UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

DOTTORATO IN SCIENZE VETERINARIE
XXXI CICLO

PhD THESIS

"*Staphylococcus pseudintermedius*-associated canine skin disorders: isolation, identification, antibiotic resistance patterns, sequence typing and alternative therapeutic strategies."

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Non basta guardare.
Occorre guardare con occhi che vogliono vedere,
che credono in quello che vedono.

Galileo Galilei

Index

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Dedicato ai miei nonni,
ai miei genitori,
alla mia famiglia,
ai miei più cari amici.

Dedicato a chi mi ha accolta nella propria vita,
a chi ha reso le mie insicurezze ali di farfalla.

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List of Abbreviations

agr	accessory gene regulator
AIP	auto-inducing peptide
AMC	amoxicillin-clavulanate
AMP	ampicillin
ATCC®	American Type Culture Collection
CD	clindamycin
CDSs	predicted protein-coding sequences
CIP	ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	gentamicin
CoNS	coagulase-negative Staphylococci
CoPS	coagulase-positive Staphylococci
CRO	ceftriaxone
DIBI	Hydroxypyridinone-based iron-chelating co-polymer
E	erythromycin
ENR	enrofloxacin
FIC	fractional inhibitory concentration
FOX	cefoxitin
IMI	imipenem
K	kanamycin
KF	cephalothin
LNZ	linezolid
Luk I	leucotoxin
MALDI-TOF-	

MS matrix assisted laser desorption ionization time of flight mass spectometry

MBC minimal bactericidal concentration

MDR multidrug resistance

MIC minimal inhibitory concentration

MLST multilocus sequence typing

MRSA methicillin-resistant *Staphylococcus aureus*

MRSP methicillin-resistant *Staphylococcus pseudintermedius*

MSA Mannitol Salt agar

MSSP methicillin-susceptible *Staphylococcus pseudintermedius*

NCCLS National Committee for Clinical Laboratory Standards

OX oxacillin

P penicillin

PBP2a modified penicillin-binding protein

PCR polymerase chain reaction

PVA immunoglobulin-binding protein

RFLP PCR-restriction fragment length polymorphism

RPMI Roswell Park Memorial Institute Medium 1640

S streptomycin

SaPI staphylococcal pathogenicity island family

sar staphylococcal accessory regulator

SCC*mec* staphylococcal cassette chromosome *mec*

SIG *Staphylococcus intermedius Group*

STs Sequence types

List of Abbreviations

SXT	sulfamethoxazole-trimethoprim
TE	tetracycline
TOB	tobramycin
VA	vancomycin
WHO	World Health Organization

List of Figures

List of Tables

Abstract

Sthaphylococcus pseudintermedius (*S. pseudintermedius*) is the main inhabitant of skin and mucosa of dogs, where it represents the major bacterial pathogen causing skin diseases.

Firstly described in 2005, *S. pseudintermedius* is a member of the *Staphylococcus intermedius Group* (SIG), together with *S. intermedius* and *S. delphini*.

The recent emergence and rapid dissemination of multidrug-resistant *S. pseudintermedius* strains, particulary methicillin-resistant *S. pseudintermedius* (MRSP), showing more and more often resistance to the antibiotic agents licensed for use in small animal practice, represent a relevant threat to small animal and public health. In the last decade, the antibiotic resistance issue has become increasingly relevant, highlighting the need to control and limit it by the means of a continuous surveillance of both methicillin-resistant and methicillin-susceptible *S. pseudintermedius* isolates as well as the development of new and alternative therapeutic options.

This study aims to describe the phenotypic and genotypic features, the antibiotic resistance patterns, the prevalence of virulence genes of both MRSP and methicillin-susceptible *S. pseudintermedius* (MSSP) strains, isolated from dogs suffering from otitis externa and pyoderma. MRSP sequence type clones circulating in Southern Italy were also investigated. Moreover, the antimicrobial activity of hydroxypiridone-based iron chelating co-polymer DIBI and abietic acid was analyzed against *S. pseudintermedius* isolates, as alternative treatment options.

From a total of 259 staphylococcal cultures, 126 *S. pseudintermedius* strains were selected during the years 2015-2017.

S. pseudintermedius strains (49%) were identified by proteomic (MALDI-TOF MS) and molecular profiling (*nuc* and *hlb* genes). *S. pseudintermedius* strains were recovered from dogs suffering from otitis externa (84%) and pyoderma (16%), originating from Naples, Campania Region (52%) and Latina, Lazio Region (48%).

Amongst the 126 *S. pseudintermedius* strains, 18% were MRSP, carrying *mec*A gene. The remaining 82% were classified as MSSP. None of the isolates was positive to *mec*C gene.

Furthermore, all the MRSP showed a complete resistance to amoxicillin-clavulanate and ampicillin (100%), while the highest resistance rates to selected non-β-lactam antibiotics were registered for erythromycin (91%); tetracycline (87%); sulfamethoxazole/trimethoprim (78%); kanamycin (78%); streptomycin (78%); clindamycin (65%) and enrofloxacin (61%). However, there was no resistance to vancomycin and linezolid. It is worth noting that 91% MRSP strains were found to be multidrug-resistant strains. The 82% MSSP isolates, showed interesting antibiotic resistant profiles, but their resistance rates were of about 50% lower than MRSP strains.

The phenotypic tetracycline- and erythromycin-resistant MRSP and MSSP strains harbored *tet*K and *tet*M genes, alone or in association, and *erm*B gene.

Referring to MRSP isolates, they mainly belonged to multidrug-resistant sequence type ST71 (26%) European clone. In this study, 9 new clones of MRSP were identified and described in Italy and worldwide for the first time. The newly described sequence types (STs) were named from ST1053 up to ST1061.

In addition, DIBI and abietic acid resulted to be effective against MRSP and MSSP strains, inhibiting their growth. Thus, they may be considered as valid non-antibiotic alternative treatment approaches for *S. pseudintermedius* infections.

The bacterial genus of *Staphylococcus* consists of many species that are part of the natural microbiota of humans and animals.

Staphylococcus intermedius, Staphylococcus pseudintermedius, and *Staphylococcus delphini* together compose the *Staphylococcus intermedius Group* (SIG). In particular, *Staphylococcus pseudintermedius* is the most common coagulase-positive staphylococcus isolated from dogs, in which this opportunistic Gram-positive bacterium can be responsible for a great variety of infections including pyoderma, otitis, abscesses, urinary tract infections (UTIs) and wound infections (Weese and van Duijkeren, 2010; Rubin *et al*., 2011; Youn *et al*., 2014).

Over the last decade, *S. pseudintermedius* has become a critically opportunistic small animal pathogen, being frequently the primary aetiological agent of pyoderma and otitis (De Martino *et al*., 2016; Loeffler and Lloyd, 2018). Even though dog is its natural host, human carriage and infections have been reported, highlighting the potential zoonotic transmission of this species (Somayaji *et al.,* 2016).

Since its first description as a novel coagulase-positive staphylococcal species in 2005, the phenotypic identification of *S. pseudintermedius* has persisted problematic. Commercial kits, commonly used in routine laboratory diagnostics, are not able to identify and distinguish *S. pseudintermedius* from the other members of the SIG and from *S. aureus*, because of their similar phenotypic characters. Moreover, as coagulase-negative Staphylococci (CoNS) also *S. pseudintermedius* generally gives negative results to rapid slide clumping and commercial latex agglutination tests. For these reasons, the real prevalence of *S. pseudintermedius* might have been underestimated in clinical

microbiology laboratories (Weese and van Duijkeren, 2010). So, molecular profiling continues to represent a necessary tool for a proper determination and diagnosis of *S. pseudintermedius*. However, Decristophoris *et al.* (2011), reported that MALDI-TOF MS, provided with a reliable database, is an effective technique for a rapid identification of S. *pseudintermedius* and the other two bacterial species of the SIG.

Antibiotic resistance is the most puzzling question of public health of the earlier decade of this 21st century and already since 2006 methicillin-resistant *S. pseudintermedius* (MRSP) strains have been identified, which have been also proved to be multidrug-resistant displaying resistance to most of the antibiotics approved for use in pet animals (Kadlec and Schwarz, 2012; Moodley *et al*., 2014).

This high prevalence of multidrug-resistant strains seems to be related to the dissemination of dominant clones, such as MRSP belonging to the clonal lineage ST71. ST71 is a widespread clone, being isolated across the European, American and Asiatic continents. As reported by Perreten *at al*. (2010) in their multicenter study, a high percentage of MRSP ST71 were resistant to at least six antimicrobial classes. Furthermore, new multidrug MRSP clones are constantly reported worldwide.

Therefore, the increasing spread of multidrug-resistant strains represents an important threat for animal and public health, since dogs, and in general pet animals, represent potential sources of spread of antimicrobial resistance due to the extensive use of antimicrobial agents; this spread may concern also humans, because of their close contact with pets. Thus, it is not only of veterinary significance, but has a zoonotic importance, with pets acting as reservoirs for humans, particularly pet owners and

veterinarians. Moreover, the lack of effective antimicrobial therapeutic treatments underlines the need of new alternative approaches to prevent and limit the dissemination of multidrug resistant bacterial isolates.

The purpose of this PhD thesis was to investigate the phenotypic and genotypic antibiotic resistance patterns of clinical methicillin-resistant and methicillin-susceptible *Staphylococcus pseudintermedius* strains associated with the most common clinical cases of otitis externa and pyoderma in dogs, attending the Veterinary Teaching Hospital (OVUD) of the Department of Veterinary Medicine and Animal Production, University of Naples "Federico II" and the Provet Lab srl of Latina. The dominant MRSP clones circulating in Southern Italy were also investigated. Furthermore, the antimicrobial activity against *S. pseudintermedius* of the two non-antibiotic agents DIBI (Chelation Parteners Inc. proprietary) and abietic acid was analyzed as alternative therapeutic options.

The topics of the thesis are organized in four chapter as follows.
Chapter 1 is focused on the:
- isolation and collection of *S. pseudintermedius* strains obtained from swabs of dogs suffering from otitis externa and pyoderma;
- comparison of Api Staph conventional biochemical test, matrix- assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) system and polymerase chain reaction (PCR) for the *S. pseudintermedius* identification.

Chapter 2 is focused on the:

- discrimination of methicillin-resistant *S. pseudintermedius* (MRSP) from methicillin-susceptible *S. pseudintermedius* (MSSP) isolated strains by PCR for *mec*A and *mec*C genes;
- evaluation of the antibiotic resistance profile to a panel of 20 antimicrobial agents by the disk diffusion method on Mueller-Hinton agar plates (Liofilchem, Teramo, Italy), according to the guidelines of the Clinical Laboratory Standard Institute (CLSI, 2015);
- determination of the presence of the different *tet* and *erm* genes in tetracycline- and erythromycin-resistant strains by PCR.

In *Chapter 3*, multilocus sequence typing (MLST) was performed to investigate the MRSP sequence type clones circulating in Southern Italy and to correlate their antibiotic resistance patterns to the belonging clones.

The aim of *Chapter 4* was to investigate new therapeutic options, in order to prevent the spread of antibiotic resistance, since it is one of the most urgent threat to the public's health. So, the antimicrobial properties of DIBI, a novel iron chelating polymer developed by Chelation Partners Inc. (Canada), and abietic acid against MRSP and MSSP strains were tested.

Clinical and Laboratory Standards Institute (CLSI) 2015. Performance Standard for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 3rd Edition CLSI VET01S (Wayne, Pennsylvannia, Clinical Laboratory and Standards Institute), 128, 2015.

Decristophoris P, Fasola A, Benagli C, Tonolla M, Petrini O, 2011. Identification of *Staphylococcus intermedius Group* by MALDI-TOF MS. Syst Appl Microbiol **34**, 45-51.

De Martino L, Nocera FP, Mallardo K, Nizza S, Masturzo E, Fiorito F, Iovane G, Catalanotti P, 2016. An update on microbiological causes of canine otitis externa in Campania Region, Italy. Asian Pac J Trop Biomed **6**, 384-389.

Kadlec K, Schwarz S, 2012. Antimicrobial resistance of *Staphylococcus pseudintermedius*. Vet Dermatol **23**, 276-282.

Loeffer A, Lyod DH, 2018. What has changed in canine pyoderma? A narrative review. Vet J **235**, 73-82.

Moodley A, Damborg P, Nielsen SS, 2014. Antimicrobial resistance in methicillin susceptible and methicillin resistant *Staphylococcus pseudintermedius* of canine origin: Literature review from 1980 to 2013. Vet Microbiol **171**, 337-341.

Perreten V, Kadlec K, Schwarz S, Grönlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerald JR, Rossano A, Guardabassi L, 2010. Clonal spread of methicillin-resistant Staphylococcus pseudintermedius in Europe and North America: an international multicenter study. J Antimicrob Chemother **65**, 1145-1154.

Rubin JE, Ball KR, Chirino-Trejo M, 2011. Antimicrobial susceptibility of Staphylococcus aureus and *Staphylococcus pseudintermedius* isolated from various animals. Can Vet J **52**, 153-157.

Somayaji R, Rubin JE, Priyantha MA, Church D, 2016. Exploring *Staphylococcus pseudintermedius*: an emerging zoonotic pathogen? Future Microbiol **11,** 1371-1374.

Weese JS, van Duijkeren E, 2010. Methicillin-resistant *Staphylococcus aureus and Staphylococcus pseudintermedius* in veterinary medicine. Vet Microbiol **140,** 418-429.

Youn JH, Park YH, Hang'ombe B, Sugimoto C, 2014. Prevalence and characterization of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from companion animals and environment in the veterinary teaching hospital in Zambia, Africa. Comp Immunol Microbiol Infect Dis **37**, 123-130.

Chapter 1

Bacterial skin disorders in dogs
Staphylococcus pseudintermedius as main bacterial agent of canine otitis and pyoderma

1. Introduction

Canine bacterial skin infections represent the mainly reason of presentation in small animal practice and are generally secondary complications of a range of canine skin diseases including endocrine, allergic, seborrheic and follicular disorders (Miller *et al*., 2013). Indeed, many bacterial species, especially Staphylococci, are part of the natural skin microbiota but particular conditions such as flea infestation, allergic reaction, immune system depletion can lead to an excessive skin bacteria proliferation with the appearance of cutaneous infection symptoms. Infected dogs can show pruritus, areas of redness, rash-like pustules and hair loss.

In dogs, skin and ear infections are very commonly caused by *Staphylococcus pseudintermedius* (*S. pseudintermedius*) since it is a normal inhabitant of the skin and mucosa of dogs and cats (Van Duijkeren *et al*., 2011). Thus, *Staphylococcus pseudintermedius* is an opportunistic pathogen and a leading cause of skin, ear and post-operative wound infections in dogs and marginally in cats (Fitzgerald, 2009; Weese and van Duijkeren, 2010).

Canine otitis externa and pyoderma are featured consistently as the major diseases affecting canine skin system.

- **Canine otitis externa**

Otitis externa is the most common ear disease seen in dogs, affecting up to 20% of the canine population (De Martino *et al.,* 2016). It has a multifactorial etiology and the otitis causes can be classified as

predisposing, primary and perpetuating factors. Moreover, the predisposing factors can be related to the host and to the environment (Fig 1.1).

Among the predisposing factors related to the host, anatomical changes of the ear canal as conformational abnormalities linked to the belonging breed seem to be the main otitis causes. The most frequently affected breeds are: german shepherds for the high moisture levels in their ear canals, cocker spaniels for their pendulous pinnae, shar-peis for their hypoplastic and stenotic ear canals and poodles for a high density of hair in their ear canals (Zur *et al.*, 2011).

Another aspect to consider is represented by the habits of the animals, that is to say dogs living outdoor and hunting dogs have more possibilities to develop otitis externa because foreign fragment of twigs, plants or dirty materials can contaminate their ear canal. Moreover, dogs that are often washed or do swimming activities are predisposed to otitis for the excessive stimulations of the ceruminous glands. Consequently, the humidity in the ear canal compromise the protective function of the epidermidis.

Some studies have shown that changes in temperature and humidity of the surrounding environment are related to temperature changes and dampness of the ear canal (Logas, 1994).

The most common primary causes of otitis externa are allergies such as atopic dermatitis and adverse food reactions. Keratinization disorders, either primary (idiopathic seborrhea) or secondary as in hypothyroidism and sex hormone imbalance, are also common primary causes affecting

the secretions of the ceruminous and sebaceous glands lining the ear canal (Rosser, 2004).

The predisposing factors and primary causes create suitable conditions for the growth and the proliferation in the ear canal of microorganisms such as bacteria and yeast, that represent both predisposing and perpetuating factors. Many studies report that the most common bacterial pathogens isolated from canine otitis externa are *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa*, *Proteus mirabilis, Streptococcus spp*. and *Escherichia coli* (Hariharan *et al*. 2000; Lyskova *et al*., 2007). Whilst among yeasts, *Malassezia pachydermatis* has been reported as the major causative agent of canine otitis externa (De Martino *et al*., 2016).

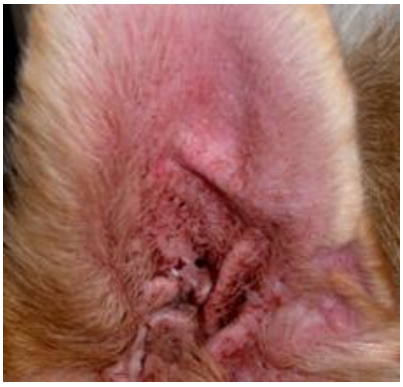

Fig. 1.1 Canine otitis externa

- **Canine pyoderma**

Pyoderma refers to a bacterial skin disorder and it comes from Greek words *pyo* meaning “pus” and *derma* meaning “skin. Pyoderma is common in dogs and less common in cats and it is one of the main

presentations leading to antimicrobial prescription in small animal practice (Hughes *et al.*, 2012). Bacterial pyoderma is usually triggered by an overgrowth/overcolonization of normal resident or transient flora. Since *Staphylococcus pseudintermedius* is a normal commensal of dog, it is the most commonly pathogen of canine pyoderma, in particular superficial pyoderma. However, normal resident bacteria in canine skin also include coagulase-negative staphylococci, streptococci, *Micrococcus spp*, and *Acinetobacter spp.* Transient bacteria in canine skin include *Bacillus spp*., *Corynebacterium spp*., *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas spp*. These organisms may play a role as secondary pathogens, but often *S. pseudintermedius* is required for a pathologic process to ensue.

Bacterial pyoderma can occur either as simple infection or complex infection. Simple infections are those occurring in young animals that are triggered by one-time or simple events, such as flea infestation. Complex infections are recurrent and are associated with underlying diseases, such as allergies, endocrinopathies, seborrheic conditions, parasitic diseases (*Demodex canis*), or anatomic predispositions. Regarding to the anatomic predisposition, it has been suggested that in dog pyoderma is partly a consequence of the thin and compact stratum corneum, of the paucity of intracellular emulsion in epidermidis and of the absence of a sebum plug in the hair follicle (Loeffler and Lloyd, 2018).

Bacterial pyoderma is generally classified as surface, superficial and deep pyoderma on the basis of the depth of infection.

Surface pyoderma remains the less known. It comprises acute moist dermatitis (hot spots, pyotraumatic dermatitis), fold pyoderma (intertrigo) and microbial/bacterial overgrowth syndrome, in which erythema is the only clinical sign.

