins assigned in this study. Each circle represents a distinct sequence type (red= MRSP, green= MSSP) together with A=*exp*A, B= *exp*B and *= number of isolates identified harboring the toxins. Size of circles reflects the number of isolates.

6 Discussion

The aim of this study was to investigate the genetic background and -relationship of a predefined set of 220 *S. pseudintermedius* isolates representing different host origins (dogs, cats), various infection sites (e.g. SSI, wounds, otitis, skin and associated structures) including methicillin-resistant and -susceptible variants. In addition, the occurrence and distribution of important virulence factors such as exfoliative toxins and leukotoxin among the isolate collection was determined and analyzed with respect to different genetic lineages within a phylogenetic tree (minimum spanning tree).

6.1 Characteristics of *S. pseudintermedius* isolates in Germany

The proportion of *S. pseudintermedius* isolates from canine origin was 88.6% within this convenience sample, while 10.5% were represented by feline and only 0.9% by equine isolates. The overwhelming proportion of *S. pseudintermedius* from canine origin mirrors the daily experience of the diagnostic lab of the Institute of Microbiology and Epizootics (personal communication) as well as the current literature [2, 12, 46, 188].

A review published in 2012 described the adaption of *S. pseudintermedius* to the canine host (*Canidae* family) [39]. Despite this apparent adaptation, the pathogen has the potential to induce severe and fulminate infectious diseases in other species including horses and cats as well [27, 44, 45].

Furthermore, the capacity of *S. pseudintermedius* to cause a wide range of different opportunistic and purulent infections is mirrored by the results of this study: The majority of isolates were obtained from cultures of ear specimens (n=60/220; 27.3%), followed by those from surgical site infections (n=38/220; 17.3 %), skin and accessory structures (n=33/220; 15%), wounds (n=25/220; 11.4%) and other body sites (64 of 220, 29.1%; Fig. 6). A similar broad panel of infectious diseases caused by *S. pseudintermedius* (including MRSP) have been reported worldwide [27, 46, 188-190].

Samples from the ear and skin (together with accessory structures) account together for 42% (27% & 15%) of the sample collection, indicating the importance of *S. pseudintermedius* as an opportunistic pathogen especially at these body sites. A recent study by Haenni et al. 2014 reported isolation rates of 55.6% *S. pseudintermedius* for samples from skin infections and 9.9% for ear swabs in France. Since a single referral clinic was the source for all isolates reported by Haenni et al., this difference in the study design might be the reason for this deviation.

6.2 Proportion and distribution of virulence factors among MRSP and MSSP

Staphylococci are able to produce a number of toxins including exotoxins like cytolytic hemolysins, exfoliative toxins, enterotoxins, toxic shock syndrome toxin and leukotoxins such as the Panton-Valentine leukocidin (PVL) factor, which are frequently involved in processes of severe tissue necrosis and furunculosis caused by *S. aureus*. A similar leukotoxin (LukI) is commonly present in *S. pseudintermedius* [27, 31].

Nonetheless, there is a lack of data regarding the occurrence, frequency and association with respect to different phylogenetic lineages of virulence factors associated with MRSP and MSSP of companion animal origin. Consequently, this study intended to evaluate the proportion of important virulence factors such as exfoliative toxins and leukocidin described for *S. pseudintermedius* in earlier studies [27, 155, 191, 192].

In this study, the presence of the two co-transcribed genes encoding *Luk*I (*luk*S and *luk*F) for each of the 220 *S. pseudintermedius* was detected. This result is in congruence with former studies describing a common occurrence of *Luk*I in *S. pseudintermedius* independently of e.g. geographic origin, host or body site affected [192, 193]. Consequently, further functional studies are needed to reveal the exact role of *Luk*I in the colonization and/or development of purulent diseases in the canine host. However, while its role in the pathogenesis of canine diseases is not clear, one report suggested an importance of this factor for causing infections in the human host [192].

Exfoliative toxins (ET) such as *Exp*A and *Exp*B (serine proteases) are considered as important virulence factors for skin-associated infections in dogs. Staphylococcal exfoliative toxins are able to induce intra-epidermal splitting by enzymatic cleavage of desmoglein 1. Current studies show that *Exp*A exhibits 67.1%, 67.9% and 65.1% amino acid similarity with other ETs from *S. aureus* (ETB, ETD) and *S. hyicus* (SHETB), respectively. *Exp*B has homologies with SHETB (70.4%), ETD (66.1%) and *Exp*A (56.9%) [55, 56].

In this study, genes encoding exfoliative toxins were more common in MSSP (*exp*A 15.9% and *exp*B 20.4%) than in MRSP (<1%). This result might reflect the predominance of a single genetic background, namely MRSP_ST71 (*exp*B-positive: 1/107) among the sample collection, but additional MRSP STs described within this study such as ST253, ST341, ST461, ST525 and ST335 were negative for both toxin variants described so far. The general observation that MRSP were rarely associated with exfoliative toxins has previously been reported in other studies [155, 194].

Conversely, studies on the occurrence of virulence factors of *S. aureus* indicated that the proportion of exfoliative toxin (ETA, ETB) harboring isolates causing bullous impetigo and staphylococcal scalded skin syndrome (SSSS) were more often associated with MRSA than MSSA [195-197].

However, results of this study indicate that *exp*A is more common among MSSP isolated from ear swabs (8/39; 20.5%) compared to an average of 15.9% for all MSSP isolates investigated here (**table** 4). A comparable result was obtained for *exp*B, which occurred in a similar frequency (8/39; 20.5%). In addition, only 4/113 (3.5%) MSSP isolates were positive for both toxins. In contrast to expectations, the occurrence of these toxins in isolates obtained from samples of skin and accessory structures (16% and 12%) were near to the average or below.

Interestingly, MSSP isolates originating from wound swabs seem to be more frequently associated with *exp*A (4/13; 30.8%) and *exp*B (6/13; 46.2%). Thus, exfoliative toxins might have a beneficial effect for the bacteria during establishment of wound infections. This aspect might be an interesting point for further investigations.

Since the presence of *exp*B in MSSP isolates obtained from wound specimens showed statistical significance (p=0.024) compared with those from other body sites, this toxin might contribute to the inflamatory progression of canine wound infections. However, further functional research is needed to evaluate this observation.

Moreover, the broad distribution of *exp*A- and *exp*B-positive isolates within the phylogenetic tree lacks obvious phylogenetic associations (**Figure** 10). This result might indicate the importance of horizontal transmission of these factors on mobile elements. However, more research on the occurrence and distribution of exfoliative toxins is needed to reveal the role and impact of these virulence factors in distinct clinical pictures. Since other staphylococcal exfoliative toxins (ETA, ETB and SHETB) were found on mobile elements such as phages and/or plasmids, horizontal transmission by such elements seems to be likely for *S. pseudintermedius*, too [198, 199]. Since other reports on the occurrence of exfoliative toxins had a different study design, a direct comparison is not possible. For instance, Gomez-Sanz and colleagues have observed a rare occurrence of *exp*A (2/16) and *exp*B (3/16) in MSSP isolated from nasal samples of healthy dogs [200], and Gharsa et al. 2013 detected three *exp*A-positive MSSP isolates (5.5%) among 55 isolates obtained from nasal swabs of healthy dogs [191]. Börjesson et al. 2015 identified the *exp*A (n=2) and *exp*B (n=1) among 12 MSSP human clinical isolates respectively [192].