Superficial pyoderma, named also superficial bacterial folliculitis, is the most recurrent type of canine pyoderma (Fig.1.2). It is limited to the epidermis that is invaded by bacteria. The clinical signs are papules, pustules and epidermal collarettes, which are typically confined to the ventral abdomen, medial thighs and to the trunk. Moreover, it is often associated with alopecia and pruritus (Loeffler and Lloyd, 2018). In fact, the most important factor in superficial pyoderma is the bacterial adherence, or "stickiness," to the keratinocytes. Warm, moist areas on the skin, such as lip folds, facial folds, neck folds, dorsal or plantar interdigital areas, vulvar fold, sand tail folds, often have higher bacterial counts than other areas of skin and are at an increased risk of infection. Pressure points, such as elbows and hocks, are prone to infections, possibly because of follicular irritation and rupture due to chronic repeated pressure. Deep pyoderma is less frequent but more serious, since it involves the dermis (deep folliculitis and furunculosis, and cellulitis). Blood vessels may also be involved with a high risk of hematogenous spread and bacteremia.

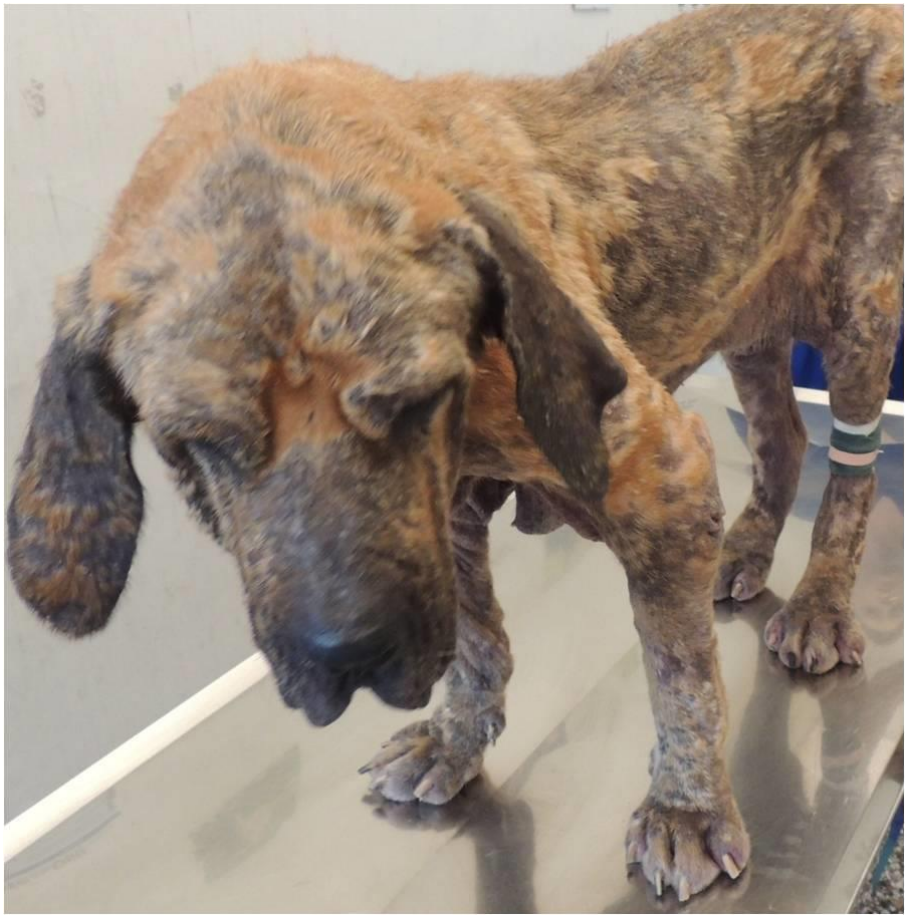

Fig. 1.2 Canine superficial pyoderma (Foglia Manzillo et al., 2016)

1.1 *Staphylococcus pseudintermedius*: the main opportunistic dog pathogen.

Staphylococcus intermedius was first described in 1976 and it has been considered as the major strain responsible of canine skin disorders for a long time (Hajek, 1976; Fitzgerald, 2009). However, the development of molecular techniques revealed a genetic diversity among *S. intermedius* isolates and in 2005 a novel staphylococcal species, *Staphylococcus pseudintermedius*, was defined based on the sequential analysis of 16S rRNA (Devriese *et al*., 2005).

Therefore, the isolates, that previously could not be distinguished by biochemical and morphological characters, in 2007 were grouped in the *Staphylococcus intermedius Group* (SIG) and differentiated thanks to the sequential analysis of *sod*A and *hsp*60. Thus, SIG comprises three distinct species: *Staphylococcus intermedius*, *Staphylococcus pseudintermedius* and *Staphylococcus delphini,* each of which has different ecological niches (Bannoehr *et al.*, 2007; Sasaki *et al.*, 2007).

Importantly, it was discovered that *S. pseudintermedius*, not *S. intermedius*, is a member of the normal skin canine microbiota and an opportunistic pathogen (Fig. 1.3).

Fig. 1.3 Staphylococcus pseudintermedius ecological niche (Bannoehr and Guardabassi, 2012)

Precisely, *Staphylococcus pseudintermedius* can be isolated from the nares, oral mucosa, pharynx, forehead, groin and anus of healthy dogs (Garbacz *et al*., 2013). So, it is the most common bacterial pathogen associated with canine infections, predominantly skin infections (Fig. 1.4).

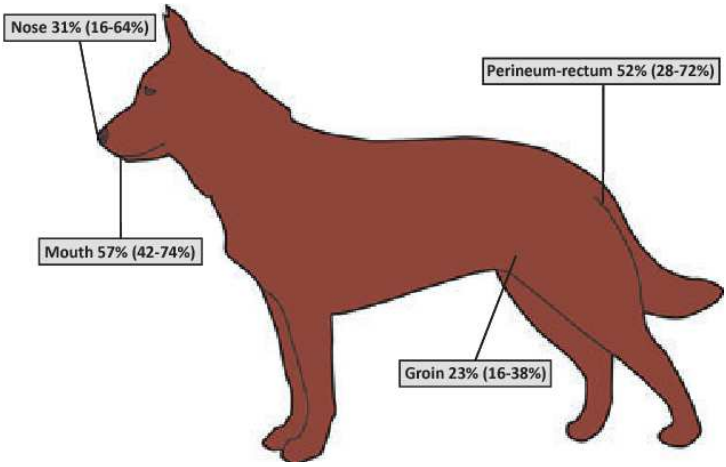

Fig. 1.4 Staphylococcus pseudintermedius colonization in dog (Bannoehr and Guardabassi, 2012)

Ben Zakour *et al.* (2011), reported the first whole-genome sequence of a canine clinical isolate of *S. pseudintermedius* (ED99). Its genome consisted of a single circular chromosome of 2,572,216 bp with an average G+C content of 37,6%, carried five ribosomal operons and 58 tRNA loci and encoded for 2401 predicted proteins coding sequences (CDSs). Moreover, *S. pseudintermedius* ED99 contained many

predicted mobile genetic elements, including insertion elements, transposons mediating resistance to antibiotics, a novel member of the staphylococcal pathogenicity island family (SaPI). The genome of other isolated *S. pseudintermedius* strains has been also sequenced (Tse *et al.*, 2011; Moodley *et al.*, 2013; Duim *et al.*, 2018). As other staphylococcal species, also *S. pseudintermedius* has various virulence factors (Fig. 1.5), including some that are closely related to *S. aureus* (Fitzgerald, 2009; Bannoehr and Guardabassi, 2012). These factors of virulence take part in almost all processes from colonization of the host to bacterial nutrition and dissemination.

S. pseudintermedius has the capability to produce some enzymes such as coagulase, protease, thermonuclease and toxins. Among toxins, mainly β-haemolysins (but some strains also α and ȝ haemolysins), exfoliative toxins and enterotoxins are produced. Exfoliative toxin seems to be the factor involved in canine pyoderma, because the exfoliative toxin gene has been harbored in many *S. pseudintermedius* isolated from dogs suffering from skin infections, that is to say chronic otitis and pyoderma (Iyori *et al.*, 2010).

Furthermore, similarly to *S. aureus,* also *S. pseudintermedius* produces a leucotoxin and an immunoglobulin-binding protein, which are known as Luk-I and staphylococcal protein A (spa), respectively (Moodley *et al*., 2009). Genes encoding virulence factors are usually located on mobile genetic elements such as pathogenicity islands. The production of virulence factors is regulated by several regulatory loci such as the accessory gene regulator (*agr*) and the staphylococcal accessory regulator (*sar*) in response to cell density, energy availability and

environmental signals. The accessory gene *agr* is a staphylococcal quorum-sensing system, which encodes the auto-inducing peptide (AIP). All staphylococcal species encode a unique to each species AIPs (Dufour *et al.,* 2002).

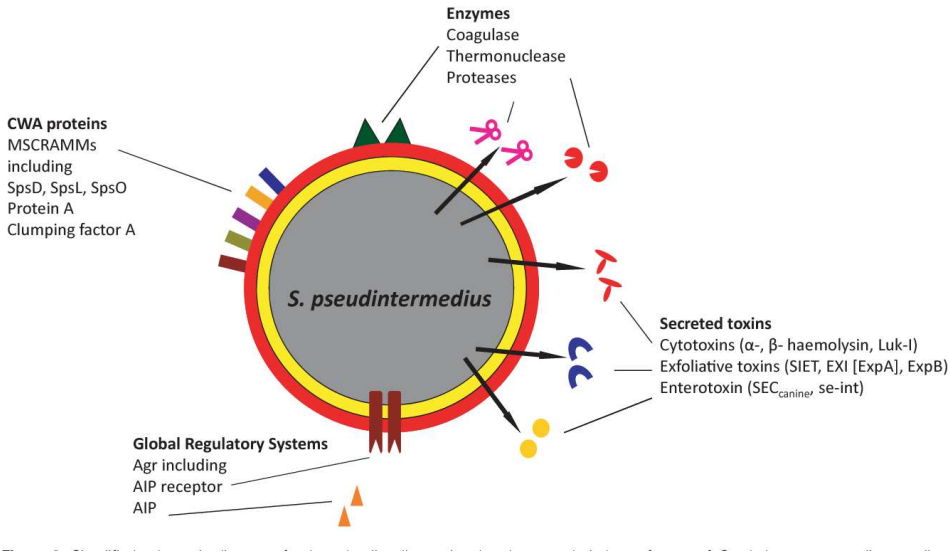

Fig. 1.5. S. pseudintermedius virulence armamentarium (Bannoehr and Guardabassi, 2012)

As other staphylococcal species, *S. pseudintermedius* strains can be biofilm producers, which represents another important virulence determinant, increasing their ability to resist to antibiotics (Casagrande Proietti *et al*., 2015; Han *et al.,* 2015).

The biofilm production by *S. pseudintermedius*, as well as *S. aureus,* may contribute to its ability to persist in the environment, cause nosocomial infections, and chronic recurrent infections that respond poorly to antimicrobial treatment.

However, the knowledge on the pathogenesis of *S. pseudintermedius* is very limited, since the majority of virulence factors have not been characterized in detail yet.

In the past, the *S. pseudintermedius* isolates were generally susceptible to β-lactams, whose major antimicrobial agent is penicillin, and many other antibiotics. Therefore, methicillin-susceptible *S. pseudintermedius* (MSSP) strains originally circulated in canine population. However, already since 2006 methicillin-resistant *S. pseudintermedius* (MRSP) strains have been isolated in Europe, becoming a relevant problem in veterinary medicine. A veterinary health issue comparable to the public health concern due to methicillin-resistant *S. aureus* (MRSA) (Deurenberg *et al*., 2007).

Referring to *S. pseudintermedius* virulence potential, its zoonotic transmission should not be underestimated, even though it is not often reported. However, *S. pseudintermedius*, particularly MRSP, has been sometimes isolated from humans, especially in pet owners. It is worth noting that infections caused by *S. pseudintermedius* in humans are often underreported due to inaccurate identification as *S. aureus* (Van Hoovels *et al.*, 2006; Stegmann *et al.*, 2010; Somayaji *et al.,* 2016; Robb *et al*., 2017; Lozano *et al.*, 2017).

1.2 Phenotypic features of *Staphylococcus pseudintermedius*

Staphylococcus pseudintermedius comes from Greek words *pseudes* or *pseudos* meaning false and from *intermedius* meaning intermediate.

Thus, the adjective *pseudintermedius* indicates a false (*Staphylococcus*) *intermedius*, because of its high phenotypic similarity to *S. intermedius*. Indeed, *S. pseudintermedius* is a Gram-positive coccus arranged in groups as the other staphylococcal species (Fig. 1.6).

During the diagnostic activity, the media used for growth and the isolation of *S. pseudintermedius* strains are different, but generally Columbia sheep blood agar is routinely used. Here colonies appear non-pigmented and always surrounded by double zone haemolysis. The outer band, which is incompletely haemolytic, becomes completely haemolytic after being put at 4°C (hot–cold haemolysis). This is due to the activity of the staphylococcal β-haemolysin, that is a sphingomyelinase (Devriese *et al*., 2005). Furthermore, also Mannitol Salt Agar (MSA) plates are used for the isolation of this strain. In particular, MSA was developed in 1945 for the selective isolation of pathogenic staphylococci. Since *S. pseudintermedius* is a mannitol negative strain, differently from *S. aureus* but similarly to coagulase-negative staphylococci, its colonies on this medium appear light pink for the missing fermentation of mannitol sugar contained in MSA plates. This is one of the reasons for *S. pseudintermedius* was often misidentified as coagulase- negative staphylococcus.

Together with the other members of the SIG, *S. pseudintermedius* is a coagulase-positive species (CoPS), producing the coagulase enzyme, which is a relevant virulence factor since it represents the way to evade the host immune system. Coagulase is an extracellular protein that binds the host prothrombin to form a complex called staphylothrombin (McAdow *et al*., 2012). The complex formation allows the conversion

of fibrinogen into fibrin (Władyka and Pustelny, 2008). Moreover, it is catalase positive and DNase positive, but it is normally negative to rapid slide clumping test and to commercial latex agglutination tests that detect the clumping factor.

The identification of *S. pseudintermedius* is problematic. Phenotypic identification is defective and there are not commercial kits that can identify *S. pseudintermedius* and distinguish it from the other members of the SIG and from *S. aureus*, because they share many phenotypic characters. Since phenotypic discrimination of CoPS species is difficult, the real prevalence of *S. pseudintermedius* might have been underestimated being misidentified with other CoPS, especially *S. intermedius* or *S. aureus,* in routine laboratory diagnostics (Weese and van Duijkeren, 2010).

Consequently, the molecular identification embodies the only reliable tool for a proper determination and diagnosis of *S. pseudintermedius*.

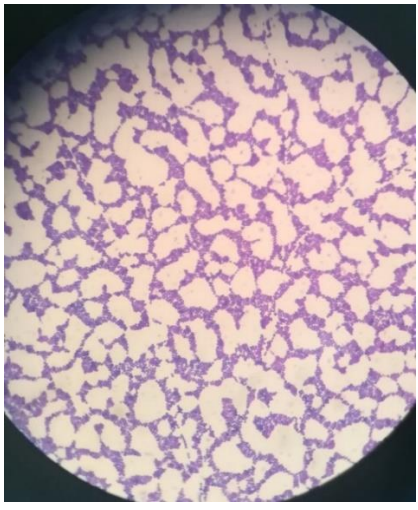

Fig. 1.6 S. pseudintermedius: Gram stain

In fact, to distinguish the belonging species of the SIG, a species-specific multiplex PCR based on the thermonuclease (*nuc*) gene is routinely performed as described by Sasaki *et al*. (2010). *S. pseudintermedius* constitutively produces β-haemolysin. Kmieciak *et al.* (2016) have designed, on the basis of the *S. pseudintermedius* ED99 complete genome deposited in Genbank, a new pair of primers for *hlb* gene, which enable the analysis of *S. pseudintermedius* strains. Their data together with species-specific *nuc* gene and multilocus sequence typing allow to better identify *S. pseudintermedius* and overcome the many difficulties in the differentiation among the species of the SIG group.

Furthermore, Bannoehr *et al*. (2009) developed a rapid, simple and inexpensive PCR-restriction fragment length polymorphism (RFLP) which allows the discrimination of *S. pseudintermedius* from the closely related SIG members through the sequence analysis of the *pta* gene (Bannoehr *et al*., 2009).

Multilocus sequence typing (MLST) is a nucleotide sequence-based method, which defines strains from the sequences of internal fragments of 7 housekeeping genes, in order to define a sequence type (ST) (Aanensen and Spratt, 2005). New sequence types are assigned when a new allelic profile is obtained. Even though MLST is a very expensive technique, it is the best method for genetic screening (Deurenberg and Stobberingh, 2008) and for a reliable discrimination of clinical isolates of *S. pseudintermedius*, which has a considerable genetic diversity within the species (Fitzgerald, 2009). So, from this point of view,

MLST appears to be the most effective method used to classify bacteria into clonal lineages and provide information about their relatedness.

On the other hand, the molecular tools can be performed only in few diagnostic and clinical laboratories and require at least 24-36 hours for the results, so in the last years the use of matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has notably increased (Fig 1.7). MALDI-TOF MS allows a rapid bacterial characterization and identification with high accuracy by a proteomic approach. Precisely, identification by MALDI-TOF MS is based on the analysis of protein spectrum from bacterial ribosome. Hence, this technique produces a fingerprint spectrum of the peptides and proteins of the analyzed bacterial strains. The extraction protocols are several, either prescribed by MALDI-TOF MS manufacturers or published in literature, and they are specific for certain groups of bacteria and yeasts. Referring to *S. pseudintermedius* or, better, to the SIG complex, Decristophoris *et al*. (2011), reported that, MALDI-TOF MS, provided with a reliable database, is a valid and effective technique for a rapid identification of the bacterial species belonging to the *S. intermedius* Group.

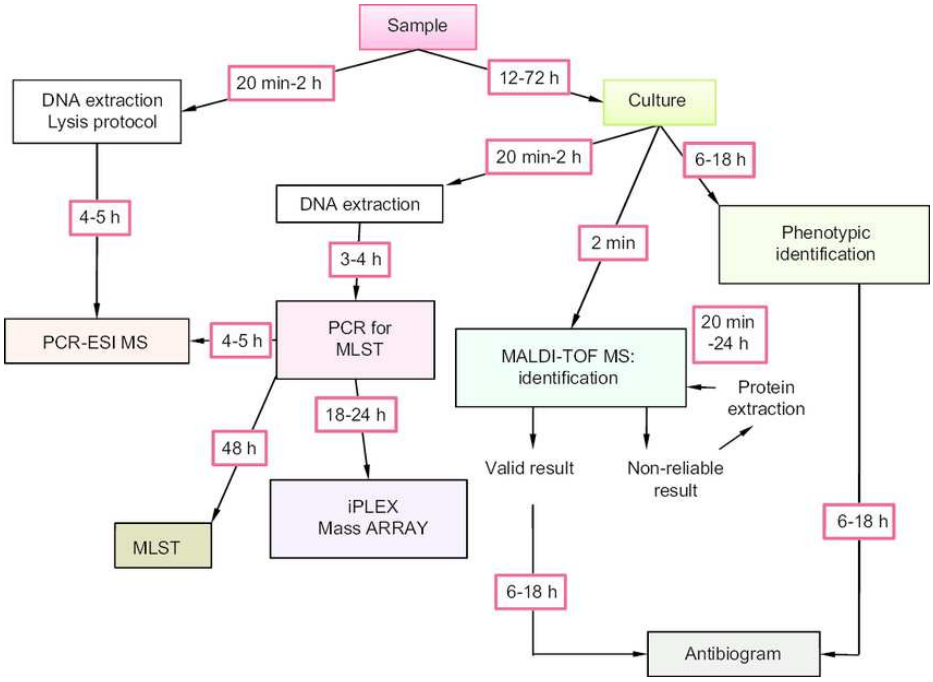

Fig. 1.7 Typical workflow of new and old methods used in clinical microbiology (Lavigne et al., 2012)

2. Materials and Methods

2.1 Collection of samples

During the years 2015-2017 the specimens, represented by auricular and cutaneous swabs, were collected from dogs suffering from otitis externa and pyoderma at the Bacteriology laboratory of the Veterinary Teaching Hospital of the Department of Veterinary Medicine and Animal Production, University of Naples "Federico II".