Only recently, Couto et al. 2015 detected an *exp*B rate of 14.3% (3/21) among MSSP isolates received from different body sites of diseased dogs. Interestingly, this rate is slightly lower than the average rate of 20.4% in this study, but the results share a similar dimension (approximately 15-20% *exp*A or *exp*B-positive MSSP isolates). Since the isolate collection studied by Couto et al. was 100-fold smaller [194], that difference might account for a greater deviation.

6.3 Current genotypes of *S. pseudintermedius* from different regions in Germany

In the past, two different genetic lineages have been described for the majority of MRSP isolates, MRSP_ST71 as the dominating lineage in Europe and MRSP_ST68 for North-America [27]. Since that time, hundreds of new entries into the MLST database for *S. pseudintermedius* (www.mlst.net) including many new MRSP_STs (**Figure** 2), indicated either a recent and on-going diversification of MRSP genotypes or a lack of representativeness of former studies. Thus, the aim of this study was to investigate the genetic relationship of a large collection of *S. pseudintermedius* isolates with respect to the host, geographic origin and methicillin resistance using macro-restriction of the whole genome followed by PFGE and MLST.

In this study, MLST analysis based on seven housekeeping genes was performed for 108 representatives, including 53 MSSP and 55 MRSP isolated from 2010 to 2012. The detailed selection criteria were explained in chapter 4.2.9.

Within this study, 51 sequence types were found being associated with MSSP isolates of different origins, while ST71 was the predominant background (87.3%) for the MRSP isolates. However, five new MRSP STs were also detected: ST258, ST335, ST341, ST461 and ST525.

A minimum spanning tree (MST) based on the MLST data base entries at (www.mlst.net) was generated including all STs identified within this study together with the Federal State the original sample originated from (**Figure** 6). No obvious clustering of certain STs with respect to their origin was observed. Furthermore, a MST based on the complete MLST database (**Figure** 7) revealed even a lack of continent-association of certain STs, especially for MSSP, and the general topology of the tree might indicate a recent expansion of the *S. pseudintermedius* population. However, more research on this subject is needed including whole genome data analysis.

The current predominance of MRSP_ST71 in Germany reported in former studies [14, 25, 27] was confirmed. However, MRSP-singletons were also assigned to ST335, ST341, ST461, ST525 and ST258 (two isolates). In the past, the latter genotype was reported as ST106 based on the former MLST-5 scheme [14, 183]. Interestingly, the ST258 clone was sporadically reported for canine MRSP in France [201], in Denmark [202] and Italy [203]. Furthermore, ST258 was also reported as an emerging clone in Norway, were this ST (35% of isolates) the most predominant background followed by ST71 (20%) identified among 49 clinical MRSP from a national canine isolate collection [12]. The authors concluded that MRSP_ST258 is currently an emerging MRSP clone in Northern Europe. In addition, polyclonal diversification seems to be underway, not only in Norway [12] but also in Germany, considering the distribution of MRSP in the MST (**Figure** 6).

Since dogs and cats travel with their owners, this might be a reason for the dissemination of distinct MRSP STs, an idea which was discussed also by other authors [12, 202].

In addition, susceptible genotypes appear to have acquired mobile elements (SCC*mec*) conferring methicillin-resistance. Interestingly, an MRSP_ST370 was already present in the MLST database, associated with a canine isolate from Korea (data base entry 29-07-2014 in mlst data base), while in this study that ST was represented by an MSSP. A deeper analysis based on WGS data might elucidate the relationship between these two isolates from different continents including insights into the genomic recombination hotspot susceptible to SCC*mec* acquisition [204].

6.4 Genetic relationship of *S. pseudintermedius* circulating in Germany: Comparison of PFGE- and MLST results

PFGE analysis has been regarded as the ‘gold standard’ for outbreak-associated molecular typing methods for a variety of clinically important bacteria for decades [83].

Here, one of the 220 *S. pseudintermedius* isolates investigated was not PFGE-typeable using *Sma*I. DNA-methylation of the restriction sites is a commonly reported reason for failure of digestion with certain endonucleases [205-207].

The putative genetic relatedness of the remaining 219 isolates representing all Federal States of Germany was investigated by PFGE and analyzed by use of Bionumerics (AppliedMath, Belgium). In total, the majority of isolates clustered into 20 distinct groups using a cut-off value of 80% with unweighed pair group method using arithmetic averages (UPGMA). Only five clusters comprised 74.4% of the whole sample collection.

A very interesting finding was the fact that PFGE analysis was not mirrored by the corresponding MLST results. The following example shows the general discrepancy between PFGE and MLST results: Clade one comprises eleven isolates, and MLST data were available for five of them, including one MRSP. Considering the seven loci defining an individual ST, the clade one STs showed two allelic variants for four loci (*fdh*, *pta*, *sar*, *tuf*), while for a further two loci (*ack* and *cpn*60), three variants, and for *pur*A, four allelic variants were found (see appendix 1). Consequently, the genetic relationship with respect to MLST results obtained for the isolates clustering in clade one is rather limited. Furthermore, the use of an *in silico* restriction analysis based on whole genome data might help to elucidate which modifications of the *Sma*I restriction sites are responsible for this incongruence between PFGE and MLST data in this study. In addition, the MLST scheme used for *S. pseudintermedius* might lack discriminatory power. Although the MLST data base entries includes 600 different MLST, however, for the gene *tuf*, only 12 variants have been identified so far.

Furthermore, isolates belonging for instance to ST438 clustered in clade two as well as in clade 12. A closer look at the data available for these isolates revealed two aspects: The two PFGE patterns differed, particularly in the first three large DNA bands, while the smaller bands below 100kb are more or less similar. Furthermore, PCR results for IMT22124 were positive for *exp*B but negative in isolate IMT27415. Since exfoliative toxins are often present on pathogenicity islands (chromosomally or extra-chromosomally) which could reach sizes between 10-200kb, substantial effects of such elements on the PFGE patterns seem to be reasonable. While recent research on this subject based on WGS data allowed more and more insights into the structure of *S. pseudintermedius* including the presence of PAIs [47, 48], the subsequent effects on PFGE restriction pattern remains unclear.

Furthermore, the majority of MRSP isolates in this work were assigned to ST71, while the corresponding PFGE pattern were assigned to six different clusters, with clade eight comprising most of them (43.8%). These finding were in agreement with the first study of MRSP_ST71 from Japan that reported a similar heterogeneity for this ST with respect to PFGE clades and an also with a further recent study by Grönthal et al. 2015 from Norway [187, 208].

Another striking finding of this study was that some canine MRSP_ST71 isolates exhibited 100% similarity with respect to their PFGE pattern. For instance, the first three isolates clustering in clade eight originated from different practices of three distinct federal states in Germany. Two further matching isolates were also obtained from two practices in Thuringia and Hessia. In consequence, some isolates seemed to be related to a single common source but widely distributed in Germany, exemplifying a clonal expansion.

Furthermore, a common geographical origin for certain MSSP genotypes was not obvious (Fig 6). However, some MSSP pattern associated with isolates obtained from different animal species originating from different federal states seem to be closely related. This finding might indicate spread of various genetic lineages across large geographic regions. A putative explanation for this observation might be the mobility of the dog owners (e.g. holiday travelling, relocation, visiting dog shows, etc.). Previous studies regarding the PFGE pattern-diversity of MSSP described also an impressive heterologous distribution, indicating a large genetic diversity for MSSP in general [82, 186, 191, 200, 209].