Moreover, further auricular swabs, screened for staphylococci growth, were obtained from Provet Lab srl of Latina, Lazio Region.

From a total of 259 staphylococcal cultures, *S. pseudintermedius* strains were selected.

2.2 Bacterial isolation and phenotypic identification of the isolates

All samples were cultured and streaked in parallel on Columbia CNA agar (Liofilchem, Teramo, Italy) and on MSA (Liofilchem, Teramo, Italy) and incubated aerobically at 37 °C for 24-48 h (Fig. 1.8). As positive control *S. pseudintermedius* ATCC® (American Type Culture Collection) 49444™ was used. Suspected *S. pseudintermedius* isolates were firstly identified by using standard, rapid screening techniques: colony morphology, β-haemolysis on Columbia CNA agar, absence of mannitol fermentation on MSA, cellular morphology (after Gram's staining method), catalase test. Additionally, each mannitol salt negative colony was also subjected to staphylocoagulase (tube coagulase) reaction (Oxoid, Ltd, UK) to confirm their capacity to produce coagulase enzyme.

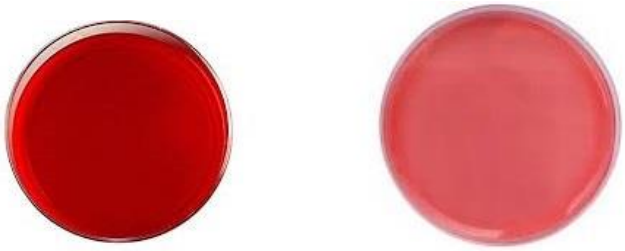

Fig. 1.8 Culture media used for S. pseudintermedius isolation
Columbia CNA agar on the left, MSA on the right

For an initial identification, a biochemically-based commercial system, manual API Staph (bioMérieux, Marcy L'Etoile, France) was performed and the presumptive *S. pseudintermedius* strains, reported as *S. intermedius*, if of animal origin, were selected. These strains were then identified by MALDI-TOF MS analysis (Bruker Daltonics, Germany), which discriminates *S. pseudintermedius* from *S. intermedius,* at the diagnostic service of the Department of Experimental Medicine, University of Campania "Luigi Vanvitelli". For MALDI-TOF MS identification, fresh colonies, grown on Columbia CNA agar, were used.

The protocol used was the following: the bacterial colony was first inoculated in the plate for mass spectrometry. Subsequently, 1 µl of the organic matrix, usually cinnamic acid, was added to the sample. Afterwards, the plate was placed in the equipment for MALDI-TOF MS analysis (Fig. 1.9). The identification by MALDI-TOF MS is based on the score value released by equipment's instructions.

Values from 2.3 to 1.9 indicated the best identification of genus and species (Santos *et al*., 2013).

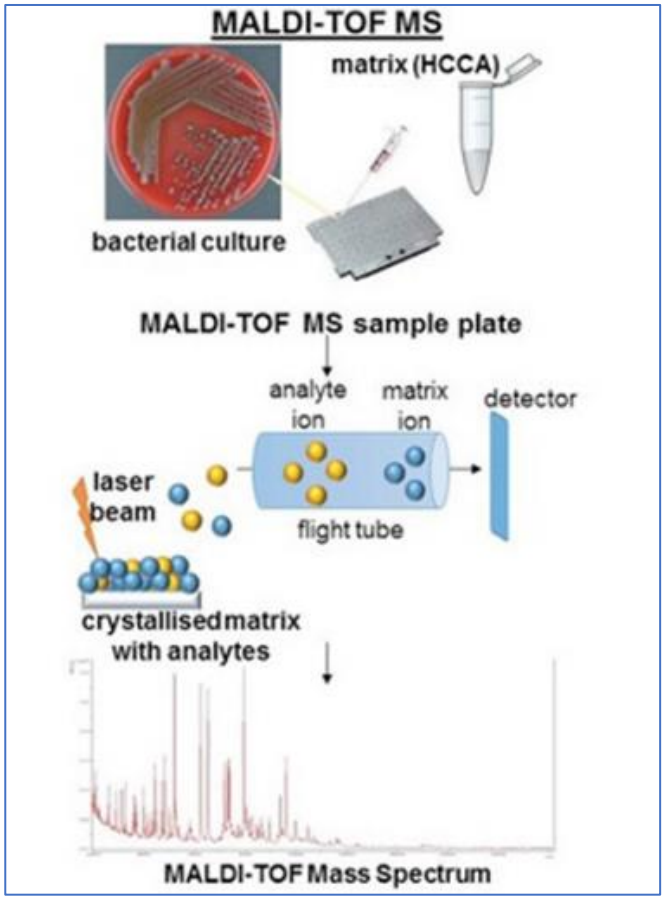

Fig. 1.9 Maldi-Tof MS technique (Navrátilová et al., 2016)

All *S. pseudintermedius* cultures were stored at -70 °C by using Microbank™ vials (Pro-lab Diagnostics, Canada) for further analysis. Microbank™ is a ready to use system designed for the long term storage and retrieval of bacterial and fungal isolates. Each Microbank™ vial contains a 25 sterile coloured beads (single colour) and the cryopreservative. The specially treated beads are of a porous nature allowing microorganisms to readily adhere onto the bead surface (Fig. 1.10). After inoculation the Microbank™ vials are kept at -70°C for extended storage. When a fresh culture is required, a single bead is easily removed from the Microbank™ vial and used to directly inoculate a suitable culture medium.

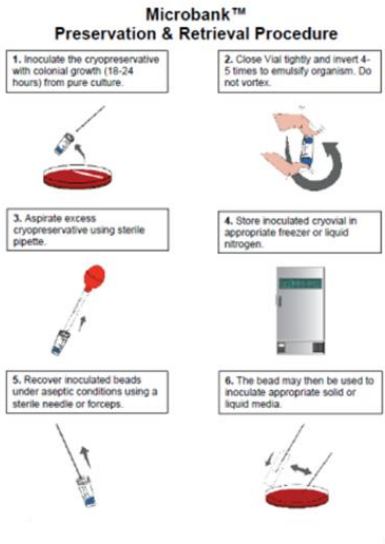

Fig. 1.10 Microbank™: preservation and retrieval procedure (Pro-lab Diagnostics)

2.3 Genotypic identification of the isolates

For the molecular characterization of the selected strains, each *S. pseudintermedius* isolate was cultured again on MSA with incubation at 37°C overnight. The bacterial DNA extraction of the isolates was carried out using two different protocols:

- by boiling;
- by using a commercial kit.

Referring to the first method, 1-5 colonies were taken from a pure culture of the isolated strain and then homogenized in 50 µL of distilled water and then denatured at 100°C for 10 minutes. Then, the obtained bacterial suspensions were stored at -20°C.

The second technique was performed by using the commercial Isolate II Genomic DNA Kit (Bioline, London, UK) and following the manufacturer's instructions. Briefly, the isolated bacterial colonies were dissolved in 180 µL of lysis buffer (buffer GL) and in 25 µL of proteinase K solution and then the obtained suspension was vortexed. At that point, the suspension was incubated at 56°C for at least one hour, in order to obtain the complete bacterial cell lysis. Subsequently, 200 µL of buffer G3 were added, followed by another incubation at 70°C for 10-15 minutes. The sample was, then, briefly vortexed and 210 µL of absolute ethanol were added to it and the suspension was vigorously vortexed. The mix of each sample, placed in the isolate II genomic DNA spin column, was transferred into a collection tube and centrifuged at 11.000 x g for 1 minute. Afterwards, the silica membrane was washed twice with two different wash buffers (500 µL of wash buffer G1 and 600 µL of wash buffer G2) and, obviously, centrifugated

at each wash at 11.000 x g for 1 minute. This last step ensured that no ethanol residue was transported in the subsequent elution step. Once placed the isolate II genomic DNA spin column in a 1.5 ml sterile microcentrifuge tube, 100 µL of elution buffer G (elution step) were directly pipetted onto the silica membrane. The last centrifugation at 11.000 x g for 1 minute allowed the collection of bacterial DNA in elution liquid in the Eppendorf tubes. Before the molecular characterization of all isolated strains, genomic DNA was tested by *Biophotometer* Eppendorf to determine the absorbance ratio (OD A260/A280). The A260/A280 was considered appreciable when ranged between 1.5-1.9 value. Bacterial DNA was stored at -20°C and used for further studies.

Molecular profiling, using species-specific *nuc* gene (Sasaki *et al*., 2010) and species-specific *hlb* gene (Kmieciak *et al*., 2016) was performed by single PCR to confirm the identification of *S. pseudintermedius* strains. Also for the genotypic characterization of the isolated strains, *S. pseudintermedius* ATCC® 49444™ was used as positive control.

Primers sequences, amplicon sizes and amplification programs are reported in Table 1.1.

The single PCR reaction mixture for each gene (*nuc* and *hlb* genes) was prepared by using the Green Hot Start PCR Master Mix Direct Load, 2x (Biotechrabbit, GmbH, Germany) as follows:

- Green Hot Start PCR Master Mix: 12,5 µL
- *primers* (F+R): 1 µL

- nuclease free water: 10,5 µL
- DNA template: 1 µL

The mixture final volume for one reaction was of 25 µL.

For every PCR reaction there was always a positive and a negative control. Genomic DNA was amplified using Biorad T100^{TM} Thermo cycler (BioRad, Hercules, CA).

Tab. 1.1. Primers sequences, amplicon sizes, amplification programs

Gene	Primer sequences (5'-3' sense and antisense)	Amplicon size (bp)	Amplification program
nuc	F: TRGGCAGTAGGATTCGTTAA R: CTTTTGTGCTYCMTTTTGG	926	94°C 5 min; 94°C 30 s, 58°C 60s, 72°C 90s, for 30 cycles; 72°C 5 min.
hlb	F: GACGAAAATCAAGCGGAA R: TCTAAATACTCTGGCGCAC	734	94°C 2:30 min; 94°C 30s, 56°c 30s, 72°C 1min, for 30 cycles; 72°C 10 min.

The amplified products were analyzed by gel electrophoresis. 1.5 g of agarose (Agarose electrophoresis grade, Gibco BRL) were placed in a flask and dissolved by heating in a microwave oven in 100 ml of TBE 1X for few minutes. Once melted the agarose, in order to stain the gel, 2 µL of Real Safe nucleic acid staining solution (Durviz s.l., Valencia, Spain) were added. At this point, the gel was poured into the appropriate bed equipped with a comb for the formation of wells and left to solidify for about 30 minutes. For each sample, 12 µL of PCR

reaction mix were loaded in each gel well. 5 µL of 100 bp DNA ladder with 6x loading dye, consisting of 100- 3000 bp, were loaded as molecular marker (Biotechrabbit, GmbH, Germany).

Electrophoresis was performed in a BioRad electrophoresis tank with 1X TBE as running buffer at 80V for 45-50 minutes. Electrophoresed gels were visualized under blue-light and their images taken using the ChemiDoc™ XRS+ with Image Lab™ Software (BioRad, Hercules, CA).

3. Results

3.1 *S. pseudintemedius* isolation and phenotypic identification

During the years 2015-2017, a total of 259 staphylococcal strains responsible for canine skin disorders were collected. The phenotypic bacterial identification by proteomic profiling defined a relevant number of 126 (49%) *S. pseudintermedius* strains. Moreover, all the isolated *S. pseudintermedius* strains were identified with good scores (1.9 or 2.0).

The other most isolated species belonged to *S. aureus* (8%), *S. schleiferi* (7%), *S. xylosus* (6%), *S. sciuri* (2%), *S. intermedius* (2%), *S. delphini* (1%).

The remaining 25% consisted of unsuspected *Staphylococcus spp*.

S. *pseudintermedius* strains were recovered from dogs suffering with otitis externa (84%) and pyoderma (16%), originating from two different Italian geographical areas (Naples, Campania Region [52%] and Latina, Lazio Region [48%]). The Table 1.2 describes the

percentage of *S. pseudintermedius* isolates from the above reported canine skin disorders.

Tab. 1.2 S. pseudintermedius strains isolated from diseased dogs of Campania and Lazio regions

Geographical area of origin	**Biological origin**	**% isolated *S.pseudintermedius* strains**
Naples	otitis	71
	pyoderma	29
Latina	otitis	98
	pyoderma	2

All the isolated *S. pseudintermedius* strains appeared to be β-heamolytic on Colombia CNA agar and mannitol negative on MSA (Fig 1.11). Furthermore, they were positive at the staphylocoagulase and catalase reactions.

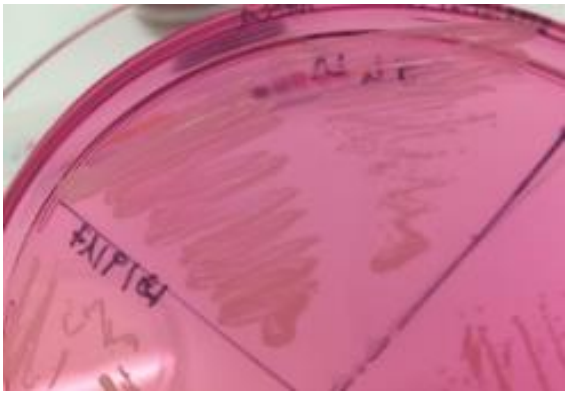

Fig. 1.11 S. pseudintermedius growth on MSA

3.2 *S. pseudintermedius* genotypic identification

All 126 *S. pseudintermedius* strains harboured the species-specific *nuc* and *hlb* genes (Fig. 1.12, 1.13) confirming, thus, the proteomic identification by MALDI TOF MS.

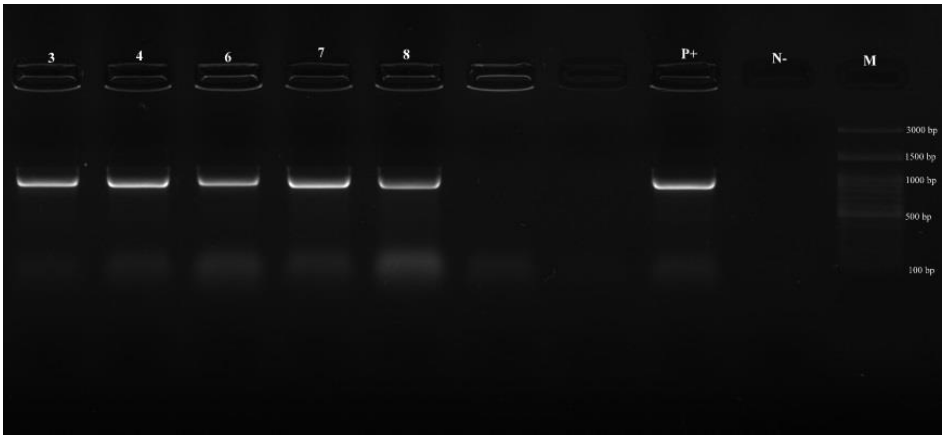

Fig. 1.12 Molecular characterization of S. pseudintermedius: PCR for detection of species-specific nuc gene. Data from one of three experiments are shown. Lanes 3,4,6,7,8, positive samples; Lane P+, positive control; lane N-, negative control; lane M, 100-bp DNA ladder.

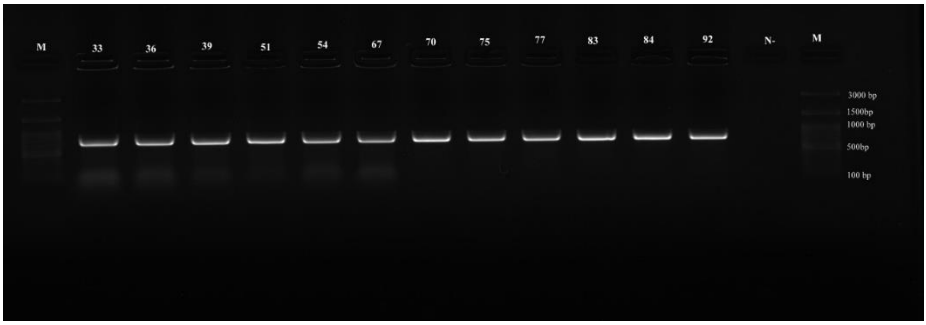

Fig. 1.13 Molecular characterization of S. pseudintermedius: PCR for detection of species-specific hlb gene. Data from one of three experiments are shown. Lanes 33,36,39,51,54,67,70,75,77,83,84,92, positive samples; lane N-, negative control; lane M, 100-bp DNA ladder.

4. Discussion and conclusions

Bacterial otitis externa and pyoderma are the most common canine skin diseases and *Staphylococcus pseudintermedius* is the staphylococcal species most frequently isolated from dogs suffering from these infections. In fact, *Staphylococcus pseudintemedius*, an opportunistic canine skin pathogen, is the major CoPS that inhabits healthy dogs (Gómez-Sanz *et al.*, 2013). In particular conditions, such as dog injures or sickness, this species can take advantage of the weakened defenses and cause infection and illness.

According to literature, the results of this study show a high prevalence (49%) of *S. pseudintermedius* among the other isolated staphylococcal species, approving its role as the main causative agent of canine skin disorders.

Due to the high rates of colonization of dogs with *S. pseudintermedius* and *S. aureus*, these species make up the majority of Staphylococcus related infections in dogs. According to this finding also the results of this research reported *S. aureus* as the second most common causative agent of staphylococcal skin infection**.**

The literature has always reported a higher interest for coagulase-positive staphylococci such as *S. aureus* and SIG, which is composed, besides *S. pseudintermdius,* by *S. intermedius* and *S. delphini* which, here, were found responsible agents of canine skin disorders with percentage in the range of 1-8%.

Even though *S. intermedius* mainly colonizes pigeons (Sasaki *et al.*, 2007), it was also isolated from dogs with otitis externa (Dziva *et al*., 2015).

Sasaki *et al.* (2007) found that *S. intermedius* can be chemically distinguished from *S. pseudintermedius* by positive arginine dihydrolase and acid production from beta-gentiobiose and D-mannitol. The two species can also be distinguished using molecular and genetic testing, as well as MALDI-TOF mass spectrometry, although this is not commonly done (Wang *et al*., 2013).

Furthermore, *S. intermedius* should be included in the differential diagnosis of invasive infection amongst human patients with close contact with dogs.

S. delphini, first isolated from a dolphin (Varaldo *et al*. 1988), was also found in several different species including horse, mink, pigeons and

camels. These data indicate that *S. delphini* may be more widespread and clinically important than was previously thought. Surprisingly, in this study, this staphylococcal species was isolated from infected dogs (3/259, 1%), therefore it should be warranted to examine the importance of *S. delphini* as a veterinary pathogen in pets.

Coagulase-Negative Staphylococci (CoNS), members of normal flora of human and animal skin, have long been considered as nonpathogenic possessing fewer virulence properties than CoPS. Recently they have assumed an important role as pathogens in skin and soft tissue infections, overall, because of their increasing multidrug-resistance profiles.

In this regard, the percentage of CoNS positivity (15%), precisely of *S. schleiferi*, *S. xylosus* and *S. sciuri*, among the processed samples, highlights a relevant dog susceptibility to these bacterial species.

In veterinary medicine, *S. schleiferi* has been repeatedly documented in literature as both an inhabitant and as a pathogen, and May *et al.* (2012) demonstrated that *S. schleiferi* could be recovered from the ears and anterior nares of healthy dogs as well as those with otitis and/or pyoderma. In this study, *S. schleiferi* was isolated from 20/259 samples (7%) followed by *S. xylosus* (6%) and *S. sciuri* (2%).

In conclusion, this study underlines that *S. pseudintermedius* is the most common bacterial isolate from dogs suffering with otitis externa and pyoderma in Naples – Campania Region, and Latina, Lazio Region.

However, its phenotypic identification remains problematic in many clinical microbiology laboratories, especially in the human ones, where it is often misidentified as *S. aureus* or CoNS. So, genotypic profiling (species-specific *nuc* and *hlb* genes) still represents the main molecular technique for the identification of this species when MALDI-TOF MS analysis is not available.

MALDI-TOF MS for the diagnosis of infectious diseases has been rapidly embraced by laboratories around the globe, and, as seen in this study, represents a valid bacterial identification method also in veterinary medicine. In fact, it represents the most accurate and rapid method for the identification of *S. pseudintermedius* and the other members of the SIG.

This study has provided a further evidence that MALDI-TOF MS is a useful and reliable technique for *S. pseudintermedius* identification. Further confirmation of proteomic profiling identification by MALDI-TOF MS was always given by genotypic profiling results, linked to the detection of species-specific *S. pseudintermdius* genes.

5. References

Aanensen DM, Spratt BG, 2005. The multilocus sequence typing network: mlst.net. Nucleic Acids Res **33**, W728–W733.

Bannoehr J, Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AH, Fitzgerald JR, 2007. Population genetic structure of the *Staphylococcus intermedius* group: insights into agr diversification and the emergence of methicillin-resistant strains. J Bacteriol **189**, 8685-8692.

Bannoehr J, Franco A, Iurescia M, Battisti A, Fitzgerald JR, 2009. Molecular diagnostic identification of Staphylococcus pseudintermedius. J Clin Microbiol 47, 469-471.

Bannoehr J, Guardabassi L, 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol **23**, 253-266.

Ben Zakour NL, Bannoehr J, van den Broek AH, Fitzgerald JR, 2011. Complete genome sequence of the canine pathogen Staphylococcus pseudintermedius. J Bacteriol **193**, 2363-2364.

Casagrande Proietti P, Stefanetti V, Hyatt DR, Marenzoni ML, Capomaccio S, Coletti M, Bietta A, Franciosini MP, Passamonti F, 2015. Phenotypic and genotypic characterization of canine pyoderma isolates of *Staphylococcus pseudintermedius* for biofilm formation. J Vet Med Sci **77**, 945-951.

Decristophoris P, Fasola A, Benagli C, Tonolla M, Petrini O, 2011. Identification of *Staphylococcus intermedius* Group by MALDI-TOF MS. Syst Appl Microbiol **34**, 45-51.

De Martino L, Nocera FP, Mallardo K, Nizza S, Masturzo E, Fiorito F, Iovane G, Catalanotti P, 2016. An update on microbiological causes of canine otitis externa in Campania Region, Italy. Asian Pac J Trop Biomed **6**, 384-389.

Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman AC, Stobberingh EE, 2007. The molecular evolution of methicillin-resistant of *Staphylococcus aureus*. Clin Microbiol Infect **13**, 222-235.

Deurenberg RH, Stobberingh EE, 2008. The evolution of *Staphylococcus aureus*. Infect Genet Evol **8**, 747-763.

Devriese LA, Vancanneyt M, Baele M, Vaneechoutte M, De Graef E, Snauwaert C, Cleenwerck I, Dawyndt P, Swings J, Decostere A, Haesebrouck F, 2005. *Staphylococcus pseudintermedius* sp. nov., a coagulase-positive species from animals. Int J Syst Evol Microbiol **55**, 1569-1573.

Dufour P, Jarraud S, Vandenesch F, Greenland T, Novick RP, Bes M, Etienne J, Lina G, 2002. High genetic variability of the *agr* locus in *Staphylococcus* species. J Bacteriol **184**, 1180-1186.

Duim B, Verstappen KMHW, Kalupahana RS, Ranathunga L, Fluit AC, Wagenaar JA, 2018. Methicillin-resistant *Staphylococcus pseudintermedius* among dogs in the description of novel SCCmec variants. Vet Microbiol **213**, 136-141.

Dziva F, Wint C, Auguste T, Heeraman C, Dacon C, Yu P, Koma LM, 2015. First identificationof methicillin-resistant *Staphylococcus pseudintermedius* strains among coagulase-positive staphylococci

isolated from dogs with otitis externa in Trinidad, West Indies. Infect Ecol Epidemiol **5**, 29170.

Fitzgerald JR, 2009. The *Staphylococcus intermedius* group of bacterial pathogens: species reclassification, pathogenesis and the emergence of methicillin resistance. Vet Dermatol **20**, 490-495.

Foglia Manzillo V, Nocera FP, De Martino L, Gizzarelli M, Oliva G, 2016. A successful vancomycin treatment of multidrug-resistant MRSA-associated canine pyoderma. J Dermatol Res Ther **1**, 12-18.

Garbacz K, Zarnowska S, Piechowcz L, Haras K, 2013. Pathogenicity potential of *Staphylococcus pseudintermedius* strains isolated from canine carriers and from dogs with infection signs. Virulence **4**, 255-259.

Gómez-Sanz E, Torres C, Lozano C, Zarazaga M, 2013. High diversity *of Staphylococcus aureus* and *Staphylococcus pseudintermedius* lineages and toxigenic traits in healthy pet-owing household members. Underestimating normal household contact? Comp Immunol Microbiol Infec Dis **36**, 83-94.

Han JI, Yang CH, Park HM, 2015. Emergence of biofilm-producing *Staphylococcus pseudintermedius* isolated from healthy dogs in South Korea. Vet Q **36**, 207-210.

Hájek V, 1976. *Staphylococcus intermedius*, a new species isolated from animals. Int J Syst Bacteriol **26**, 401-406.

Hariharan H, Coles M, Poole D, Lund L, Page R, 2006. Update on antimicrobial susceptibilities of bacterial isolates from canine and feline otitis externa. Can Vet J **47**, 253-255.

Iyori K, Hisatsune J, Kawakami T, Shibata S, Murayama N, Ide K,

Nagata M, Fukata T, Iwasaki T, Oshima K, Hattori M, Sugai M, Nishifuji K, 2010. Identification of a novel *Staphylococcus pseudintermedius* exfoliative toxin gene and its prevalence in isolates from canines with pyoderma and healthy dogs. FEMS Microbiol Lett **312**, 169-175.

Kmieciak W, Szewczyk EM, Ciszewski M, 2016. Searching for beta-haemolysin hlb gene in *Staphylococcus pseudintermedius* with species-specific primers. Curr Microbiol **73**,148-152.

Lavigne JP, Espinal P, Dunyach-Remy C, Messad N, Pantel A, Sotto A, 2013. Mass spectrometry: a revolution in clinical microbiology? Clin Chem Lab Med **51**, 257-270.

Loeffer A, Lyod DH, 2018. What has changed in canine pyoderma? A narrative review. Vet J **235**, 73-82.

Logas DB, 1994. Diseases of the ear canal. Vet Clin North Am Small Anim Pract **24**, 905-919.

Lozano C, Rezusta A, Ferrer I, Pérez-Laguna V, Zarazaga M, Ruiz-Ripa L, Revillo MJ, Torres C, 2017. *Staphylococcus pseudintermedius* human infection cases in Spain: dog-to-human transmission. Vector Borne Zoonotic Dis **17**, 268-270.

Liskova P, Vydrzalova M, Mazurova J, 2007. Identification and antimicrobial susceptibility of bacteria and yeasts isolated from healthy dogs and dogs with otitis externa. J Vet Med **54**, 559-563.

McAdow M, Missiakas DM, Schneewind O, 2012. *Staphylococcus aureus* secretes coagulase and von Willebrand factor binding protein to modify the coagulation cascade and establish host infections. J Innate Immun **4**, 141–148.

May ER, Kinyon JM, Noxon JO, 2012. Nasal carriage of *Staphylococcus schleiferi* from healthy dogs and dogs with otitis, pyoderma or both. Vet Microbiol **160**, 443-448.

Miller WH, Griffin CG, Campbell KL, 2013. Muller and Kirk's small animal dermatology. 7th edn. Elsevier, St Louis,195.

Moodley A, Stegger M, Ben Zakour NL, Fitzgerald JR, 2009. Tandem repeat sequence analysis of staphylococcal protein A (spa) gene in methicillin-resistant *Staphylococcus pseudintermedius*. Vet Microbiol **135**, 320–326.

Moodley A, Riley MC, Kania SA, Guardabassi L, 2013. Genome sequence of *Staphylococcus pseudintermedius* strain E140, an ST71 European associated methicillin-resistant isolate. GenomeA **1**, e00207-12.

Navrátilová L, Procházková P, Bardoň J, Novotný R, Zápalka M, Jakubec P, Zatloukal J, Kolek V, Kopřiva F, Flodrová P, Raclavský V, 2016. Performance of pyrosequencing versus MALDI-TOF MS in bacteria identification in chronic lung disease. J Biol Methods **3**, e52.

Robb AR, Wright ED, Foster AME, Walker R, Malone C, 2017. Skin infection caused by a novel strain of *Staphylococcus pseudintermedius* in a Siberian husky dog owner. JJM Case Rep 4, jmmcr005087.

Rosser EJ, 2004. Causes of otitis externa. Vet Clin North Am Small Anim Pract **34**, 459-468.

Santos AF, Cayô R, Schandert L, Gales AC, 2013. Evaluation of

MALDI-TOF MS in the microbiology laboratory. J Bras Patol Med Lab **49**, 191-197.

Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K, 2007. Reclassification of phenotypically identified *Staphylococcus intermedius* strains. J Clin Microbiol **45**, 2770-2778

Sasaki T, Tsubakishita S, Tanaka Y, Sakusabe A, Ohtsuka M, Hirotaki S, Kawakami T, Fukata T, Hiramatsu K, 2010. Multiplex-PCR method for species identification of coagulase-positive staphylococci. J Clin Microbiol **48**, 765-769.

Somayaji R, Rubin JE, Priyantha MA, Church D, 2016. Exploring *Staphylococcus pseudintermedius*: an emerging zoonotic pathogen? Future Microbiol **11**, 1371-1374.

Stegmann R, Burnens A, Maranta CA, Perreten V, 2010. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius* ST71. J Antimicrob Chemoter **65**, 2047-2048.

Tse H, Tsoi HW, Leung SP, Urguhart IJ, Lau SK, Woo PC, Yuen KY. Complete genome sequence of the veterinary pathogen *Staphylococcus pseudintermedius* strain HKU10-03, isolate in a case of canine pyoderma. J Bacteriol **193**, 1783-1784.

van Duijkeren E, Kamphuis MIC, Mije V, Laarhoven LM, Duim B, Wagenaar JA, Houwers DJ, 2011. *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet Microbiol **150**, 338-343.

Van Hoovels L, Vankeerberghen A, Boel A, Van Vaerenbergh K, De

Beenhouwer H, 2006. First case of *Staphylococcus pseudintermedius* infection in a human. J Clin Microbiol **44**, 4609-4612.

Varaldo PE, Kilpper-Balz R, Biavasco F, Satta G, Schleifer KH, 1988. *Staphylococcus delphini* sp. nov. a coagulase-positive species isolated from dolphins. Int J Syst Bacteriol **38**, 436-439.

Wang N, Neilan AM, Klompas M, 2013. *Staphylococcus intermedius* infections: case report and literature review. Infect Dis Rep **22**, e3.

Weese JS, van Duijkeren E, 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. Vet Microbiol **140**, 418-429.

Władyka B, Pustelny K 2008. Regulation of bacterial protease activity. Cell Mol Biol Lett **13**, 212-229.

Zur G, Lifshitz B, Bdolah-Abram T, 2011. The association between signalment, common causesof canine otitis externa and pathogens. J Small Anim Pract **52**, 254-258.

Chapter 2

Antibiotic resistance in *Staphylococcus pseudintermedius*

The spread of methicillin-resistant and methicillin-susceptible *S. pseudintermedius* strains

1. Introduction

The rate and the spread of antimicrobial resistance in bacteria has increased in recent years. The World Health Organization (WHO) defines antimicrobial resistance as the "…resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it" (WHO, 2014). Precisely, it is worth noting that the use of antimicrobial drugs has a tendency to select for resistant bacteria already present in the population, rather than inducing resistance (Hirsh *et al*., 2004; Quinn *et al*., 2011).

Resistance to antimicrobial drugs can be due to acquired resistance, either by mutation in the chromosomal DNA, or by acquiring genetic material. Chromosomal mutation often leads to a change in the bacterial structure, for example altered antimicrobial target proteins. These rare, spontaneous changes in the bacterial DNA are linked to mistakes during DNA replication and are not caused by the presence of antibiotics. Mutations in genes leading to expression of efflux proteins can induce multiple antibiotic resistances (Fig. 2.1) (Hirsh *et al*., 2004; Quinn *et al*., 2011).

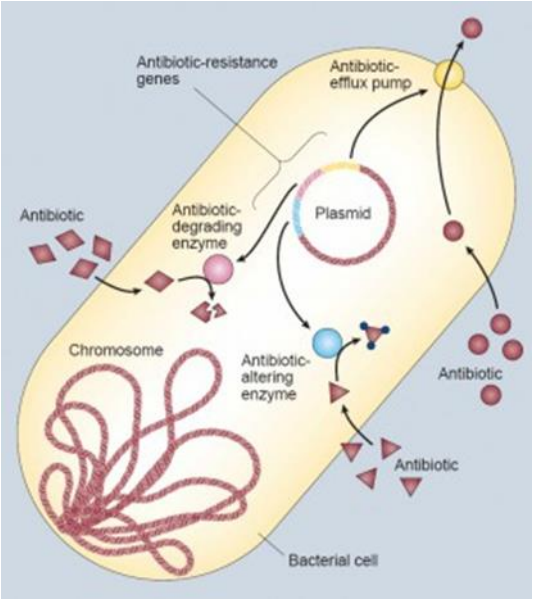

Fig. 2.1 Bacterial antimicrobial resistance mechanisms

The mechanism that has an important role in the development of antibiotic resistance is the exchange of transferable DNA structures, usually plasmids, operons and transposons. These mobile gene elements carrying resistance genes can be spread to other bacteria by different mechanisms (Fig. 2.2):

• Transduction – DNA incorporated in bacterial phages (virus that infects bacteria) and transferred between bacteria by phages.

• Conjugation – The transfer of plasmid or chromosomal DNA from donor to recipient through a sex pilus.

• Transformation – The ability of certain bacteria genera to pick up naked DNA from the environment.

• Transposition – Gene segments can change position within the bacterial genome.

• Integrons – Consists of an intergrase gene that can capture gene cassettes coding for antibiotic resistance.

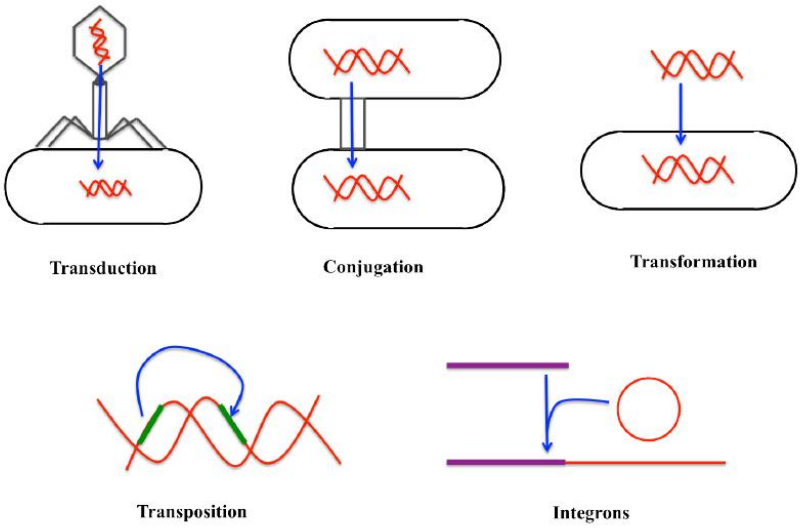

Fig. 2.2 Mobile gene elements (Fang, 2015)

1.1 Antimicrobial resistance in *Staphylococcus pseudintermedius*

Referring to *S. pseudintermedius*, in the past the isolates were generally susceptible to β-lactam antibiotics (MSSP), but, since 2006, MRSP has emerged as a significant health problem in pet animals. The first phenotypic MRSP strains were isolated in France in the mid-1980 from healthy dogs and dogs affected by pyoderma (Pellerin *et al*., 1998). In 1999, the first *mec*A positive strain was described as responsible of canine pyoderma in US (Gortel *et al*., 1999) and in Europe in 2005 (Loeffler *et al*., 2007). Compared with MRSA, the emergence of MRSP is of greater concern for veterinary patients as *S. pseudintermedius* is the primary staphylococcal species inhabiting healthy dogs and cats, where it can cause a great variety of infections. Moreover, MRSP has been reported with an increasing frequency (Loeffler *et al*., 2007; van Duijkeren *et al*., 2011; Kasai *et al*., 2016) and MRSP strains show often multidrug resistance profiles worldwide, including resistance to several classes of antimicrobial drugs (Perreten *et al*., 2010). This limits the treatment options and represents a relevant threat to small animal therapy in veterinary medicine, challenging infection control measures (van Duijkeren *et al*., 2011, Bannoehr and Guardabassi, 2012; Bond and Loeffler, 2012). In fact, there are several reports on isolates resistant almost to all antimicrobials authorized for use in veterinary medicine (Wettstein *et al*., 2008; Perreten *et al*., 2010) inducing veterinarians to use antimicrobials authorized for human medicine (Weese and van Duijkeren, 2010).

1.1.1 Methicillin resistance in *S. pseudintermedius*

In *Staphylococcus pseudintermedius*, as in MRSA, methicillin resistance is mediated by the *mec*A gene, which is conferred by the acquisition of one of the several staphylococcal cassette chromosome *mec* (SCC*mec*) elements (Weese and van Duijkeren, 2010; Cartwright *et al*., 2013). The SCC*mec* element can be transferred between different staphylococcal species (Wielders *et al*., 2001). Differently from MRSA, in MRSP only 5 SCCmec elements have been completely characterized. The different types of SCCmec elements found in MRSP are SCCmec II-III, SCCmec III, SCCmec IV, SCCmec V, SCCmec VII and other nontypeable cassettes (Perreten *et al.*, 2013; McCarthy *et al.*, 2015).