7 Summary

Staphylococcus pseudintermedius, a member of the *Staphylococcus intermedius*-group, is known as a common colonizer of canine skin and mucosa, occasionally detected in specimens of other animal species and humans as well. Being a representative of a typical opportunistic pathogen, *S. pseudintermedius* is often associated with a wide variety of purulent or toxin-associated infectiouse diseases in animals as well as occasionally in humans. Despite increasing reports concerning the occurence of *S. pseudintermedius*, including its methicillin resistant variant (MRSP), in recent years, knowledge about the occurrence and distribution of genotypes together with important toxins in specimens from diseased dogs, cats and horses in Germany is scarce. MRSP are often associated with a multi-drug resistant phenotype, limiting effective antibiotic therapy options in veterinary medicine. In some cases, animals have to be put down due to reasons of animal welfare.

The aim of this study was to investigate the genetic background and relationships among a predefined set of 220 *S. pseudintermedius* isolates representing different host origins (dog, cat, horse), various infected sites (e.g. surgical site infection (SSI), wound, otitis, skin and skin-associated structures) including methicillin-resistant (n=107) and -susceptible (n=113) variants.

Genotypic characterization was performed by conducting pulsed-field gel electrophoresis (PFGE) for all *S. pseudintermedius* as well as multi-locus sequence typing (MLST) for 53 methicillin-susceptible (MSSP) and 55 methicillinresistant *S. pseudintermedius* (MRSP).

In addition, the occurrence and distribution of important virulence factors such as exfoliative toxins (*Exp*A, *Exp*B) and leukotoxin (LukI) among the isolate collection was determined by PCR detection of the respective encoding genes and subsequently analyzed with respect to different genetic lineages.

All investigated MSSP and MRSP yielded a positive PCR-result for *luk*I. Furthermore, *exp*A was detected in 18/113 of MSSP (15.9%), but not among MRSP. In addition, *exp*B was identified in 23/113 of MSSP (20.4%) and 1/107 of MRSP (0.9%), respectively.

With regard to the sample site origin distribution, MSSP isolates obtained from either “skin” or “skin-associated structures” were with 16% (*exp*A) and 12% (*exp*B) equal or even below the overall average. Interestingly, MSSP isolated from wound swabs yielded positive PCR signals above the overall average, 30.8% for *exp*A and 46.2% for *exp*B. The putative impact of these toxins during development of wound infections might be an interesting aspect and should be further investigated in functional studies.

In total, 219 of 220 *S. pseudintermedius* were typeable using the PFGE technique. Subsequent PFGE analysis and dendrogramm construction was carried out with Bionumerics 7.5 (Applied Maths, Belgium) using the Unweighted Pair Group Method with

Arithmetic mean (UPGMA) employing an 80% cut-off for cluster dedication. In total, 20 distinct clusters as well as three singletons were assigned. Pattern of MSSP isolates clustered within 19 of the 20 clades and were associated with two of the three singletons described in this work. However, some MSSP patterns associated with isolates obtained from different animal species originating from different federal states seem to be closely related. This finding might indicate spread of various genetic lineages across large geographic regions. A putative explanation for this observation might be the mobility of the dog owners (e.g. holidays, relocation, dog shows etc.).

Interestingly, the PFGE pattern of MRSP showed a remarkable diversity, an observation which has been published in different studies from other countries as well. Several MRSP revealed identical patterns, although these isolates originated from distinct animal patients living in different federal states. MLST analysis revealed 50 novel sequence types (ST) associated with the MSSP and one sequence type (ST370) that has been reported in a previous study.

In contrast, the predominant genotype assigned to 48 of 55 MRSP isolates obtained from various German regions, animals and infected sites determined in this study was ST71. PFGE analysis revealed that the majority of MRSP investigated by MLST (39 of 55) clustered within the largest clade (clade 8). However, further PFGE patterns associated with MRSP_ST71 clustered within other clades as well. The apparent incongruence of MLST and PFGE data for *S. pseudintermedius,* especially for MRSP_ST71, has previously been described in other studies as well. The use of an *in silico* restriction analysis based on whole genome data might elucidate DNA sequence modifications of the *Sma*I restriction sites possibly responsible for this discrepancy between results of MLST and PFGE analysis. In addition, the MLST scheme currently used for *S. pseudintermedius* might lack discriminatory power. At present, the MLST database includes more than 600 different data base entries. However, for the gene *tuf* only 12 variants have so far been identified.

Although ST71 is the predominant genetic lineage for MRSP investigated in this study, a diversification of MRSP sequence types was observed as well, consistent with recent publications. In addition, two novel MRSP genotypes (ST461 and ST525) were identified during this work. Furthermore, ST258 – an emerging genetic lineage identified in Northern Europe - was also detected in the present MRSP collection. This current evolvement seems to be similar to the evolution of methicillin-resistant *S. aureus* (MRSA) in the past, where several different genetic lineages seem to be capable of aquiring and integrating the mobile genetic element(s) harboring the methicillin resistance encoding gene. The future will show, whether MRSP infections will remain largely restricted to veterinary medicine or if serious infections in humans will occur more frequently.

8 Zusammenfassung

Vergleichende genotypische Charakterisierungen von Methicillin-resistenten und -empfindlichen *Staphylococcus pseudintermedius* Isolaten von Katzen und Hunden in Deutschland

Staphylococcus pseudintermedius, ein Vertreter der *Staphylococcus intermedius*-Gruppe (SIG), gehört zu den gewöhnlichen Besiedlern der Haut und Schleimhäute von Hunden und kommt gelegentlich auch bei anderen Haustieren vor. Als typischer opportunistischer Infektionserreger kann *S. pseudintermedius* eine Vielzahl von purulenten sowie Toxin-vermittelten Erkrankungen bei Tieren und gelegentlich auch bei Menschen induzieren bzw. sekundär ein bereits bestehendes Krankheitsbild verschlechtern. Trotz zahlreicher Berichte über das Auftreten von *S. pseudintermedius* sowie der Methicillin-resistenten Variante (MRSP) in der jüngsten Vergangenheit ist über das Vorkommen bestimmter Genotypen und deren Toxine bei Erkrankungen von Hunden, Katzen und Pferden in Deutschland bislang wenig bekannt. MRSP-Infektionen sind für Veterinärmediziner ein therapeutisches Problem, da die betroffenen Tiere aufgrund des meist multi-resistenten Phänotyps dieser Infektionserreger oftmals nur schwer antibiotisch zu versorgen sind und in Einzelfällen sogar eingeschläfert werden müssen.

Im Rahmen dieser Arbeit sind daher 220 Isolate aus ganz Deutschland, einschließlich 113 Methicillin-empfindlicher (MSSP) und 107 -resistenter (MRSP) Isolate zunächst mittels PFGE typisiert worden. Anschließend wurden insgesamt 108 Isolate (53 MSSP und 55 MRSP), welche das gesamte Spektrum der auftretenden PFGE-Cluster repräsentieren, mittels MLST charakterisiert. Zudem sind alle Isolate auf Gene für die bedeutenden Virulenzfaktoren Leukozidin (LukI) sowie Varianten eines exfoliativen Toxins (*Exp*A, *Exp*B) untersucht worden.

Alle MSSP und MRSP waren *luk*I positiv, während für *exp*A nur 18 der MSSP und keiner der MRSP (15,9% / 0%) und für *exp*B 23 der MSSP und ein MRSP positiv waren (20,4% / 0.9%). Insgesamt wurde bei detaillierter Betrachtung der Ergebnisse für MSSP deutlich, dass die Isolate mit Vorbericht „Abstrich von Haut oder Haut-assoziierten Strukturen“nur nahe bzw. unter dem Gesamt-Durchschnitt von ca. 16% für *exp*A und 20% für *exp*B lagen, während der Anteile für Isolate aus Wundabstrichen mit 30,8% (*exp*A) und 46,2% (*exp*B) deutlich über dem Durchschnitt lagen. Der potentielle Einfluss dieser Toxine auf die Entwicklung einer Wundinfektion ist ein interessanter Aspekt, der in Rahmen von weiteren Untersuchungen zur Virulenz von *S. pseudintermedius* aufgegriffen werden sollte.

Mittels Pulsfeld-Gelelektrophorese (PFGE) konnten Bandenmuster von 219 der 220 Isolate dargestellt werden. Die Auswertung der PFGE-Bandenmuster mittels unweighed pair group method using arithmetic averages (UPGMA) ergab insgesamt 20 Cluster sowie

3 Einzelmuster bei Nutzung eines cut-offs bei 80%. Die Bandenmuster der MSSP-Isolate verteilten sich auf 19 der insgesamt 20 Cluster und stellten zwei der drei Einzelmuster. Dennoch sind in mehreren Fällen sehr ähnliche Muster bei MSSP-Isolaten von unterschiedlichen Patienten aus verschiedenen Bundesländern nachgewiesen worden, was die Verbreitung zahlreicher Genotypen über ein großes geographisches Gebiet andeutet. Gründe hierfür könnten z.B. in der allgemeinen Mobilität der Hundehalter (Urlaub, Umzug, Hundetreffen) liegen.

Die Diversität der Bandenmuster von MRSP aus Deutschland ist hoch, eine Beobachtung, die auch in anderen Ländern gemacht wurde. Während für MSSP eine Vielzahl von Sequenztypen detektiert wurde (davon war einer bereits aus einer anderen Studie bekannt und 50 neu) dominierte bei den MRSP der ST71, der offensichtlich in ganz Deutschland verbreitet ist. 48 der mittels MLST untersuchten 55 MRSP aus unterschiedlichen Bundesländern und medizinischen Indikationen sind dieser genetischen Linie zuzuordnen. Die überwiegende Mehrzahl dieser Isolate (39 von 55) ließ sich dem PFGE Cluster 8 zuordnen, aber MRSP_ST71 sind auch in anderen PFGE Clustern aufgetreten. Diese Beobachtung der Inkongruenz zwischen PFGE und MLST Daten für *S. pseudintermedius* und insbesondere für MRSP_ST71 ist in der Literatur bereits beschrieben. Eine *in silico* Restriktionsanalyse auf Basis von Ganzgenomdaten könnte zeigen, welche Änderungen an den Schnittstellen der hier gewählten Endonuklease SmaI möglicherweise zu der weltweit beobachteten Diskrepanz zwischen PFGE und MLST-Analysen führen. Auch das verwendete MLST Schema könnte möglicherweise eine zu geringe Repräsentativität aufweisen, z.B. gibt es für das Gen *tuf* bei über 600 Datenbankeinträgen nur 12 Varianten.

Die in aktuellen Studien beschriebene Diversifikation der MRSP-STs. spiegelt sich jedoch auch in dieser Studie wider. Zwei bislang nicht beschriebene STs für MRSP wurden identifiziert (ST461, ST525). Ferner ist ein in Nordeuropa als epidemisch beschriebener Genotyp (ST258) auch in dieser Studie nachgewiesen worden. Diese aktuelle Entwicklung scheint für MRSP ähnlich zu verlaufen wie für Methicillin-resistente *Staphylococcus aureus* (MRSA) in der Vergangenheit, eine Vielzahl von genetischen Linien ist scheinbar auch in dieser Spezies in der Lage, das Resistenz-tragendende und mobilisierbare genetische Element aufzunehmen. Die weitere Zukunft wird zeigen, ob diese Problematik hauptsächlich auf den Bereich Veterinärmedizin beschränkt bleibt oder ob sich auch die Fälle der schweren Infektionen beim Menschen durch MRSP häufen.

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10 Appendix 1

An overview on all *S. pseudintermedius* isolates associated with novel STs with respect to geographic and host origins

IMT	Original ID	Region	Clade	Body Site	Isolation date	Host	LukI	*mec* A	*exp*A	*exp*B	*ack*	*cpn* 60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	MLST	novel
29940	VB965373	Bavaria	1	RS	11.07.2012	dog	pos	pos	neg	neg	7	2	4	1	1	1	1	ST341	
29899	VB966611	Hessia	1	SSI	17.07.2012	dog	pos	neg	pos	neg	5	9	4	4	8	1	1	ST462	yes
29921	VB965700	Hamburg	1	RS	12.07.2012	cat	pos	neg	neg	neg	5	2	2	1	18	1	2	ST463	yes
30135	VB966813	Brandenburg	1	ear	17.07.2012	cat	pos	neg	neg	neg	1	8	4	4	11	2	1	ST466	yes
29926	VB965918	NRW	1	eye	13.07.2012	dog	pos	neg	neg	neg	5	2	2	1	1	1	1	ST475	yes
27253	VB971693	Bavaria	1	RS	05.03.2010	cat	pos	neg	neg	neg									
27370	VB961874	Bavaria	1	SAS	07.09.2010	dog	pos	neg	neg	neg									
29893	VB966166	Hessia	1	UT	14.07.2012	dog	pos	neg	neg	neg									
29896	VB966261	Bavaria	1	wound	14.07.2012	dog	pos	neg	neg	neg									
29900	VB966656	LS	1	SAS	17.07.2012	dog	pos	neg	neg	neg									
27446	VB973185	Bavaria	1	SAS	12.03.2010	dog	pos	neg	pos	neg									
Number of allelic variants/ MLST loci											**3**	**3**	**2**	**2**	**4**	**2**	**2**		
22124	VB998932	Bavaria	2	UT	06.07.2010	dog	pos	neg	neg	pos	2	20	1	1	3	1	1	ST438	yes
25758	VB967549-2	BW	2	UT	04.10.2010	dog	pos	neg	neg	neg	5	7	2	2	5	1	1	ST443	yes
26519	VB998802	Saarland	2	ear	05.07.2010	dog	pos	neg	pos	pos	5	24	1	1	1	1	1	ST450	yes
25816	VB960566	Hessia	2	SAS	31.08.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25848	VB964247	BW	2	UT	17.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29769	VB951438	NRW	2	eye	15.05.2012	dog	pos	neg	neg	pos									
30139	VB967180-2	BW	2	ear	18.07.2012	dog	pos	neg	neg	neg									
29922	VB965735	Hessia	2	RS	12.07.2012	dog	pos	neg	neg	pos									
27356	VB963089	Hessia	2	UT	11.09.2010	dog	pos	neg	neg	pos									
27357	VB964576-2	LS	2	ear	18.09.2010	cat	pos	neg	neg	neg									
27348	VB963817	Bavaria	2	ear	15.09.2010	dog	pos	neg	neg	pos									
27397	VB977654	NRW	2	eye	31.03.2010	dog	pos	neg	neg	neg									