*Mec*A gene encodes a modified penicillin-binding protein (PBP2a) with low affinity for β-lactams, including cephalosporins. Normally, β-lactam antibiotics interact with the PBP of *S. pseudintermedius* to prevent cell wall construction. However, in MRSP, the antibiotics cannot inhibit the enzyme and fail to exercise its inhibitory effect on bacterial growth (van Duijkeren *et al*., 2011). Although methicillin is no longer used in clinical practice, the term "methicillin-resistant" has persisted and has been used to indicate strains that are resistant to all beta-lactams (Morris *et al.*, 2017). So, it still represents a marker for broad resistance to all β- lactams and an indicator of likely nosocomial epidemiology and additional multidrug resistance. Precisely, in most veterinary diagnostic laboratories the methicillin resistance is generally evaluated by phenotypic methods. Commonly, oxacillin or cefoxitin is used in place of the no more produced methicillin. However, in *S.*

pseudintermedius cefoxitin disc diffusion testing as screening test for methicillin resistance has been reported as a poorer predictor (Bemis *et al*., 2009). So, it appears to be inappropriate for its many false negative results and it is not recommended currently by the Clinical Laboratory Standards Institute (CLSI). Thus, oxacillin disc diffusion testing is the one to prefer and use. In particular, as suggested by CLSI, an oxacillin MIC breakpoint R ≥ 0.5 mg/L and disc diffusion breakpoint R ≤ 17 mm are highly correlated with the detection of *mec*A in *S. pseudintermedius* (van Duijkeren *et al*., 2011). In any case, the *gold standard*, or better, the most reliable test for the detection of *mec*A gene is PCR (Paterson *et al*., 2014). In recent years, another gene responsible for methicillin resistance has been described in *S. aureus*: *mec*A homologue termed mecC (formerly $mecA_{LGA251}$). Since its original description, it has been reported in *S. aureus* isolates of many countries (Paterson *et al.*, 2012; Cartwright *et al.*, 2013), but until now never described in *S. pseudintermedius* isolates.

As MRSA is considered to be a major nosocomial pathogen amongst humans worldwide (Graffunder and Venezia, 2002), MRSP embodies the corresponding threat amongst dogs in veterinary medicine. Indeed, many studies underline that hospitalization, frequent visits to veterinary practices, and prior antimicrobial administration are recognized risk factors for canine MRSP infection and carriage (Nienhoff *et al.*, 2011; Weese *et al*., 2012; Eckholm *et al*., 2013; Lehner *et al*., 2014; Grönthal *et al*., 2017). One reason could be the direct transmission between dogs in the veterinary clinics, since dogs can carry MRSP for at least six months after acquisition (Bergström *et al*., 2012). Another reason is the

possible risk of indirect transmission between dogs via environmental surfaces in hospitals.

1.1.2 Resistance to non-β-lactam antibiotics in *S. pseudintermedius*

It is well known that MRSP isolates are resistant to several of non-β-lactam antibiotics, showing multidrug resistance profiles. Characteristically, MRSP are more often multidrug-resistant strains (MDR) than MSSP. However, an increasing trend of resistance to the penicillinase-labile penicillins amongst MSSP has occurred in the last years (Moodley *et al*., 2014). The Clinical and Laboratory Standards Institute (CLSI) defined that multidrug resistance should exclusively be referred to acquired resistance properties. Particularly, MRSP, as MRSA, is defined as MDR when it is not susceptible to at least one agent in three or more antimicrobial categories (Magiorakos *et al*., 2012).

Multiresistance in *S. pseudintermedius* includes resistance to tetracyclines, macrolides, lincosamides, aminoglycosides, streptogramins, trimethoprim-sulfamethoxazole and fluoroquinolones, as reported worldwide (Perreten *et al.*, 2010; Weese and van Duijkeren, 2010; van Duijkeren *et al*., 2011; Cain, 2013). Currently, it is common to isolate MRSP strains that are susceptible to very few antimicrobials. Generally, they are susceptible only to amikacin, rifampicin, vancomycin and linezolid. This denotes a true therapeutic dilemma, due both to potential drug toxicities (amikacin and rifampicin) and

ethical use considerations (vancomycin and linezolid) (Morris *et al*. 2017).

Referring to tetracycline resistance, it is common in staphylococci and genotypically mediated by *tet* genes, whose acquisition is generally associated with transposons or plasmids. *Tet*K, *tet*L, *tet*M and *tet*O are the four major genes associated with tetracycline resistance amongst Gram-positive bacteria. *Tet*K and *tet*L genes encode for efflux proteins, which prevent the accumulation of tetracycline within the cell. *Tet*M and *tet*O encode for ribosomial protection proteins, reducing the affinity of tetracycline to the ribosome. The distribution of *tet* genes is different among Gram-positive cocci: *tet*L and *tet*O are generally detected in streptococci and enterococci, while *tet*K is often found alone or in combination with *tet*M in staphylococci (Bismuth *et al.*, 1990). Precisely, *tet*K gene is located in small plasmids (from 4.3 to 4.7 kbps). The prototype of these plasmids, called pT181, is commonly isolated in *S. aureus*. Plasmids closely related to pT181 have been identified in all staphylococcal species of animal origin (Schwarz *et al.*, 2000).

The *tet*M gene is located on conjugative transposons of enterococcal origin, such as the Tn916 or Tn1545 (Roberts, 1996).

The most commonly genes expressed by *S. pseudintermedius* are *tet*M and *tet*K. Generally, *S. pseudintermedius* strains which harbour only *tet*K maintain susceptibility to minocycline but not to other tetracyclines, while strains carrying *tet*M tend to exhibit cross resistance to minocycline and other tetracyclines (Youn *et al*., 2014; Morris *et al.*, 2017).

Staphylococcal resistance against erythromycin (class of macrolides) is commonly based on three mechanisms: modification of the target site, active efflux, enzymatic inactivation (Sutcliffe *et al*., 1996; Schwarz *et al*., 2000). The target modification, encoded by the *erm* genes, is the main resistance mechanism and occurs with the demethylation of an adenyl residue at the level of the 23S portion of the rRNA (Zou *et al.*, 2011). According to literature in animal and human staphylococci, resistance to erythromycin is due to *erm* genes. In staphylococci of animal origin *erm*A, *erm*B and *erm*C have been identified as responsible for resistance to erythromycin (Zou *et al.*, 2011) and generally present individually.

*Erm*A and *erm*B genes are commonly found in small transposons such as the Tn544 and the Tn917 / Tn551 (Werckenthin and Schwarz, 2001). *Erm*C gene is generally found in small multi-copy plasmids and this gene is predominant in staphylococcal isolates from both humans and animals, particularly in *S. hyicus* and other porcine staphylococci (de Vries *et al*., 2012).

In contrast, *erm*B gene is the most widespread in canine isolates of *Staphylococcus pseudintermedius* (Jensen *et al*., 1999; Boerlin *et al*., 2001; Lüthje and Schwarz, 2007; Youn *et al*., 2014).

Ben Zakour *et al*. (2012) identified four transposons harboring one or more antibiotic resistance genes in the genome of *S. pseudintermedius* ED99 (Table 2.1).

In contrast to the other two species of the SIG, *S. pseudintermedius* comes across an extensive antibiotic selective pressure, which has allowed the spread of mobile genetic elements encoding antibiotic

resistance. Notably, *S. pseudintermedius* strains, harboring antibiotic resistance determinants on their mobile genetic elements, act as a reservoir of resistance genes that could easily spread to human skin flora (Guardabassi *et al*., 2004).

Tab. 2.1 Mobile genetic elements associated with antibiotic resistance in S. pseudintermedius ED99 (Ben Zakour et al., 2012)

Name	**Resistance factors**	**Closest homologs**
Tn5801	*tet*M	*S. aureus*
Tn552	*bla* operon	*S. haemolyticus, S.epidermidis*
Tn554-like	*bla* operon	*S. aureus*
Tn5405	*aad*6-*sat*4-*aph*A-3, *erm*B	*Streptococcus sp., Enterococcus faecium*

2. Materials and Methods

2.1 Antimicrobial susceptibility testing

The antimicrobial susceptibility testing is a rapid and reliable technique, which is needed in order to reduce the risk of the spread of multidrug-resistant strains, often responsible for chronic infections. In fact, its results allow to choose the most appropriate therapeutic protocol for the treatment of the infection caused by a specific bacterial pathogen.

The importance of the antibiogram is based on the principle that *in vitro* susceptibility prefigures the *in vivo* efficacy of an antibiotic therapy. Minimal inhibitory concentration (MIC) is objectively determined by antimicrobial susceptibility testing. MIC is defined as the lowest concentration of an antibacterial agent, that inhibits the growth of a bacterial isolate (Quinn *et al*., 2011).

However, many phenotypic methods used in diagnostic laboratory, such as Kirby-Bauer disc diffusion method, give a semi-quantitative estimate of the MIC, which is obtained from two antibiotic concentration namely breakpoints. These breakpoints are established by the National Committee for Clinical Laboratory Standards (NCCLS), so that bacteria can be classified as susceptible (S), intermediate (I), or resistant (R) to specific antimicrobial agent.

In this study, the antimicrobial susceptibility testing was performed on 126 canine *S. pseudintermedius* isolates by using Kirby-Bauer disk diffusion method (Bauer *et al*., 1966) on Mueller-Hinton agar plates.

Kirby-Bauer disc diffusion method is the most used test, since it is a relatively simple and economical method, most suitable for testing rapidly aerobic bacteria. Herein, the bacterial suspension was prepared by selecting several morphologically similar colonies with a sterile cotton swab and homogenizing the colonies in a sterile saline solution (0.85% NaCl) (bioMérieux, Marcy L'Etoile, France) to the density of a McFarland 0.5 standard. Then, the bacterial sample was spread over the entire surface of Mueller-Hinton agar plate (Liofilchem, Teramo, Italy) and antimicrobial discs with specific amounts of antibiotics were placed on the inoculated surface plates. After incubation at 37°C for 24 hours, the diameter of the inhibition zone of each tested antibiotic was measured in millimeters and interpreted as susceptible (S), intermediate (I) or resistant (R) compared to international standards.

Based on Kirby-Bauer method, all the identified *S. pseudintermedius* strains, confirmed by the species-specific *nuc* and *hlb* PCR, respectively, were tested to the following 20 antibiotics: amoxicillin-clavulanate (AMC, 20/10 µg), ampicillin (AMP, 10 µg), oxacillin (OX, 1 µg), penicillin (P, 10 IU), cephalothin (KF, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), clindamycin (CD, 2 µg), ciprofloxacin (CIP, 30 µg), enrofloxacin (ENR, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), tobramycin (TOB, 10 µg), imipenem (IMI, 10 µg), linezolid (LNZ, 30 µg), sulfamethoxazole-trimethoprim (SXT, 1.25/23.75 µg), tetracycline (TE, 30 µg), vancomycin (VA, 30 µg). The isolates were classified as susceptible (S), intermediate (I) or resistant (R) according

to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2015). For streptomycin breakpoints employed were those recommended by the French Society for Microbiology (http://www.sfm-microbiologie.fr). The antibiotic classes chosen and tested in this study are reported in Table 2.2.

Tab. 2.2 Antibiotics and antibiotic classes tested for canine S. pseudintermedius isolates

Antibiotics	**µg**	**Antibiotics by class**
Amoxicillin + clavulanate (AUG)	30 (20/10)	Penicillin combination
Ampicillin (AMP)	10	Penicillins
Oxacillin (OX)	1	
Penicillin (P)	10 UI	
Cephalothin (KF)	30	Cephalosporins (first generation)
Cefoxitin (FOX)	30	Cephalosporins (second generation)
Ceftriaxone (CRO)	30	Cephalosporins (third generation)
Clindamycin (CD)	2	Lincosamides
Ciprofloxacin (CIP)	30	Fluoroquinolones
Enrofloxacin (ENR)	5	
Erythromycin (E)	15	Macrolides
Gentamicin (CN)	10	Aminoglycosides
Kanamycin (K)	30	
Streptomycin (S)	10	
Tobramycin (TOB)	10	
Imipenem (IMI)	10	Carbapenems
Linezolid (LNZ)	30	Oxazolidinones
Sulfamethoxazole-Trimethoprim (SXT)	25 (1,25/23,75)	Sulfonamides
Tetracyclin (TE)	30	Tetracyclines
Vancomycin (VA)	30	Glycopeptides

2.2 Genotypic characterization of antibiotic resistance

After bacterial DNA extraction from *S. pseudintermedius* strains as already described in chapter 1, genetic profiles of antibiotic resistance by PCR for *mec*A and *mec*C genes and multiplex PCR for *tet*K, M, L, O and *erm*A, B, C genes were systematically performed on all 126 *S. pseudintermedius* isolates.

2.2.1 Methicillin resistance determinants – molecular characterization of MRSP

Methicillin-resistant strains (MRSP) were assessed by single PCR for *mec*A (Chovanovà *et al*., 2015) and *mec*C (Stegger *et al*., 2012) genes. *S. aureus* ATCC® BAA44TM was used as *mec*A positive control.

Primers sequences, amplicon sizes and amplification programs are reported in Table 2.3.

The single PCR reaction mixture for each gene (*mec*A and *mec*C genes) was prepared by using the Green Hot Start PCR Master Mix Direct Load, 2x (Biotechrabbit, GmbH, Germany) as follows:

- Green Hot Start PCR Master Mix: 12,5 µL
- primers (F+R): 1 µL
- nuclease free water: 10,5 µL
- DNA template: 1 µL

The mixture final volume for one reaction was of 25 µL.

For every PCR reaction there was always a positive and a negative control. Genomic DNA was amplified using Biorad T100TM Thermo cycler (BioRad, Hercules, CA).

Tab. 2.3 Primers sequences, amplicon sizes, amplification programs of mec genes

Gene	Primer sequences (5'-3' sense and antisense)	Amplicon size (bp)	Amplification program
*mec*A	F: TCCACCCTCAAACAGGTGAA R: TGGAACTTGTTGAGCAGAGGT	139	94°C 5 min; 94°C 30 s, 55°C 40s, 72°C 30s, for 30 cycles; 72°C 5 min.
*mec*C	F: GAAAAAAAGGCTTAGAACGCCTC R: GAAGATCTTTTCCGTTTTCAGC	138	94°C 5 min; 94°C 30s, 59°C 60s,72°C 60s, for 30 cycles; 72°C 10 min

The amplified products were analyzed by gel electrophoresis and electrophoresed gels were visualized under blue-light and their images taken using the ChemiDocTM XRS+ with Image LabTM Software (BioRad, Hercules, CA), as previously described.

2.2.2 Genotypic characterization of tetracycline and erythromycin resistance

S. pseudintermedius strains showing phenotypic resistance to tetracycline were subjected to multiplex PCR to confirm, genotypically, the presence of the tetracycline resistance genes: *tet*K, *tet*M, *tet*L, *tet*O. Primers used for multiplex PCR were selected from primer sequences described by Ullah *et al*., (2012). The primer sequences used are shown

in Table 2.4. Moreover, single PCR were performed for each tetracycline resistance genes.

According to literature in staphylococci of animal origin *erm*A, *erm*B and *erm*C have been identified as responsible for resistance to erythromycin (Zou *et al*., 2011). So, *S. pseudintermedius* isolates phenotypically erythromycin-resistant were analyzed with multiplex PCR to detect the presence of these three genes. Primers used (Table 2.4) were chosen from published primer sequences (Sutcliffe *et al*., 1996; Zou *et al*., 2011). Furthermore, single PCR were performed for each erythromycin resistance genes.

The multiplex PCR reaction mixture for *tet*K, M, L, O genes was prepared by using the Green Hot Start PCR Master Mix Direct Load, 2x (Biotechrabbit, GmbH, Germany) as follows:

- Green Hot Start PCR Master Mix: 12,5 µL
- primers *tet*K (F+R): 1 µL
- primers *tet*M (F+R): 1 µL
-primers *tet*L (F+R): 1 µL
- primers *tet*O (F+R): 1 µL

} 4 µL

- nuclease free water: 7,5 µL
- DNA template: 1 µL

The mixture final volume for one reaction was of 25 µL.

The multiplex PCR reaction mixture for *erm*A, B, C genes was prepared by using the Green Hot Start PCR Master Mix Direct Load, 2x (Biotechrabbit, GmbH, Germany) as follows:

- Green Hot Start PCR Master Mix: 12,5 µL
- primers *erm*A (F+R): 1 µL
- primers *erm*B (F+R): 1 µL
-primers *erm*C (F+R): 1 µL

} 3 µL

- nuclease free water: 8,5 µL
- DNA template: 1 µL

The mixture final volume for one reaction was of 25 µL.

For every multiplex PCR reaction there was always a positive and a negative control. Genomic DNA was amplified using Biorad T100TM Thermo cycler (BioRad, Hercules, CA).

Tab. 2.4 Primers sequences, amplicon sizes, amplification programs of tet and erm genes

Gene	Primers sequences (5'-3' sense and antisense)	Amplicon size (bp)	Amplification programs
*tet*K	F: GTAGCGACAATAGGTAATAGT	360 bp	
	R: GTAGTGACAATAAACCTCCTA		
*tet*M	F: AGTTTTAGCTCATGTTGATG	1862 bp	94°C 15s;
	R: TCCGACTATTTAGACGACGG		94°C 1min, 52°C
*tet*L	F: ATAAATTGTTTCGGGTCGGTAAT	1077 bp	1min, 72°C 90s,
	R: AACCAGCCAACTAATGACAAGAT		for 30 cycles;
*tet*O	F: AACTTAGGCATTCTGGCTCAC	515 bp	72°C 5 min
	R: TCCCACTGTTCCATATCGTCA		
*erm*A	F: TCTAAAAGCATGTAAAAGAA	645 bp	
	R: CTTCGATAGTTTATTAATATTAGT		94°C 2min;
*erm*B	F: GAAAAGGTACTCAACCAAATA	639 bp	94°C 1min, 55°C
	R: AGTAACGGTACTTAAATTGTTTAC		1min, 72°C 90s,
*erm*C	F: TCAAAACATAATATAGATAAA	642 bp	for 30 cycles;
	R: GCTAATATTGTTTAAATCGTCAAT		72°C 5 min

The amplified products were analyzed by gel electrophoresis and Electrophoresed gels were visualized under blue-light and their images taken using the ChemiDocTM XRS+ with Image LabTM Software (BioRad, Hercules, CA), as previously described (Chapter 1).

3. Results

3.1 Phenotypic and genotypic characterization of MRSP antibiotic resistance profiles

Amongst the 126 *S. pseudintermedius* strains collected from dogs suffering from otitis externa or pyoderma during the years 2015-2017, 23 strains (18%) were MRSP, being positive to *mec*A gene. The remaining 103 strains (82%) were classified as MSSP. None of the isolates carried *mec*C gene.