IMT	Original ID	Region	Clade	Body Site	Isolation date	Host	LukI	*mec* A	*exp*A	*exp*B	*ack*	*cpn* 60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	MLST	novel
30132	VB967022	NRW	2	SSI	18.07.2012	dog	pos	neg	neg	neg									
25954	VB998937	BW	2	ear	06.07.2010	dog	pos	neg	neg	neg									
30136	VB966925	Hessia	2	ear	18.07.2012	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci											**3**	**4**	**2**	**2**	**3**	**2**	**1**		
23967	VB995007-3	RP	3	ear	17.06.2010	dog	pos	neg	neg	pos	1	6	2	4	7	1	1	ST439	yes
25753	VB962867	Bavaria	3	ear	10.09.2010	dog	pos	neg	neg	neg									
29945	VB965235-2	Hessia	3	ear	11.07.2012	dog	pos	pos	neg	neg									
Number of allelic variants/ MLST loci																			
21648	VB973713-2	SH	4	UT	13.03.2010	cat	pos	neg	neg	neg	7	7	2	1	5	1	1	ST436	yes
27389	VB961457	NRW	4	wound	03.09.2010	dog	pos	neg	neg	pos	2	13	2	1	13	1	1	ST474	yes
24470	VB972371	LS	4	eye	09.03.2010	dog	pos	neg	neg	neg	1	2	12	25	5	2	1	ST478	yes
24073	VB998818	Hessia	4	RS	07.07.2010	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci											**3**	**4**	**2**	**3**	**3**	**2**	**1**		
25957	VB966757	NRW	5	SAS	30.09.2010	cat	pos	neg	neg	neg									
26284	VB965620-2	Bavaria	5	UT	24.09.2010	dog	pos	neg	neg	neg									
26524	VM895139	BW	5	SAS	13.09.2010	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci																			
30534	VB996958-2	Bavaria	6	SSI	20.11.2012	dog	pos	neg	neg	neg	5	7	7	1	8	1	1	ST470	yes
29767	VB951053	Hessia	6	ear	15.05.2012	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci																			
25855	VB974070	NRW	7	ear	16.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
27214	VB972266	NRW	7	SSI	09.03.2010	dog	pos	neg	neg	neg									
29755	VB951134	RP	7	RS	15.05.2012	cat	pos	neg	pos	neg									
Number of allelic variants/ MLST loci																			
29925	VB965913	BW	8	wound	13.07.2012	dog	pos	neg	neg	pos	11	21	4	1	19	1	2	ST186	yes
25841	VB997253-2	LS	8	SAS	26.06.2010	dog	pos	neg	neg	neg	1	21	1	1	23	1	1	ST444	yes
26490	VB971189	NRW	8	ear	03.03.2010	dog	pos	neg	neg	neg	5	6	2	1	5	1	1	ST446	yes

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26509	VB999815	LS	8	SAS	09.07.2010	dog	pos	neg	neg	neg	4	2	1	4	13	12	1	ST447	yes
26510	VB971143	Bavaria	8	UT	03.03.2010	cat	pos	neg	neg	neg	2	6	2	1	8	2	1	ST448	yes
26518	VB962757	Hessia	8	SAS	10.09.2010	dog	pos	neg	neg	pos	3	7	2	2	3	1	2	ST449	yes
27221	VB961383-3	NRW	8	wound	03.09.2010	dog	pos	neg	neg	neg	4	24	2	1	2	2	2	ST454	yes
30128	VB966549	BW	8	eye	17.07.2012	dog	pos	neg	neg	pos	1	7	4	1	42	1	1	ST464	yes
30129	VB966950	Hessia	8	SAS	18.07.2012	dog	pos	neg	neg	pos	5	13	4	4	1	2	1	ST465	yes
21647	VB973066-2	MV	8	UT	11.03.2010	cat	pos	pos	neg	neg	1	24	2	1	1	2	1	ST525	yes
30544	VB997719	NRW	8	SAS	23.11.2012	dog	pos	neg	neg	neg	1	2	3	1	11	1	2	ST527	yes
25843	VB972404	NRW	8	SSI	09.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
21470	VB971531	Thuringia	8	wound	04.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
30123	VB967225	Thuringia	8	SAS	18.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29939	VB965253	BW	8	wound	11.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29947	VB965977	Bavaria	8	SSI	13.07.2012	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25858	VB972533	MV	8	ear	09.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29950	VB965976	Bavaria	8	SSI	13.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25836	VB973150-3	MV	8	ear	11.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25825	VB963000	Hessia	8	SSI	11.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25852	VB974271	Bavaria	8	wound	17.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29943	VB965230-4	BW	8	ear	11.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25809	VB999161	SH	8	wound	06.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25808	VB998965	Bavaria	8	ear	06.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
22123	VB999720	Bavaria	8	SSI	08.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25798	VB975586	RP	8	UT	23.03.2010	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25813	VB972077	Brandenburg	8	SAS	06.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
21473	VB973843	NRW	8	SSI	15.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25750	VB968540	LS	8	RS	08.10.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25817	VB962118-3	Hamburg	8	wound	07.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25827	VB964513-2	Saxony	8	RS	18.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	

IMT	Original ID	Region	Clade	Body Site	Isolation date	Host	LukI	*mec* A	*exp*A	*exp*B	*ack*	*cpn* 60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	MLST	novel
25837	VB994901	BW	8	ear	16.06.2010	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25818	VB963091	NRW	8	RS	11.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25842	VB999111-2	NRW	8	UT	06.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25826	VB998900	NRW	8	UT	06.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
22121	VB999400	RP	8	SSI	07.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
21474	VB977000	Hessia	8	SSI	27.03.2010	dog	pos	pos	neg	pos	3	9	1	2	1	2	1	ST71	
22119	VB995121	NRW	8	SSI	17.06.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25847	VB964677	Bavaria	8	UT	20.09.2010	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25806	VB963335	Hessia	8	wound	14.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
24872	VB999756-2	BW	8	UT	08.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25822	VB961306	Bavaria	8	SSI	03.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
30678	VB961047	Thuringia	8	SAS	28.12.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25811	VB999036	RP	8	eye	06.07.2010	horse	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
24469	VB972185	NRW	8	RS	08.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
30121	VB966928	BW	8	ear	18.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25851	VB962295	NRW	8	UT	08.09.2010	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
24473	VB975813	SA	8	RS	23.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
30120	VB966803-2	Bavaria	8	wound	17.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
24471	VB975610	BW	8	SSI	23.03.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25838	VB976308	BW	8	wound	25.03.2010	dog	pos	pos	neg	neg									
25849	VB963731	Hessia	8	eye	15.09.2010	dog	pos	pos	neg	neg									
25807	VB962346	Thuringia	8	UT	08.09.2010	dog	pos	pos	neg	neg									
29752	VB952332	Hessia	8	UT	19.05.2012	dog	pos	pos	neg	neg									
21650	VB973379	Bavaria	8	ear	12.03.2010	dog	pos	neg	neg	neg									
25712	VB977639-2	Hessia	8	UT	22.11.2010	cat	pos	pos	neg	neg									
25805	VB963410	Hessia	8	UT	14.09.2010	dog	pos	pos	neg	neg									
29742	VB950132	Bavaria	8	SSI	10.05.2012	dog	pos	pos	neg	neg									
25840	VB997214	Hessia	8	UT	26.06.2010	dog	pos	pos	neg	neg									