The antimicrobial susceptibility results of the 23 MRSP, obtained by Kirby-Bauer disk diffusion testing on Mueller-Hinton agar (Liofilchem, Teramo, Italy), showed a complete resistance to amoxicillin-clavulanate and ampicillin (100%), whilst the highest resistance rates to selected non-β-lactam antibiotics were: erythromycin (91%); tetracycline (87%); sulfamethoxazole/trimethoprim (78%); kanamycin (78%); streptomycin (78%); clindamycin (65%) and enrofloxacin (61%). However, no resistance was observed to vancomycin and linezolid. Furthermore, 91% (n=21) MRSP strains were found to be multidrug-resistant, showing resistance to at least 3 different antibiotic classes (Table 2.5).

The 20 (87%) phenotypic tetracycline-resistant MRSP strains harbored *tet*K and *tet*M genes, alone or in association. Precisely, 2/20 (10%) tetracycline-resistant strains carried *tet*K and *tet*M together, 6/20 (30%) strains carried only *tet*K gene, while 12/20 (60%) harbored *tet*M gene alone.

Erythromycin resistance gene *erm*B was found positive in all the 21 (91%) MRSP isolates, which were phenotypically erythromycin-resistant.

The distribution of *tet*K and *tet*M genes, *erm*B gene among MRSP and MSSP is described in Table 2.7.

Tab. 2.5 Antibiotic resistance profiles of canine MRSP

MRSP	Biological source	AMC	AMP	FOX	KF	CRO	CD	CIP	ENR	E	CN	K	IMI	LNZ	OX	P	S	SXT	TE	TOB	VA
4	otitis externa	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	S	**R**	S	S	**R**	**R**	**R**	**R**	**R**	S	S
22	otitis externa	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
70	otitis externa	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	**R**	S	S	S	**R**	**R**	**R**	**R**	S	S	S
75	pyoderma	**R**	**R**	S	S	S	S	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	**R**	**R**	**R**	S	S
94	otitis externa	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
102	otitis externa	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
115	pyoderma	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**	S
147	otitis externa	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
155	otitis externa	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	**R**	S	**R**	S	S
166	otitis externa	**R**	**R**	S	S	S	S	S	S	S	**R**	**R**	S	S	**R**	**R**	**R**	**R**	**R**	S	S
169	otitis externa	**R**	**R**	**R**	S	S	**R**	**R**	S	**R**	S	**R**	S	S	**R**	**R**	**R**	S	**R**	S	S
171	pyoderma	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	**R**	S
183	otitis externa	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	S	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
184	pyoderma	**R**	**R**	S	S	S	S	S	S	**R**	S	S	**R**	S	**R**	**R**	S	**R**	S	S	S
199	otitis externa	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
218	otitis externa	**R**	**R**	S	S	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
224	otitis externa	**R**	**R**	S	S	S	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	S	**R**	S	S
227	otitis externa	**R**	**R**	S	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	**R**	S	S
239	otitis externa	**R**	**R**	S	S	S	S	S	S	**R**	S	S	S	S	**R**	**R**	S	S	**R**	S	S
246	otitis externa	**R**	**R**	**R**	S	S	S	S	S	S	S	S	S	S	**R**	**R**	S	S	**R**	S	S
250	otitis externa	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	S	**R**	**R**	S	S
251	otitis externa	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	S	S	**R**	**R**	S	**R**	**R**	S	S
258	otitis externa	**R**	**R**	S	S	S	**R**	S	S	**R**	**R**	**R**	S	S	**R**	**R**	**R**	**R**	S	**R**	S
N°R		23	23	14	8	11	15	12	14	21	12	18	12	0	23	23	18	18	20	3	0

**R: resistant **S: susceptible*

3.2 Phenotypic and genotypic characterization of MSSP antibiotic resistance profiles.

The 103 MSSP (strains negative for both *mec*A and *mec*C genes) isolates, showed interesting antibiotic resistant profiles, but their resistance rates were of about 50% lower than MRSP strains to the same antibiotics, confirming the trends described worldwide.

Precisely, the isolated MSSP showed, as predictable, high resistance rates to penicillin (77%), ampicillin (76%) and amoxicillin-clavulanate (56%). Among non-β-lactam antibiotics the most frequently found resistance phenotypes were tetracycline (50%), streptomycin (46%), kanamycin (44%), erythromycin (37%), sulfamethoxazole-trimethoprim (35%). Also, for MSSP no resistance was observed for vancomycin and linezolid. Multidrug resistance was detected in 34% of MSSP (35/103) and the most prevalent association included amoxicillin-clavulanate, ampicillin, penicillin, tetracycline, erythromycin, kanamycin, streptomycin and sulfamethoxazole-trimethoprim. 10% of MSSP isolates (11/103) were susceptible to all tested antibiotics.

The resistance rates to the 20 tested antibiotics of both MRSP and MSSP are summarized in Table 2.6 and in Fig. 2.3.

The 52 (50%) phenotypic tetracycline-resistant MSSP strains harbored *tet*K and *tet*M genes, alone or together. Precisely, 21% (11/52) of tetracycline-resistant MSSP carried *tet*K and *tet*M together, 33% (17/52) of the strains carried only *tet*K gene, while 46% (24/52) of MSSP isolates carried *tet*M gene alone.

The multiplex PCR for *erm* genes detected in 37% (38/103) phenotypic erythromycin-resistant MSSP the presence of *erm*B gene.

The distribution of *tet*K, *tet*M, *erm*B genes among both MRSP and MSSP is reported in Table 2.7.

Tab. 2.6 Antibiotic resistance rates of MRSP and MSSP isolates of canine origin

Antibiotics	µg	MRSP % resistance	MSSP % resistance	Antibiotics by class
Amoxicillin + clavulanate (AUG)	30 (20/10)	100	56	Penicillin combination
Ampicillin (AMP)	10	100	76	Penicillins
Oxacillin (OX)	1	100	0	
Penicillin (P)	10 UI	100	77	
Cephalothin (KF)	30	35	6	Cephalosporins (first generation)
Cefoxitin (FOX)	30	61	0	Cephalosporins (second generation)
Ceftriaxone (CRO)	30	48	12	Cephalosporins (third generation)
Clindamycin (CD)	2	58	33	Lincosamides
Ciprofloxacin (CIP)	30	52	9	Fluoroquinolones
Enrofloxacin (ENR)	5	61	16	
Erythromycin (E)	15	91	37	Macrolides
Gentamicin (CN)	10	52	10	Aminoglycosides
Kanamycin (K)	30	78	44	
Streptomycin (S)	10	78	46	
Tobramycin (TOB)	10	13	4	
Imipenem (IMI)	10	52	36	Carbapenems
Linezolid (LNZ)	30	0	0	Oxazolidinones
Sulfamethoxazole-Trimethoprim (SXT)	25 (1,25/23,75)	78	35	Sulfonamides
Tetracycline (TE)	30	87	50	Tetracyclines
Vancomycin (VA)	30	0	0	Glycopeptides

Tab. 2.7 Molecular profiles of tetracycline and erythromycin resistance in canine S. pseudintermedius isolates

Molecular profiles of tetracycline and erythromycin resistance	**MRSP (23 strains)**	**MSSP (103 strains)**
*tet*K gene	6 (26%)	17 (16%)
*tet*M gene	12 (52%)	24 (23%)
*tet*K and *tet*M genes	2 (9%)	11 (11%)
e*rm*B gene	21 (91%)	38 (37%)

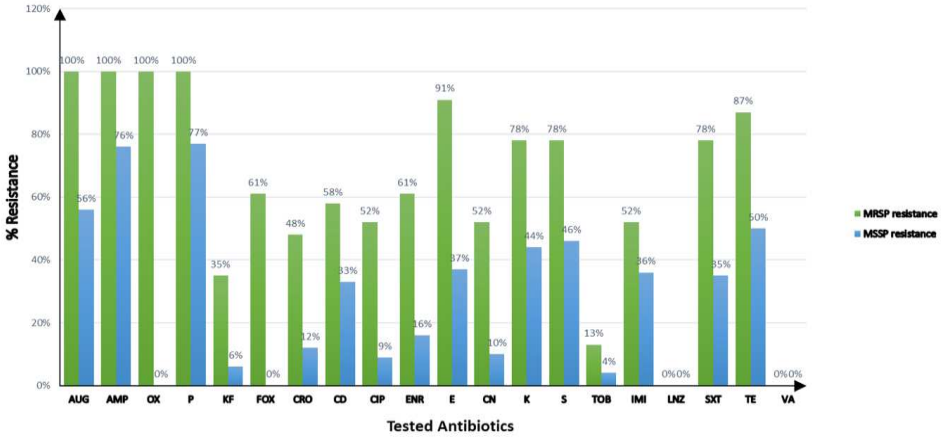

Fig. 2.3 Antibiotic resistance rates of MRSP and MSSP to the 20 tested antibiotics

4. Discussion and conclusions

Antibiotic resistance is the most puzzling question in recent years and the spread of multidrug resistant Staphylococci of animal origin, principally MRSP strains, have assumed new public health relevance (Deurenberg *et al.*, 2007; Corrente *et al*., 2013). This is due to their resistance not only to all β-lactams, but also to a wide range of other antibiotics with a limited susceptibility sometimes only to vancomycin and linezolid, considered as 'last resort antibiotics'. In fact, their use in veterinary medicine appears questionable, since they are used for treatment of methicillin-resistant and multidrug-resistant staphylococci infections in human medicine (Stefani and Varaldo, 2003; Perreten *et al*., 2010; Vysakh and Jeya, 2013).

In this context, the emergence in dogs of MRSP, often associated with even broader drug-resistance, has become a great veterinary challenge.

This study confirms that *S. pseudintermedius* is the main causative staphylococcal agent of canine otitis externa and pyoderma. Precisely, among the 126 isolated *S. pseudintermedius* strains, 18% (23/126) were MRSP presenting almost all worrying multidrug-resistant profiles. Besides β-lactam antibiotics, the MDR profile of MRSP strains showed relevant resistance rates to all classes of antibiotics approved in veterinary medicine and used for systemic treatment in dogs (tetracycline, aminoglycosides, macrolides, sulfamethoxazole-trimethoprim, lincosamides), confirming the multidrug resistance trend reported for MRSP worldwide (Osland *et al*., 2012; Haenni *et al*.; 2014; Moodley *et al*., 2014; Stefanetti *et al*., 2017).

It is known that MRSP originates from an animal reservoir, consequently pet animals might act as potential reservoir for the emergence of novel methicillin-resistant clones in human beings. Even though infection in humans with MRSP happens rarely, the potential transfer of antimicrobial resistance genes from MRSP to other staphylococci, such as *S. aureus*, might be possible. Furthermore, in last years it has been reported an increase of the zoonotic transmission of MRSP, probably due to a more appropriate identification of this strain, even though it still remains limited. The veterinary environments (hospitals, clinics) seem to play an important role in the dissemination of MRSP between pet animals and humans, particularly people who have a constant contact with pets (veterinary personnel and pet owners) (Paul *et al*., 2011; van Duijkeren *et al*., 2011). However, human colonization with MRSP appears to be unusual and transient, as reported for methicillin-susceptible *S. intermedius*. So human acquisition might be transiently related to increased colonization pressure or antibiotic exposure when they interact, especially for long time, with pets acutely ill (Wang *et al*., 2013). Veterinarians and owners of infected pets are exposed to a higher risk of being MRSP positive. Despite there are few reports of veterinarians, particularly pet animal dermatologists, colonized by MRSP, it could be considered as an occupational risk (Paul *et al*., 2011).

In this study, interesting antibiotic resistance profiles were also showed by MSSP isolated strains.

Although their multiresistance pattern was lower than MRSP, the studied MSSP strains displayed a significant increasing trend of resistance compared to the data of some years ago (Ganiere *et al*., 2005; Norström *et al*., 2009). Moreover, the reported increasing trend is also confirmed by more recent studies of other European countries (Haenni *et al*. 2014; Moodley *et al*., 2014).

The MSSP strains showed high resistance rates to antibiotics commonly used to treat canine infections, such as the penicillinase-labile penicillins, tetracycline, aminoglycosides, macrolides and sulfamethoxazole-trimethoprim. It is worth noting that 34% of MSSP were multidrug-resistant strains (resistant to ≥ 3 different antibiotic classes), whilst only 10% of MSSP isolates were susceptible to all tested antibiotics.

None of both MRSP and MSSP isolates showed resistance to vancomycin and linezolid, which use in veterinary medicine is disputed and in many authors' opinion they should be strictly reserved for use in human beings, since they represent the last chance antibiotics to treat severe infections caused by methicillin-resistant staphylococci.

Furthermore, in accordance with literature, this study shows that *tet*K and *tet*M genes were the most prevalent tetracycline resistance determinants in both MRSP and MSSP (Schmitz *et al*., 2001, Youn *et al*., 2014). Among the three macrolide resistance genes, all the erythromycin-resistant strains harbored only *erm*B gene, responsible

for erythromycin resistance in canine *S. pseudintermedius* strains (Boerlin *et al.*, 2001; Lüthje and Schwarz, 2007; Youn *et al*., 2014).

Even though MRSP are generally more resistant than MSSP and their high prevalence of multidrug resistance is probably related to the dissemination of dominant clones (for instance MRSP belonging to the clonal lineage ST71), according to literature the obtained results underline an increasing resistance in MSSP (Moodley *et al*., 2014). Therefore, the spread of multidrug-resistant MSSP should be monitored and their pathogenic role, related to antibiotic resistance, deserves further studies.

Since the emergence and dissemination of methicillin-resistant staphylococci has posed a major challenge in the treatment of staphylococcal infections, when antibiotics are prescribed to small animals, it is important to remember that most of antibiotics are used also in human medicine, either as identical or related molecules, so they should be prescribed and administered wisely and whenever possible supported by antimicrobial susceptibility testing. Current EU Summary of Products Characteristics guidance for the responsible use of antimicrobials in veterinary medicine recommends a restricted use of fluoroquinolones, third-and fourth-generation cephalosporins to clinical cases always supported by antibiogram, because they are defined critically important antimicrobials by WHO (Pomba *et al*., 2017; Loeffler *et al*., 2018).

In conclusion, the increasing spread of multidrug-resistant MRSP and MSSP strains and the lack of effective, new, conventional antibiotics highlight the limited therapeutic possibilities and the need of new treatment approaches, such as antimicrobial peptides, to prevent and control staphylococcal infections.

5. References

Bannoehr J, Guardabassi L, 2012. *Staphylococcus pseudintermedius* in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. Vet Dermatol **23**, 253-266.

Bauer AW, Kirby WM, Sherris JC, Turck M, 1966. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol, **45**, 493-496.

Bemis DA, Jones RD, Frank LA, Kania SA, 2009. Evaluation of susceptibility test breakpoints used to predict *mec*A-mediated resistance in *Staphylococcus pseudintermedius* isolated from dogs. J Vet Diagn Invest **21**, 53-58.

Ben Zakour NL, Beatson SA, van den Broek AHM, Thoday KL, Fitzgerald JR, 2012. Comparative genomics of the *Staphylococcus intermedius* group of animal pathogens. Front Cell Infect Microbiol **2**, 44.

Bergström A, Gustafsson C, Leander M, Fredriksson M, Grönlund U, Trowald-Wigh G, 2012. Occurrence of methicillin-resistant Staphylococci in surgically treated dogs and the environment in a Swedish animal hospital. J Small Anim Practice **53**, 404-410.

Bismuth R, Zilhao R, Sakamoto H, Guesdon JL, Courvalin P, 1990. Gene heterogeneity for tetracycline resistance in *Staphylococcus* spp. Antimicrob Agents Chemother,1611-1614.

Boerlin P, Burnens AP, Frey J, Kuhnert P, Nicolet J, 2001. Molecular

epidemiology and genetic linkage of macrolide and aminoglycoside resistance in Staphylococcus intermedius of canine origin. Vet Microbiol **79**, 155-169.

Bond R, Loeffler A, 2012. What's happened to *Staphylococcus intermedius*? Taxonomic revision and emergence of multi-drug resistance. J Small Anim Pract **53**, 147-154.

Cain CL, 2013. Antimicrobial resistance in staphylococci in small animals. Vet Clin North Am Small Anim **43**, 19-40.

Cartwright E JP, Paterson GK, Raven K E, Harrison EM, Gouliouris T, Kearns A, Pichon B, Edwards G, Skov RL, Larsen AR, Holmes MA, Parkhill J, Peacock SJ, Török ME, 2013. Use of Vitek 2 antimicrobial susceptibility profile to identify *mec*C in methicillin-resistant *Staphylococcus aureus*. J Clin Microbiol **51**, 2732-2734.

Chovanová R, Mikulášová M, Vaverková S, 2016. Modulation of *mec*A gene expression byessential oil from *Salvia sclarea* and synergism with oxacillin in methicillin resistant *Staphylococcus epidermidis* carrying different types of Staphylococcal chromosomal cassette *mec.* Inter J Microbiol, doi.10.1155/2016/6475837.

Clinical and Laboratory Standards Institute (CLSI) 2015. Performance Standard for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 3rd Edition CLSI VET01S (Wayne, Pennsylvannia, Clinical Laboratory and Standards Institute), 128, 2015.

Corrente M, Ventrella G, Greco MF, Martella V, Parisi A, Buonavoglia

D, 2013. Characterisation of a catalse-negative methicillin-resistant *Staphylococcus aureus* isolate from a dog. Vet Microbiol **167**, 734-736.

de Vries LE, Christensen H, Agersø, Y, 2012. The diversity of inducible and constitutively expressed *erm*(C) genes and association to different replicon types in staphylococci plasmids. Mob Genet Elements **2**, 72-80.

Deurenberg RH, Vink C, Kalenic S, Friedrich AW, Bruggeman AC, Stobberingh EE, 2007. The molecular evolution of methicillin-resistant of *Staphylococcus aureus*. Clin Microbiol Infect **13**, 222-235.

Eckholm NG, Outerbridge CA, White SD, Sykes JE, 2013. Prevalence and risk factors for isolation of methicillin-resistant *Staphylococcus spp*. from dogs with pyoderma in northern California, USA. Vet Dermatol **24**, 154-161.

Fang Y, 2015. A literature study on methicillin-resistant *Staphylococcus pseudintermedius* (MRSP): classification, resistance mechanisms, risk factors and clonal distribution. Web source.

Ganiere JP, Medaille C, Mangion C, 2005. Antimicrobial drug susceptibility of *Staphylococcus intermedius* clinical isolates from canine pyoderma. J Vet Med B Infect Dis Vet Public Health **52**, 25-31.

Gortel K, Campell KL, Kakoma I, Whittem T, Schaeffer DJ, Weisiger RM, 1999. Methicillin resistance among staphylococci isolated from dogs. Am J Vet Res **60**, 1526-1530.

Graffunder EM, Venezia RA, 2002. Risk factors associated with

nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infection including previous use of antimicrobials. J Antimicrob Chemother **49**, 999-1005.

Grönthal T, Eklund M, Thomson K, Piiparinen H, Sironen T, Rantala M, 2017. Antimicrobial resistance in *Staphylococcus pseudintermedius* and the molecular epidemiology of methicillin-resistant *S. pseudintermedius* in small animals in Finland. J Antimicrob Chemother **72**, 1021-1030.

Guardabassi L, Schwarz S, Lloyd DH, 2004. Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother **54**, 321-332.

Haenni M, de Moraes NA, Châtre P, Médaille C, 2014. Characterisation of clinical canine methicillin-resistant and methicillin-susceptible *Staphylococcus pseudintermedius* in France. J Glob Antimicrob Res **2**, 119-123.

Hirsh DC, MacLachlan NJ, Walker R.L, 2004. Antimicrobial drugs: a strategy for rational use and the ramifications of misuse. Veterinary Microbiology 2nd edition, Wiley.

Jensen LB, Frimodt-Moller N, Aarestrup FM, 1999. Presence of erm gene classes in gram-positive bacteria of animal and human origin in Denmark. FEMS Microbiol Letters **170**, 151-158.

Kasai T, Saesuga S, Shirai M, Murakami M, Kato Y, 2016. New categories designated as healthcare-associated and community-associated methicillin-resistant *Staphylococcus pseudintermedius*. Microbiol Immunol **60**, 540-551.

Lehner G, Linek M, Bond R, Lloyd DH, Prenger-Berninghoff E, Thom

N, Straube I, Verheyen K, Loeffler A, 2014. Case–control risk factor study of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) infection in dogs and cats in Germany. Vet Microbiol **168**, 154-160.

Loeffler A, Linek M, Moodley A, Guardabassi L, Sung JM, Winkler M, Weiss R, Lloyd DH, 2007. First report of multiresistant, *mec*A-positive *Staphylococcus intermedius* in Europe: 12 cases from a veterinary dermatology referral clinic in Germany. Vet Dermatol **18**, 412-421.

Loeffler A, Llyod DH, 2018. What has changed in canine pyoderma. A narrative review. Vet J **235**, 73-82.

Lüthje P, Schwarz S, 2007. Molecular basis of resistance to macrolides and lincosamides among *staphylococci* and *streptococci* from various animal sources collected in the resistance monitoring program BfT-GermVet. Inter J Antimicrob Agents **29**, 528-535.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL, 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect **18**, 268-281.

McCarthy AJ, Harrison EM, Stanczak-Mrozek K, Leggett B, Waller A, Holmes MA, Lloyd DH, Lindsay JA, Loeffler A, 2015. Genomic insights into the rapid emergence and evolution of MDR in

Staphylococcus pseudintermedius. J Antimicrob Chemother **70**, 997-1007.

Moodley A, Damborg P, Nielsen SS, 2014. Antimicrobial resistance in methicillin susceptible and methicillin resistant *Staphylococcus pseudintermedius* of canine origin: Literature review from 1980 to 2013. Vet Microbiol **171**, 337-341.

Morris DO, Loeffler A, Meghan FD, Guardabassi L, Weese JS 2017. Recommendations for approaches to meticillin-resistant staphylococcal infections of small animals: diagnosis, therapeutic considerations and preventative measures. Vet Dermatol **28**, 304-e69.

Nienhoff U, Kadlec K, Chaberny IF, Verspohl J, Gerlach GF, Kreienbrock L, Schwarz S, Simon D, Nolte I, 2011. Methicillin-resistant *Staphylococcus pseudintermedius* among dogs admitted to a small animal hospital. Vet Microbiol **150**, 191-197.

Norström M, Sunde M, Tharaldsen H, Mørk T, Bergsjø B, Kruse H, 2009. Antimicrobial resistance in *Staphylococcus pseudintermedius* in the Norwegian dog population. Microb Drug Resist **15**, 55-59.

Osland AM, Vestby LK, Fanuelsen H, Slettemeås JS, Sunde M, 2012. Clonal diversity and biofilm-forming ability of methicillin-resistant *Staphylococcus pseudintermedius*. J Antimicrob Chemother **67**, 841-848.

Paterson GK, Larsen AR, Robb A, Edwards GE, Pennycott TW, Foster G, Mot D, Hermans K, Baert K, Peacock SJ, Parkhill J, Zadoks RN, Holmes MA 2012. The newly described *mec*A homologue, *mec*$A_{LGA251,}$ is present in methicillin-resistant Staphylococcus aureus

isolates from a diverse range of host species. J Antimicrob Chemother **67**, 2809-2813.

Paterson GK, Harrison EM, Holmes MA, 2014. The emergence of *mec*C methicillin-resistant *Staphylococcus aureus*. Trends Microbiol **22**, 42-47.

Paul NC, Moodley A, Ghibaudo G, Guardabassi L, 2011. Carriage of methicillin-resistant *Staphylococcus pseudintermedius* in small animal veterinarians: Indirect evidence of zoonotic transmission. Zoonoses and Public Health **58**, 533-539.

Pellerin JL, Bordeau P., Sebbag H., Person JM, 1998. Epidemiosurveillance of antimicrobial compound resistance of *Staphylococcus intermedius* clinical isolates from canine pyodermas. Comp Immunol Microbiol Infect Dis **21**, 115-133.

Perreten V, Kadlec K, Schwarz S, Grönlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerald JR, Rossano A, Guardabassi L, 2010. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in Europe and North America: an international multicenter study. J Antimicrob Chemother **65**, 1145-1154.

Perreten V, Chanchaithong P, Prapasarakul N, Rossano A, Blum SE, Elad D, Schwendener S, 2013. Novel pseudo-staphylococcal cassette chromosome *mec* element (*ΨSCCmec57395*) in methicillin-resistant *Staphylococcus pseudintermedius* CC45. Antimicrob Agents Chemother **57**, 5509-5515.

Pomba C, Rantala M, Greko C, Baptiste KE, Catry B, van Duijkeren E,

Mateus A, Moreno MA, Pyörälä S, Ružauskas M, Sanders P, Teale C, Threlfall EJ, Kunsagi Z, Torren-Edo J, Jukes H, Törneke K, 2017. Public health risk of antimicrobial resistance transfer from companion animals. J Antimicrob Chemother **72**, 957-968.

Quinn PJ, Markey B K, Leonard FC, Hartigan P, Fanning S, FitzPatrick ES, 2011. Cultivation, preservation, and inactivation of bacteria, bacterial genetics, mechanisms of genetic variation and gene databases, laboratory diagnosis of bacterial disease, antibacterial agents, antibacterial resistance. Veterinary Microbiology and Microbial Disease. 2nd edition. John Wiley & Sons, 123-157.

Roberts MC, 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol **19**, 1-24.

Schmitz FJ, Krey A, Sadurski R, Verhoef J, Milatovic D, Fluit AC, European SENTRY Participants, 2001. Resistance to tetracycline and distribution of tetracycline genes in European *Staphylococcus aureus* isolates. J Antimicrob Chemother **47**, 239-240.

Schwarz S, Cardoso M, Wegener HC, 2000. Nucleotide sequence and phylogeny of the tet(L) tetracycline resistance determinant encoded by plasmid pSTE1 from *Staphylococcus hyicus.* Antimicrob Agents Chemother **36**, 580-588.

Stefanetti V, Bietta A, Pascucci L, Marenzoni ML, Coletti M, Franciosini MP, Passamonti F, Casagrande Proietti P, 2017. Investigation of the antibiotic resistance and biofilm formation of *Staphylococcus pseudintermedius* strains isolated from canine pyoderma. Vet Ital **53**, 289-296.

Stefani S, Varaldo PE, 2003. Epidemiology of methicillin resistant-staphylococci in Europe. Clin Microbiol Infect **9**, 1179-1186.

Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR, 2012. Rapid detection, differentiation and typing of methicillin-resistant *Staphylococcus aureus* harbouring either mecA or the new *mec*A homologue *mec*A_{LGA251}. Clin Microbiol Infect **18**, 395-400.

Sutcliffe J, Tait-Kamradt A, Wondrack L, 1996. *Streptococcus pneumoniae and Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux System. Antimicrob Agents Chemother **40**, 1817-1824.

Ullah F, Malik SA, Ahmed J, Ullah F, Shah SM, Ayaz M, Hussain S, Khatoon L, 2012. Investigation of the genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. Trop J Pharm Res **11**, 925-931.

van Duijkeren E, Catry B, Greko C, Moreno MA, Pomba MC, Pyörälä S, Ruzauskas M, Sanders S, Threlfall EJ, Torren-Edo J, Törneke K [Scientific Advisory Group on Antimicrobials (SAGAM)], 2011. Review on methicillin-resistant Staphylococcus pseudintermedius. J Antimicrob Chemother **66**, 2705-2714.

van Duijkeren E, Kamphuis MIC, Mije V, Laarhoven LM, Duim B, Wagenaar JA, Houwers DJ, 2011. *Staphylococcus pseudintermedius* between infected dogs and cats and contact pets, humans and the environment in households and veterinary clinics. Vet Microbiol **150**, 338-343.

Vysakh PR, Jeya M, 2013. A comparative analysis of community

acquired and hospital acquired methicillin resistant *Staphylococcus aureus*. J Clin Diagn Res **7**, 1339-1342.

Wang N, Neilan AM, Klompas M, 2013. Staphylococcus intermedius infections: case report and literature review. Infect Dis Reports, 5, e3.

Weese JS, van Duijkeren E, 2010. Methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* in veterinary medicine. Vet Microbiol **140**, 418-429.

Weese JS, Faires MC, Frank LA, Reynolds LM, Battisti A, 2012. Factors associated with methicillin-resistant versus methicillin-susceptible *Staphylococcus pseudintermedius* infection in dogs. J Am Vet Med Assoc **240**, 1450-1455.

Wettstein K, Descloux S, Rossano A, Perreten V, 2008. Emergence of methicillin-resistant *Staphylococcus pseudintermedius* in Switzerland: three cases of urinary tract infections in cats. Schweiz Arch Tierheilkd **150**, 339-343.

Werckenthin C, Cardoso M, Martel JL, Schwarz S, 2001. Antimicrobial resistance in Staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. Vet Res **32**, 341-362.

Wielders CL, Vriens MR, Brisse S, de Graaf-Milternburg LA, Troelstra A, Fleer A, Schmitz FJ, Verhoef J, Fluit AC, 2001. *In vivo* transfer of *mec*A DNA to *Staphylococcus aureus*. Lancet **357**, 1674-1675.

WHO, 2014. Antimicrobial resistance http://www.who.int/mediacentre/factsheets/fs194/en/

Youn JH, Park YH, Hang'ombe B, Sugimoto C, 2014. Prevalence and characterization of *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from companion animals and environment in the veterinary teaching hospital in Zambia, Africa. Comp Immunol Microbiol Infect Dis **37**, 123-130.

Zou LK, Wang HN, Zeng B, Li JN, Li XT, Zhang AY, Zhou YS, Yang X, Xu CW, Xia QQ, 2011. Erythromycin resistance and virulence genes in Enterococcus feacalis from swine in China. New Microbiol **34**, 73-80.

Chapter 3

Multilocus sequence typing for characterization of MRSP strains

The discovery of new sequence types in Southern Italy

1. Introduction

Multilocus sequence typing (MLST) is a nucleotide sequence-based method, which defines strains from the sequences of internal fragments of 7 housekeeping genes, in order to define a sequence type (ST) (Aanensen and Spratt, 2005). New sequence types are assigned when a new allelic profile is obtained. The 7 loci for *S. pseudintermedius* MLST are: *tuf*, *cpn*60 (*hps*60), *pta*, *pur*A, *fdh*, *ack*, *sar* (Solyman *et al*., 2013). The sequencing products (around 450-500 bp in size) are then analyzed thanks to the available database at the MLST Web-site (https://pubmlst.org/spseudintermedius/), in order to obtain an allele number for each gene. The combination of the seven allele numbers gives an allelic profile of the strains, defined sequence type (ST) (Aanensen and Spratt, 2005). Even though MLST is a very expensive technique, it is the best method for genetic screening (Deurenberg and Stobberingh, 2008) and for a reliable discrimination of clinical isolates of *S. pseudintermedius*, which has a considerable genetic diversity within the species (Fitzgerald, 2009). So, from this point of view, MLST appears to be the most effective method used to classify bacteria into clonal lineages and provide information about their relatedness, giving the possibility to define a specific bacterial population structure and its long-term epidemiological trends worldwide. Referring to the distribution of the main MRSP lineages, ST71, previously described as the epidemic European clone, is now widespread worldwide; while ST68, previously described as the epidemic North American clone, is now frequently reported also in Europe (Perreten *et al*., 2010) (Fig. 3.1). Furthermore, ST45, ST258, ST261, ST112, ST265, ST169, ST181

clones have been reported in many countries (Pires dos Santos *et al*., 2016). Recently, the newly ST497 and ST496, identified for the first time in Australia, have been reported (Worthing *et al.,* 2018), confirming the high genetic variability of this species.

The first species-specific MLST scheme was launched in 2013 (Solyman *et al*., 2013), and its publicly available database (http://pubmlst.org/spseudintermedius/) contains records of 1173 sequence types (STs) at present. Since 2007, numerous scientific papers have been reported MLST data on MRSP carriage and infection in a variety of countries and continents.

Web of Science is searched systematically for articles reporting data on multilocus sequence typing (MLST) of *S. pseudintermedius* isolates from dogs or other animal or human patients and carriers. The results of this study regarding the finding of new clones have been added to data of the MLST database for this species.

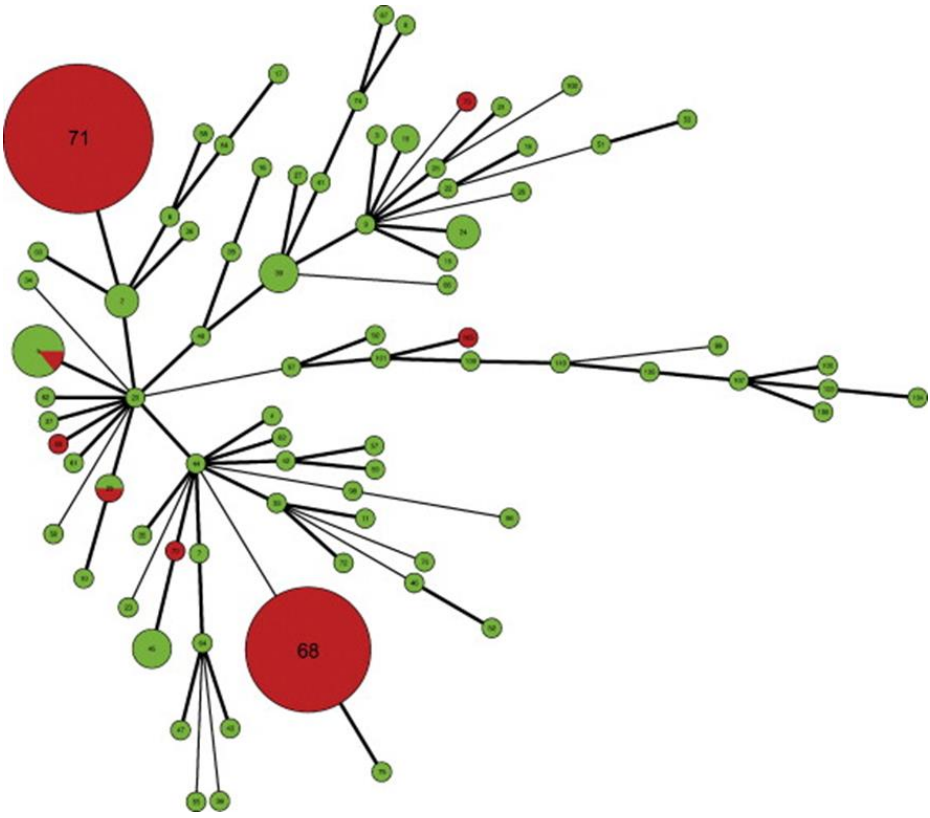

Fig. 3.1 Population genetic structure of S. pseudintermedius lineage (Ruscher et al., 2010)
On the top, the European endemic clone ST71.

2. Materials and Methods

2.1 Multilocus sequence typing of MRSP isolates

During 2015-2017, 126 clinical isolates of *S. pseudintermedius* were collected from two different cities of Southern Italy, Naples and Latina. The bacterial strains were obtained from dogs suffering from otitis externa and pyoderma.

As reported in chapter 1, the identification of *S. pseudintermedius* strains was performed by MALDI-TOF MS and confirmed by single PCR for the species-specific *nuc* and *hlb* genes.

As already described in chapter 2, 18% (23/126) were MRSP strains, since harboured *mec*A gene, assessed by PCR.

The genetic diversity of the 23 MRSP isolates was determined by multilocus sequence typing (MLST) of seven genes (*tuf*, *cpn*60, *pta*, *pur*A, *fdh*, *sar*, and *ack*), as proposed by Solyman *et al.* (2013). MLST was performed in collaboration with Dr. Antonio Parisi at the Istituto Zooprofilattico Sperimentale di Puglia e Basilicata, Putignano-Bari, Italy.

After bacterial DNA extraction by commercial kit as described in chapter 1, PCR were performed, and the amplicons were purified and subsequently sequenced.

The primers sequences, previously employed by Soliman *et al*., (2013) and the amplicon sizes are reported in Table 3.1

Alleles and sequence types (STs) were determined using the MLST database for *S. pseudintermedius*

(https://pubmlst.org/spseudintermedius/) and new sequence types were assigned by the curator Vincent Perreten.

As described in chapter 2, the antibiotic resistance profiles of all 23 MRSP isolates were evaluated by disk diffusion test on Mueller-Hinton agar plates. The following antibiotics were tested: amoxicillin-clavulanate (AMC, 20/10 µg), ampicillin (AMP, 10 µg), oxacillin (OX, 1 µg), penicillin (P, 10 IU), cephalothin (KF, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), clindamycin (CD, 2 µg), ciprofloxacin (CIP, 30 µg), enrofloxacin (ENR, 5 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), tobramycin (TOB, 10 µg), imipenem (IMI, 10 µg), linezolid (LNZ, 30 µg), sulfamethoxazole-trimethoprim (SXT,1.25/23.75 µg), tetracycline (TE, 30 µg), vancomycin (VA, 30 µg) according to CLSI guidelines (2015), while breakpoints for streptomycin were the ones recommended by the French Society for Microbiology (http://www.sfm-microbiologie.fr).

The 23 MRSP were also subjected to multiplex PCR (chapter 2) to determine genetic profiles of tetracycline (Ullah *et al*., 2012) and erythromycin resistance (Sutcliffe *et al*., 1996; Zou *et al*., 2011).

Tab. 3.1 Primers for MLST-7 of S. pseudintermedius and amplicon sizes

Locus	Primers sequences (5'-3') sense and antisense	Amplicon size (bp)
tuf	F: CAATGCCACAAACTCG	500
	R: GCTTCAGCGTAGTCTA	
cpn60	F: GCGACTGTACTTGCACAAGCA	552
	R: AACTGCAACCGCTGTAAATG	
pta	F: GTGCGTATCGTATTACCAGAAGG	570
	R: GCAGAACCTTTTGTTGAGAAGC	
purA	F: GATTACTTCCAAGGTATGTTT	490
	R: TCGATAGAGTTAATAGATAAGTC	
fdh	F: TGCGATAACAGGATGTGCTT	408
	R: CTTCTCATGATTCACCGGC	
ack	F: CACCACTTCACAACCCAGCAAACT	680
	R: AACCTTCTAATACACGCGCACGCA	
sar	F: GGATTTAGTCCAGTTCAAAATTT	521
	R: GAACCATTCGCCCCATGAA	

3. Results

3.1 MSRP clones

The most frequently identified MRSP lineage in this study was ST71, with 6/23 strains (26%), whereas 2/23 (9%) strains were ST118, 2/23 (9%) belonged to ST181 and one strain (4%) for each following STs: ST45, ST258, ST496, ST862 were identified.