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25799	VB973997	BW	8	ear	16.03.2010	dog	pos	neg	neg	pos									
25803	VB963898	RP	8	SSI	16.09.2010	dog	pos	pos	neg	neg									
29747	VB951080	BW	8	SSI	15.05.2012	dog	pos	pos	neg	neg									
29743	VB950620	BW	8	eye	12.05.2012	dog	pos	pos	neg	neg									
25823	VB962986	Hessia	8	ear	11.09.2010	dog	pos	pos	neg	neg									
27344	VB963716	NRW	8	eye	15.09.2010	dog	pos	pos	neg	neg									
21649	VB973742	NRW	8	SSI	13.03.2010	dog	pos	pos	neg	neg									
25845	VB997177	RP	8	SSI	26.06.2010	dog	pos	pos	neg	neg									
25810	VB972256	LS	8	wound	09.03.2010	dog	pos	pos	neg	neg									
29741	VB950062	NRW	8	RS	10.05.2012	dog	pos	pos	neg	neg									
25839	VB950129	NRW	8	ear	10.07.2010	dog	pos	pos	neg	neg									
29744	VB950877	Bavaria	8	ear	12.05.2012	dog	pos	pos	neg	neg									
21651	VB975527	NRW	8	SSI	23.03.2010	dog	pos	pos	neg	neg									
30681	VM895430	Bavaria	8	SSI	27.12.2012	dog	pos	pos	neg	neg									
25833	VB962879	SH	8	RS	10.09.2010	dog	pos	pos	neg	neg									
21471	VB972590	BW	8	SSI	09.03.2010	dog	pos	pos	neg	neg									
23966	VB995574-2	NRW	8	ear	19.06.2010	dog	pos	pos	neg	neg									
25814	VB999535	Hessia	8	ear	08.07.2010	dog	pos	pos	neg	neg									
26527	VB996403	Bavaria	8	ear	23.06.2010	dog	pos	pos	neg	neg									
29745	VB950635	BW	8	RS	12.05.2012	cat	pos	pos	neg	neg									
25804	VB963710	Hessia	8	SSI	15.09.2010	dog	pos	pos	neg	neg									
25821	VB961384	NRW	8	SAS	03.09.2010	dog	pos	pos	neg	neg									
25850	VB963642-2	Hessia	8	ear	15.09.2010	dog	pos	pos	neg	neg									
30682	VM895499 2-2	Bavaria	8	RS	20.12.2012	cat	pos	pos	neg	neg									
24070	VB997600	BW	8	SSI	29.06,2010	dog	pos	pos	neg	neg									
29746	VB950878	Bavaria	8	ear	12.05.2012	cat	pos	pos	neg	neg									
30517	VB996853-3	NRW	8	SSI	20.11.2012	dog	pos	pos	neg	neg									
29946	VB965881	BW	8	SSI	13.07.2012	dog	pos	pos	neg	neg									

IMT	**Original ID**	**Region**	**Clade**	**Body Site**	**Isolation date**	**Host**	**LukI**	***mec* A**	***exp*A**	***exp*B**	***ack***	***cpn* 60**	***fdh***	***pta***	***pur*A**	***sar***	***tuf***	**MLST**	**novel**
30520	VB997799	Saxony	8	UT	23.11.2012	dog	pos	pos	neg	neg									
25846	VB997344-2	Bavaria	8	ear	26.06.2010	dog	pos	pos	neg	neg									
24874	VB961597	BW	8	SAS	04.09.2010	dog	pos	pos	neg	neg									
25844	VB974411	Hamburg	8	SSI	17.03.2010	dog	pos	pos	neg	neg									
30523	VB998127	LS	8	SAS	24.11.2012	dog	pos	pos	neg	neg									
25824	VB962902-2	Hessia	8	SSI	10.09.2010	dog	pos	pos	neg	neg									
30518	VB997570	SH	8	SSI	22.11.2012	cat	pos	pos	neg	neg									
30676	VB961025	NRW	8	SSI	28.12.2012	dog	pos	pos	neg	neg									
25834	VB963732	Hessia	8	wound	15.09.2010	dog	pos	pos	neg	neg									
24072	VB999033	Bavaria	8	SSI	06.07.2010	dog	pos	pos	neg	neg									
24878	VB972407	Hessia	8	UT	09.03.2010	dog	pos	pos	neg	neg									
25820	VB950082	NRW	8	SSI	10.07.2010	dog	pos	pos	neg	neg									
25828	VB962907	Hessia	8	SSI	10.09.2010	dog	pos	pos	neg	neg									
25801	VB977833	Bavaria	8	SSI	31.01.2010	dog	pos	pos	neg	neg									
Number of allelic variants/ MLST loci											**6**	**7**	**4**	**3**	**10**	**3**	**2**		
27263	VB964175	NRW	9	ear	17.09.2010	dog	pos	neg	neg	pos	2	43	3	1	11	1	2	ST472	yes
29935	VB964832	BW	9	UT	10.07.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
30677	VB961039	SA	9	SAS	28.12.2012	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
27349	VB997559	NRW	9	wound	29.06.2010	dog	pos	neg	pos	neg									
27423	VB971248	Saxony	9	ear	03.03.2010	dog	pos	neg	pos	neg									
Number of allelic variants/ MLST loci											**2**	**2**	**2**	**2**	**2**	**2**	**2**		
27205	VB998664	LS	10	UT	03.07.2010	dog	pos	neg	neg	neg	1	7	2	1	3	1	2	ST453	yes
27394	VB998935	BW	10	ear	06.07.2010	dog	pos	neg	neg	neg									
27393	VB977839	BW	10	wound	31.08.2010	dog	pos	neg	pos	pos									
Number of allelic variants/ MLST loci																			
25754	VB977080-2	Bavaria	11	RS	18.11.2010	cat	pos	neg	neg	neg									
30692	VB960811-3	NRW	11	SAS	28.12.2012	dog	pos	neg	pos	neg									
Number of allelic variants/ MLST loci																			

IMT	Original ID	Region	Clade	Body Site	Isolation date	Host	LukI	*mec* A	*exp*A	*exp*B	*ack*	*cpn* 60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	MLST	novel
21480	VB972390	SH	12	SAS	09.03.2010	dog	pos	neg	neg	neg	1	10	1	4	11	1	1	ST187	yes
27382	VB998948	Hessia	12	UT	06.07.2010	dog	pos	neg	neg	neg	2	25	2	32	7	2	1	ST189	yes
21478	VB971638	Bavaria	12	ear	05.03.2010	dog	pos	neg	neg	neg	1	57	2	32	2	2	2	ST435	yes
22120	VB996401	Thuringia	12	ear	23.06.2010	dog	pos	neg	neg	neg	5	13	3	1	13	1	2	ST437	yes
27415	VB971682	SH	12	UT	05.03.2010	dog	pos	neg	neg	neg	2	20	1	1	3	1	1	ST438	yes
24467	VB961600	Bavaria	12	SAS	04.09.2010	dog	pos	neg	neg	neg	2	2	4	1	13	1	1	ST440	yes
26522	VB963480	Hessia	12	SSI	14.09.2010	dog	pos	neg	neg	pos	3	7	2	1	3	2	2	ST452	yes
27355	VB963048	LS	12	ear	11.09.2010	dog	pos	neg	neg	neg	1	7	1	1	14	1	1	ST456	yes
27381	VB999505-2	BW	12	ear	07.07.2010	dog	pos	neg	pos	neg	11	18	2	4	7	1	2	ST457	yes
27388	VB961566	NRW	12	ear	04.09.2010	dog	pos	neg	neg	neg	5	2	2	1	11	1	2	ST458	yes
30525	VB997751	Bavaria	12	wound	23.11.2012	dog	pos	neg	neg	neg	1	2	1	1	11	1	2	ST469	yes
27391	VB960497	Bavaria	12	UT	31.08.2010	dog	pos	neg	neg	neg	5	13	1	1	5	2	1	ST528	yes
25802	VB996387	NRW	12	wound	23.06.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
27265	VB997326	LS	12	ear	26.06.2010	dog	pos	neg	pos	neg									
21652	VB972966	LS	12	ear	11.03.2010	dog	pos	neg	pos	pos									
27430	VB961593	Bavaria	12	SAS	04.09.2010	dog	pos	neg	neg	neg									
21477	VB971163	NRW	12	SAS	09.03.2010	dog	pos	neg	neg	neg									
21472	VB972288	NRW	12	UT	09.03.2010	dog	pos	neg	neg	neg									
27431	VB961230	Hamburg	12	ear	02.09.2010	dog	pos	neg	neg	neg									
27418	VB973740	RP	12	SAS	13.03.2010	dog	pos	neg	neg	neg									
26526	VB963726	Hessia	12	RS	15.09.2010	dog	pos	neg	neg	neg									
29760	VB950770-2	SH	12	ear	12.05.2012	dog	pos	neg	neg	neg									
26506	VB960095-2	Hessia	12	ear	27.08.2010	dog	pos	neg	neg	neg									
22122	VB998885	Berlin	12	ear	06.07.2010	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci											**5**	**9**	**4**	**4**	**8**	**2**	**2**		
30701	VB960947	LS	13	wound	28.12.2012	cat	pos	pos	neg	neg	5	13	4	1	11	2	2	ST258	
30691	VB960719	Bavaria	13	ear	27.12.2012	dog	pos	neg	neg	neg	1	2	2	1	11	3	1	ST471	yes
27378	VB975480	NRW	13	SAS	22.03.2010	dog	pos	neg	pos	neg									

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30703	VB960960	Saxony	13	SAS	28.12.2012	dog	pos	neg	pos	neg									
29764	VB950477	LS	13	eye	11.05.2012	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loc											**2**	**2**	**2**	**1**	**1**	**2**	**2**		
30149	VB967499	Bavaria	14	wound	19.07.2012	dog	pos	neg	pos	pos	1	7	4	1	1	1	1	ST370	
27419	VB973202	Hessia	14	wound	12.03.2010	dog	pos	neg	pos	neg	2	2	2	1	6	1	1	ST459	yes
30524	VB997742	NRW	14	ear	23.11.2012	dog	pos	neg	neg	neg	1	18	1	22	2	1	2	ST468	yes
27448	VB962805	NRW	14	SAS	10.09.2010	dog	pos	neg	neg	neg	5	14	1	1	11	1	1	ST526	yes
24873	VB999707	Bavaria	14	ear	08.07.2010	dog	pos	neg	neg	neg									
27358	VB964231	LS	14	eye	17.09.2010	cat	pos	neg	neg	neg									
29901	VB966710	BW	14	eye	17.07.2012	dog	pos	neg	neg	neg									
30704	VB960961	Saxony	14	SAS	28.12.2012	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci											**3**	**4**	**3**	**2**	**4**	**1**	**2**		
21475	VB978053	NRW	15	ear	01.04.2010	dog	pos	pos	neg	neg	1	7	2	2	13	1	2	ST335	
30521	VB997059	NRW	15	ear	21.11.2012	dog	pos	pos	neg	neg	1	7	2	2	13	1	2	ST335	
30146	VB967393	Hessia	15	SAS	19.07.2012	dog	pos	neg	neg	neg	1	2	4	1	14	2	1	ST467	yes
Number of allelic variants/ MLST loci											**1**	**2**	**2**	**2**	**2**	**2**	**2**		
27226	VB973192-3	Bavaria	16	ear	12.03.2010	dog	pos	pos	neg	neg	5	13	4	1	11	2	2	ST258	
21476	VB971245	LS	16	wound	03.03.2010	dog	pos	neg	neg	neg	5	21	4	2	11	1	2	ST441	yes
26520	VB963460	NRW	16	ear	14.09.2010	dog	pos	neg	pos	neg	1	9	1	4	8	2	1	ST451	yes
29932	VB965933	BW	16	RS	13.07.2012	dog	pos	neg	neg	neg	1	10	4	8	7	1	2	ST476	yes
24472	VB975461	Saxony	16	RS	22.03.2010	dog	pos	neg	neg	neg	1	58	7	1	19	2	1	ST530	yes
27441	VB962983	RP	16	ear	11.09.2010	dog	pos	neg	neg	neg									
24468	VB961312	Bavaria	16	ear	03.09.2010	dog	pos	neg	neg	pos									
27437	VB999397	BW	16	wound	07.07.2010	dog	pos	neg	neg	pos									
30542	VB997709	LS	16	SAS	23.11.2012	dog	pos	neg	neg	neg									
24071	VB997462	Bavaria	16	ear	29.06.2010	dog	pos	neg	neg	neg									
30118	VB967267	BW	16	UT	19.07.2012	dog	pos	pos	neg	neg									
29933	VB965952-2	Bavaria	16	ear	13.07.2012	dog	pos	neg	neg	neg									

IMT	Original ID	Region	Clade	Body Site	Isolation date	Host	LukI	*mec* A	*exp*A	*exp*B	*ack*	*cpn* 60	*fdh*	*pta*	*pur*A	*sar*	*tuf*	MLST	novel
Number of allelic variants/ MLST loci											**2**	**5**	**3**	**4**	**4**	**2**	**2**		
21479	VB972190	Saxony	17	wound	08.03.2010	dog	pos	neg	neg	pos	11	21	4	1	19	1	2	ST186	yes
27207	VB971890	Bavaria	17	ear	06.03.2010	dog	pos	neg	pos	neg	7	7	2	8	1	1	1	ST188	yes
27350	VB997467	BW	17	wound	29.06.2010	dog	pos	neg	neg	neg	1	2	2	1	20	19	2	ST455	yes
27449	VB963894	Hessia	17	eye	16.09.2010	dog	pos	neg	neg	neg	4	63	2	1	1	2	2	ST460	yes
25853	VB974573	Bavaria	17	ear	18.03.2010	dog	pos	neg	pos	neg									
27361	VB962363	LS	17	SAS	08.09.2010	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci											**4**	**4**	**2**	**2**	**3**	**3**	**2**		
29748	VB951114	LS	18	SSI	15.05.2012	dog	pos	pos	neg	neg	7	8	4	1	1	1	1	ST461	yes
29770	VB951480	Hessia	18	ear	16.05.2012	dog	pos	neg	neg	neg									
Number of allelic variants/ MLST loci																			
25812	VB998708	Hessia	19	SSI	03.07.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
25856	VB963468	NRW	19	ear	14.09.2010	dog	pos	pos	neg	neg									
Number of allelic variants/ MLST loci																			
24474	VB975342	Saxony	20	SAS	20.03.2010	dog	pos	neg	neg	neg	5	25	4	1	7	1	1	ST442	yes
25952	VB999370	Bavaria	20	RS	07.07.2010	horse	pos	neg	neg	neg	5	2	2	1	19	1	1	ST445	yes
Number of allelic variants/ MLST loci											**1**	**2**	**2**	**1**	**2**	**1**	**1**		
27362	VB962494	LS	Single	ear	09.09.2010	dog	pos	neg	neg	pos	5	8	4	4	11	5	2	ST473	yes
25800	VB976146	Thuringia	Single	UT	24.03.2010	cat	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	
29754	VB952387	Bavaria	Single	SAS	19.05.2012	dog	pos	neg	neg	pos									
25819	VB962374	Saxony	Un-typable.	UT	08.09.2010	dog	pos	pos	neg	neg	3	9	1	2	1	2	1	ST71	

Abbreviations: LS: Lower Saxony, SH: Schleswig-Holstein, MP: Mecklenburg Western Pomerania, SA: Saxony-Anhalt, RP: Rhineland-Palatinate, BW: Baden-Württemberg, NRW: North-Rhine-Westphalia, SAS: skin and accessory structures; SSI: surgical site infections; UT: uro-genital tract; RS: respiratory system, LukI: *luk*S and *luk*F encoding leukotoxin I; *exp*A: gene encoding exfoliative toxin A; *exp*B: gene encoding exfoliative toxin B *mec*A: gene encoding methicillin resistance; neg: negative, pos: positive; Un-typable: strain was not digested by *Sma*I enzyme.

11 List of Publications

Journal articles

Species differentiation within the *Staphylococcus intermedius* group (SIG) using a refined MALDI-TOF MS database

Murugaiyan, J.; Walther, B.; Stamm, I.; Abou-Elnaga, Y.; Brüggemann-Schwarze, S.; Wieler, L.H.; Lübke-Becker, A.; Semmler, T.; Roesler, U.: Clin Microbiol Infect. 2014 May 8. doi: 10.1111/1469-0691.12662

Sharing more than friendship - nasal colonization with coagulase-positive staphylococci (CPS) and co-habitation aspects of dogs and their owners

Walther, B.; Hermes, J.; Cuny, C.; Wieler, L.H.; Vincze, S.; Abou Elnaga, Y.; Stamm, I.; Kopp, P.A.; Kohn, B.; Witte, W.; Jansen, A.; Conraths, F.J.; Semmler, T.; Eckmanns, T.; Lübke-Becker, A.: PLoS One. 2012; 7(4): e35197. doi: 10.1371/journal.pone.0035197

Poster presentations

Genetic relationship of methicillin- resistant (MRSP) and methicillin-susceptible (MSSP) *Staphylococcus pseudintermedius* of small animal origin

Abou-Elnaga, Y.; Lübke-Becker, A.; Brandenburg, A.; Kopp, P.A.; Stamm, I.; Wieler, L.H.; Walther, B.: 6. Doktorandensymposium und DRS Präsentationsseminar "Biomedical Sciences" am Fachbereich Veterinärmedizin der Freien Universität Berlin. Berlin – 01.07.2011

In: Von Doktoranden für Doktoranden: 6. Doktoranden-Symposium & DRS Präsentationsseminar "Biomedical Sciences" am Fachbereich Veterinärmedizin der FU Berlin; Programm und Abstracts. Berlin: Mensch-und-Buch-Verl. ISBN: 978-3-86664-980-4

Unravelling the clonal relationship of methicillin resistant and susceptible *S. pseudintermedius* strains of companion animal origin.

Abou Elnaga, Y.; Lübke-Becker, A.; Brandenburg, A.G.; Kopp, P.A.; Stamm, I.; Wieler, L.H.; Walther, B.: DVG-Fachgruppe "Bakteriologie und Mykologie "Leipzig. 27. -29.06.2012

In: Tagung der DVG-Fachgruppe "Bakteriologie und Mykologie "(1. Aufl.) Gießen: DVG-Service, S. 83–84 ISBN: 978-3-86345-080

Distribution of genes encoding exfoliative toxins and leukotoxin among methicillin-resistant and susceptible *Staphylococcus pseudintermedius* strains from dogs
Abou-Elnaga, Y.; Lübke-Becker, A.; Vincze, S.; Brandenburg, A.G.; Kopp, P.A.; Stamm, Y.; Wieler, L.H.; Walther, B.: 16. International Symposium of the World Association of Veterinary Laboratory Diagnosticians (WAVLD) Berlin, Germany. 05. - 08.06.2013

Insights into genetic variability of methicillin resistant *Staphylococcus aureus* (MRSA) from companion animals in Germany
Vincze, S.; Lübke-Becker, A.; Stamm, I.; Kopp, P.A.; Abou Elnaga, Y.; Semmler, T.; Wieler, L.H.; Walther, B.: National Symposium on Zoonoses Research Berlin. 06.-07.10.2011

The genetic structure of multidrug-resistant *Staphylococcus pseudintermedius* strains in Germany.
Walther, B.; Lübke-Becker, A.; Stamm, I.; Abou-Elnaga, Y.; Vincze, S.; Brandenburg, A.G.; Wieler, L.H.; Semmler: German Symposium on Zoonoses Research 16.10.2014

Optimization of Matrix-assisted laser desoption ionizationtime of flight mass spectrometry database for rapid identification of staphylocolocci composing the *Staphylococus intermedius*-group.
Murugaiyan, J.; Walther, B.; Stamm, I.; Abou-Elnaga, Y.; Vincze, S.; Wieler, L.H.; Lübke-Becker, A.; Semmler, T.; Rösler, U.: VAAM-Jahrestagung 2014, 66. Jahrestagung der DGHM Dresden. 05.-08.10.2014. In: Tagungsband zur 4. GemeinsamenKonferenz von DGHM und VAAM; VAAM-Jahrestagung 2014, 66. Jahrestagung der DGHM. Springer. BIOspektrum Tagungsband, S. 212

Matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS)-based rapid species identification within the *Staphylococcus intermedius*-group (SIG).
Murugaiyan, J.; Walther, A.; Stamm, I.; Abou-Elnaga, Y.; Brüggemann, S.; Vincze, S.; Wieler, L.H.; Lübke-Becker, A.; Semmler, T.; Rösler, U.: German Symposium on Zoonoses Research 16. 10. 2014

Presentations:

Evaluation of *S. pseudintermedius* differentiation using molecular marker analysis

Murugaiyan, J.; Rösler, U.; Semmler, T.; Vincze, S.; Abou-Elnaga, Y.; Stamm, I.; Kopp, P.; Wieler, L.; Lübke-Becker, A.; Walther, B.: 63rd Annual Meeting of the German Society for Hygiene and Microbiology Essen 25.-28.09.2011. In: International journal of medical microbiology, S. 9

The frequency of Livestock-associated MRSA of CC398 in clinical samples from small animals and horses is on the rise

Vincze, S.; Lübke-Becker, A.; Stamm, I.; Kopp, P.A.; Abou-Elnaga, Y.; Semmler, T.; Wieler, L.H.; Walther, B.: 63. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie (DGHM) Essen 25. -28. 09. 2011, ISSN: 1438-4221

12 Acknowledgements

I would like to thanks all people, colleagues and my family who helped and supported me to finish this work.

First of all, I would like to thank my chef supervisor Prof. Dr. Wieler for his patience, motivation and guidance for helping me during my doctoral study. I could not have imagined having a better advisor and mentor for my dissertation. I really appreciate all your efforts in helping me.

My sincere thanks goes to Dr. Birgit Walther for the continuous support of my thesis and immense knowledge. Without her assistance and dedicated involvement in every step throughout the process, this doctoral thesis would have never been accomplished. I would like to thank her very much for her understanding over these past five years.

I would like to express my special appreciation and thanks to Dr. Antina Lübke-Becker for supervision and provided me an opportunity to join her team and gave access to the laboratory and research facilities. Without her precious support it would not be possible to conduct this doctoral study.

I would especially like to thank our project partner IDEXX Vet Med Labor Ludwigsburg particularly, Mrs. Antje Brandenburg and Ivonne Stamm for your highly appreciated cooperation in this thesis.

A huge thanks to everyone in the Institute of Microbiology and Epizootics who helped, welcomed and taught me immensely at the lab all these years and for incited me to strive towards my goal.

I need to further thank all my friends who have supported me over the last years particularly, Szilvia Vincze for your kindness and encouragement in my difficult times.

A special thanks to my family. Words cannot express how grateful I am to my mother, and father for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far and to you this thesis is dedicated.

An extra warm thank you to my lovely children Mariam, Jana and Youssef for all their love and support, for putting up with my moods and grumpiness at times.

At the end I would like express my heartfelt thanks to my husband Wael who spent sleepless nights with, and was always my support in the hard moments. All my love to him, for finding me the light whenever it was far way.

13 Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Berlin, den, 31.03.2017

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39,90 Euro | ISBN: 978-3-86387-868-9