Herein, 9 new clones of MRSP out of 23 MRSP strains (39%) were found, as reported in Table 3.2. The newly described sequence types (STs) named from ST1053 up to ST1061, which were submitted and assigned by the curator of the PubMLST database, were for the first time reported in Italy and worldwide.

Tab. 3.2 Geographical, biological origin and clones of methicillin-resistant S. pseudintermedius

Sample n.	Animal host	Biological source	Geographical area	Sequence types (STs)
4	dog	otitis externa	Naples	118
22	dog	otitis externa	Naples	71
70	dog	otitis externa	Naples	71
75	dog	pyoderma	Naples	1053
94	dog	otitis externa	Naples	71
102	dog	otitis externa	Naples	181
115	dog	pyoderma	Naples	71
147	dog	otitis externa	Latina	1054
155	dog	otitis externa	Latina	1055
166	dog	otitis externa	Naples	118
169	dog	otitis externa	Latina	1056
171	dog	pyoderma	Latina	71
183	dog	otitis externa	Naples	71
184	dog	pyoderma	Naples	496
199	dog	otitis externa	Naples	45
218	dog	otitis externa	Latina	862
224	dog	otitis externa	Latina	1057
227	dog	otitis externa	Naples	181
239	dog	otitis externa	Latina	1058
246	dog	otitis externa	Latina	1059
250	dog	otitis externa	Naples	1060
251	dog	otitis externa	Naples	1060
258	dog	otitis externa	Naples	1061

The isolated MRSP strains showed interesting antibiotic resistance profiles (Table 3.3). In particular, most of them (91%) were multidrug-resistant strains (resistant to at least 3 different antibiotic classes) and only the isolates belonging to the new STs 1058 and 1059 had a low degree of resistance. Moreover, only two ST71 strains, as well as two ST1060 strains, presented the same antibiotic resistance profiles.

All 23 MRSP strains, analyzed by MLST, were resistant to amoxicillin-clavulanate and ampicillin. High resistance rates were found for erythromycin (91%) and tetracycline (87%). Interestingly, these clones were associated with carriage of the *erm*B gene and tetracycline resistance genes, precisely, *tet*M (60%) and *tet*K (30%) alone or together (10%).

In addition, high percentages of resistance were the following: sulfamethoxazole/trimethoprim (78%); kanamycin (78%); streptomycin (78%); clindamycin (65%) and enrofloxacin (61%). However, no resistance was observed to vancomycin and linezolid.

Tab. 3.3 Origin, MLST analysis, phenotypic and genotypic antimicrobial resistance of MRSP isolates from canine cutaneous swabs

MRSP origin	STs	Resistance phenotype	*erm* and *tet* genes
Naples/otitis externa	45	AMC, AMP, FOX, KF, CRO, CD, E, CN, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*M
Naples/otitis externa	71	AMC, AMP, FOX, KF, CRO, ENR, E, CN, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*K
Naples/otitis externa	71	AMC, AMP, FOX, CRO, ENR, E, CN, OX, P, S, SXT	*erm*B
Naples/otitis externa	71	AMC, AMP, FOX, KF, CRO, CD, CIP, ENR, E, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*M
Naples/ pyoderma	71	AMC, AMP, FOX, KF, CRO, CD, CIP, ENR, E, CN, K, IMI, OX, P, S, SXT, TE, TOB	*erm*B, *tet*M
Latina/ pyoderma	71	AMC, AMP, FOX, KF, CRO, CD, CIP, ENR, E, CN, K, IMI, OX, P, S, SXT, TE, TOB	*erm*B, *tet*M
Naples/otitis externa	71	AMC, AMP, FOX, KF, CRO, CIP, ENR, E, CN, IMI, OX, P, S, SXT, TE	*erm*B, *tet*K
Naples/otitis externa	118	AMC, AMP, CN, K, OX, P, S, SXT, TE	*tet*K
Naples/otitis externa	118	AMC, AMP, FOX, KF, CRO, CD, E, K, OX, P, S, SXT, TE	*erm*B, *tet*K, *tet*M
Naples/otitis externa	181	AMC, AMP, KF, CRO, CD, CIP, ENR, E, CN, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*M
Naples/otitis externa	181	AMC, AMP, CRO, CD, CIP, ENR, E, CN, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*M,
Naples/ pyoderma	496	AMC, AMP, E, IMI, OX, P, SXT	*erm*B
Latina/otitis externa	862	AMC, AMP, CRO, CD, ENR, E, CN, K, IMI, OX, P, S, SXT, TE, TOB	*erm*B, *tet*M
Naples/ pyoderma	1053	AMC, AMP, CIP, ENR, E, K, OX, P, S, SXT, TE	*erm*B, *tet*M
Latina/otitis externa	1054	AMC, AMP, FOX, CD, CIP, ENR, E, K, IMI, OX, P, S, SXT, TE	*erm*B, *tet*K
Latina/otitis externa	1055	AMC, AMP, FOX, CD, CIP, ENR, E, K, OX, P, S, TE	*erm*B, *tet*K
Latina/otitis externa	1056	AMC, AMP, FOX, CD, CIP, E, K, OX, P, S, TE	*erm*B, *tet*K
Latina/otitis externa	1057	AMC, AMP, CD, E, CN, K, IMI, OX, P, S, TE	*erm*B, *tet*K, *tet*M
Latina/otitis externa	1058	AMC, AMP, E, OX, P, TE	*erm*B, *tet*M
Latina/otitis externa	1059	AMC, AMP, FOX, OX, P, TE	*tet*M
Naples/otitis externa	1060	AMC, AMP, FOX, CD, CIP, ENR, E, K, OX, P, SXT, TE	*erm*B, *tet*M
Naples/otitis externa	1060	AMC, AMP, FOX, CD, CIP, ENR, E, K, OX, P, SXT, TE	*erm*B, *tet*M
Naples/otitis externa	1061	AMC, AMP, CD, E, CN, K, OX, P, S, SXT, TOB	*erm*B

4. Discussion and conclusions

In this study, MLST revealed 15 STs of which 9 were new clones. The most frequently isolated clone is represented by MRSP ST71, which is the largely predominant ST in Europe (Osland *et al*., 2012; Haenni *et al*., 2014; Damborg *et al*. 2016; Ventrella *et al*., 2017) and has diffused worldwide (Pires dos Santos *et al*., 2016). ST45, ST118, ST181, ST258 were already reported in other countries, precisely ST 258 was described for the first time in Southern Italy by Ventrella *et al*. (2017). Referring to ST496, it was described only in Australia (Worthing *et al*., 2018), so here is reported its first finding in Italy; as for the ST862 that was described only in USA, both cataloged in PubMLST.org.

It is well known that MRSP are resistant to several non-β-lactam antibiotics (Ruscher *et al*., 2010; Perreten *et al*., 2010) and this was confirmed also by the results of this study, since the isolated MRSP clones exhibited interesting antibiotic resistance profiles, with high rate of resistance to non-β-lactam antibiotics. Precisely, in this study the isolates were almost all multidrug-resistant strains. In particular, MRSP strains belonging to ST71 clone were the most resistant, expressing worrying multidrug resistance patterns. These results, together with other reports, highlight the role of ST71 as a relevant pathogen not only for dogs, but also for other pets and humans, since infections by ST71 have already been described in humans (Stegmann *et al*., 2010). Differently from ST71 clones, the isolates belonging to the new STs 1058 and 1059 had a low degree of resistance. However, the strains of this study showed resistance to the classes of antibiotics commonly

used for systemic treatment in dogs. Moreover, none of the MRSP clones were resistant to vancomycin or linezolid, that in human medicine are considered as the 'last resort antibiotic' for treatment of infections caused by methicillin-resistant staphylococci.

S. pseudintermedius propensity to horizontal transfer of resistance genes is considered one of the reasons for the rapid spread of multidrug-resistant strains (McCarthy *et al*., 2015; Pires dos Santos *et al*. 2016), displaying, thus, an epidemic population structure and a high genetic diversity.

According to a recent multicenter study (Marques *et al.,* 2016), MRSP and, in general, multidrug-resistant bacteria are more commonly disseminated in Southern Europe than in Northern Europe. This seems to be a consequence of the lack of national antibiotic usage guidelines and surveillance programs in Southern European countries. So, surveillance initiatives in pet animals would be useful in order to obtain data on antibiotic resistance patterns of pet pathogens, with particular attention for those showing multidrug-resistant phenotypes and zoonotic potential, such as *S. pseudintermedius*.

In conclusion, the results of this study demonstrated the circulation of different and new MRSP lineages from clinically diseased dogs in Southern Italy. Considering that few information on genetic lineages and clonal spread of MRSP are currently available, these findings justify the need of a constant epidemiological monitoring of MRSP. Moreover, according to literature, some clones isolated in this study have been already detected both in pets and humans, so an increased surveillance should be addressed with a One Health approach.

5. References

Aanensen DM, Spratt BG, 2005. The multilocus sequence typing network: mlst.net. Nucleic Acids Res **33**, W728–W733.

Clinical and Laboratory Standards Institute (CLSI) 2015. Performance Standard for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, 3rd Edition CLSI VET01S (Wayne, Pennsylvannia, Clinical Laboratory and Standards Institute), 128, 2015.

Damborg P, Moodley A, Aalbæk B, Ventrella G, dos Santos TP, Guardabassi L, 2016. High genotypic diversity among methicillin-resistant *Staphylococcus pseudintermedius* isolated from canine infections in Denmark. BMC Vet Res **12**, 131.

Deurenberg RH, Stobberingh EE, 2008. The evolution of *Staphylococcus aureus*. Infect Genet Evol **8**, 747-763.

Fitzgerald 2009. The *Staphylococcus intermedius* group of bacterial pathogens: species reclassification, pathogenesis and the emergence of methicillin resistance. Vet Dermatol **20**, 490-495.

Haenni M, de Moraes NA, Châtre P, Médaille C, 2014. Characterisation of clinical canine methicillin-resistant and methicillin-susceptible *Staphylococcus pseudintermedius* in France. J Glob Antimicrob Res **2**, 119-123.

Kadlec K, Schwarz S, Perreten V, Andersson UG, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerald JR, Rossano A, Guardabassi L, 2010. Molecular analysis of methicillin-resistant *Staphylococcus pseudintermedius* of feline

origin from different European countries and North America. J Antimicrob Chemother **65**, 1826-1828.

Marques C, Gama LT, Belas A, Bergström K, Beurlet S, Briend-Marchal A, Broens EM, Costa M, Criel D, Damborg P, van Dijk MAM, van Dongen AM, Dorsch R, Espada CM, Gerber B, Kritsepi-Konstantinou M, Loncaric I, Mion D, Misic D, Movilla R, Overesch G, Perreten P, Roura X, Steenbergen J, Timofte D, Wolf G, Zanoni RG, Schmitt S, Guardabassi L, Pomba C, 2016. European multicenter study on antimicrobial resistance in bacteria isolated from companion animal urinary tract infections. BMC Vet Res **12**, 213.

McCarthy AJ, Harrison EM, Stanczak-Mrozek K, Leggett B, Waller A, Holmes MA, Lloyd DH, Lindsay JA, Loeffler A, 2015. Genomic insights into the rapid emergence and evolution of MDR in *Staphylococcus pseudintermedius*. J Antimicrob Chemother **70**, 997-1007.

Osland AM, Vestby LK, Fanuelsen H, Slettemeås JS, Sunde M, 2012. Clonal diversity and biofilm-forming ability of methicillin-resistant *Staphylococcus pseudintermedius*. J Antimicrob Chemother **67**, 841-848.

Perreten V, Kadlec K, Schwarz S, Grönlund Andersson U, Finn M, Greko C, Moodley A, Kania SA, Frank LA, Bemis DA, Franco A, Iurescia M, Battisti A, Duim B, Wagenaar JA, van Duijkeren E, Weese JS, Fitzgerarld JR, Rossano A, Guardabassi L, 2010. Clonal spread of methicillin-resistant *Staphylococcus pseudintermedius* in

Europe e North America: an international multicenter study. J Antimicrob Chemother **65**, 1145-1154.

Pires dos Santos T, Damborg P, Moodley A, Guardabassi L, 2016. Systematic review on global epidemiology of methicillin-resistant *Staphylococcus pseudintermedius*: inference of population structure from multilocus sequence data typing. Front Microbiol **7**, 1599.

Ruscher C, Lübke-Becker A, Semmler T, Wleklinski CG, Paasch A, Soba A, Stamm I, Kopp I, Wieler LH, Walther B, 2010. Widespread rapid emergence of a distinct methicillin- and multidrug-resistant *Staphylococcus pseudintermedius* (MRSP) genetic lineage in Europe. Vet Microbiol **144**, 340-346.

Solyman SM, Black CC, Duim B, Perreten V, van Duijkeren E, Wagenaar JA, Eberlein LC, Sadeghi LN, Videla R, Bemis DA, Kania SA, 2013. Multilocus sequence typing for characterization of *Staphylococcus pseudintermedius*. J Clin Microbiol **51**, 306-310.

Stegmann R, Burnens A, Maranta CA, Perreten V, 2010. Human infection associated with methicillin-resistant *Staphylococcus pseudintermedius* ST71. J Antimicrob Chemoter **65**, 2047-2048.

Sutcliffe J, Tait-Kamradt A, Wondrack L, 1996. *Streptococcus pneumoniae and Streptococcus pyogenes* resistant to macrolides but sensitive to clindamycin: a common resistance pattern mediated by an efflux System. Antimicrob Agents Chemother **40**, 1817-1824.

Ullah F, Malik SA, Ahmed J, Ullah F, Shah SM, Ayaz M, Hussain S, Khatoon L, 2012. Investigation of the genetic basis of tetracycline resistance in *Staphylococcus aureus* from Pakistan. Trop J Pharm Res **11**, 925-931.

Ventrella G, Moodley A, Grandolfo E, Parisi A, Corrente M, Buonavoglia D, 2017. Frequency, antimicrobial susceptibility and clonal distribution of methicillin-resistant *Staphylococcus pseudintermedius* in canine clinical samples submitted to a veterinary diagnostic laboratory in Italy: a 3-year retrospective investigation. Vet Microbiol **211**, 103-106.

Worthing KA, Abraham S, Coombs GW, Pang S, Saputra S, Jordan D, Trott DJ, Norris JM, 2018. Clonal diversity and geographic distribution of methicillin-resistant *Staphylococcus pseudintermedius* from Australian animals: Discovery of novel sequence types. Vet Microbiol **213**, 58-65.

Zou LK, Wang HN, Zeng B, Li JN, Li XT, Zhang AY, Zhou YS, Yang X, Xu CW, Xia QQ, 2011. Erythromycin resistance and virulence genes in *Enterococcus faecalis* from swine in China. New Microbiol **34**, 73-80.

Chapter 4

Alternative therapeutic options

DIBI and abietic acid antimicrobial activity against *S. pseudintermedius*

1. Introduction

Widespread emergence of multidrug-resistant bacterial pathogens is a problem of global dimension. And multidrug-resistant MRSP and MSSP strains represent an important problem to animal health and an increasing therapeutic challenge in veterinary medicine. The regulatory pressure to limit antibiotic use and the everincreasing incidence of multidrug-resistant bacterial strains among companion animals with otitis externa, pyoderma and other infections create a need for new treatment approaches.

Among new therapeutic strategies, there is the one based on the combination antibiotic therapy which is practiced for different reasons such as broadening antibacterial spectrum, polymicrobial infection treatment and overall for its synergistic action.

Another strategy could be the use of adjuvants able to act in concert with the known conventional antibiotics, thus enhancing their activity, especially against resistant isolates.

A non-antibiotic treatment approach has particular interest as do agents that can improve response to conventionally used antibiotics. Alternative treatments for bacterial infections as phage therapy, use of probiotics and prebiotics, antibacterial peptides, nanoparticles and so on, represent noteworthy ongoing developments in the field of antibacterials.

Alternative non-antibiotic treatment strategies need to be explored to ensure that a robust pipeline of effective therapies is available to both human and veterinary medicine (Ruiz *et al*., 2017).

In this study the antibacterial activity of two different agents, hydroxypiridone-based iron chelating co-polymer DIBI and abietic acid, against MRSP and MSSP was evaluated. Moreover, the synergistic interaction between these agents and specific antibiotics was also investigated.

DIBI, a novel water-soluble hydroxypyridinone-containing iron chelating polymer, developed by Chelation Partners Inc. (Canada), provides a potential new antibacterial treatment by denying pathogens of iron as needed for their growth (Fig. 4.1.).

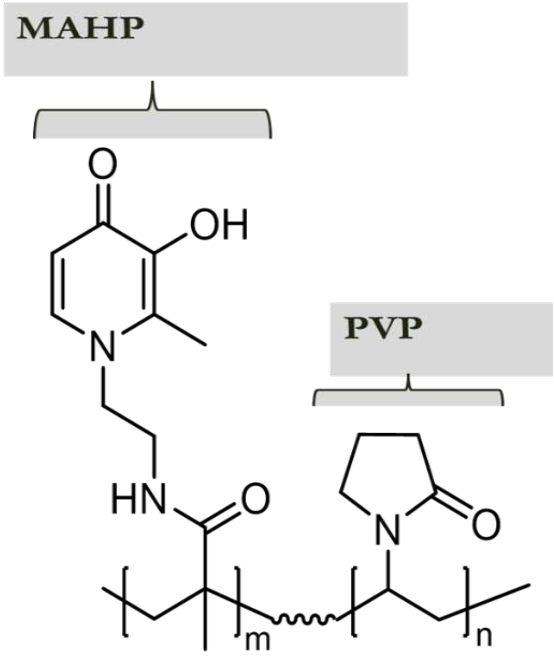

Fig. 4.1 DIBI

Previous minimum inhibitory concentration (MIC) studies has already shown that DIBI is effective against representative reference strains for Gram-positive and Gram-negative bacteria *Staphylococcus aureus* and *Acinetobacter baumannii*, and the yeast *Candida albicans and Malassezia pachydermatis* (Ang *et al.*, 2018; Del Carmen Parquet *et al*., 2018; Savage *et al*., 2018). All pathogens implicated in otitis (and all other non-viral infectious agents) require iron to grow and to infect their host, in fact the animal host already attempts to limit infection through active partial iron sequestration, i.e, as part of the innate immune response, early in infections when microbial iron requirements are highest.

DIBI has been conceived to bolster the existing innate iron related host defenses and has already been shown to be non-toxic when administrated at repeated high dosage to the ear canal of healthy dogs (Kingfisher 2017).

Abietic acid, extracted from tree rosin, contains both a conjugated diene and a carboxylic acid, both of which are readily reacted on a commercial scale. The resin of pine trees (*Pinus sp.*) is the main source of abietic acid, which can be also obtained from many other coniferous trees. In the past, natural products from plants, fungi and bacteria have been successfully used for their antimicrobial activity. Pine resin has been used for several millennia to treat external skin conditions including wounds, boils and pyodermas (San Feliciano *et al*., 1993).

Precisely, abietic acid is a diterpenoid that vaunts a well-studied bioactivity profile, including antiviral, antibiotic and antifungal activity

(Fig. 4.2). In particular, abietic acid has shown an intrinsic activity mainly against Gram-positive bacteria, such as *Staphylococcus aureus*, and yeasts (Gigante *et al*., 2003; Gu *et al*., 2010; Caetano da Silva *et al*., 2014; Leandro *et al*., 2014).

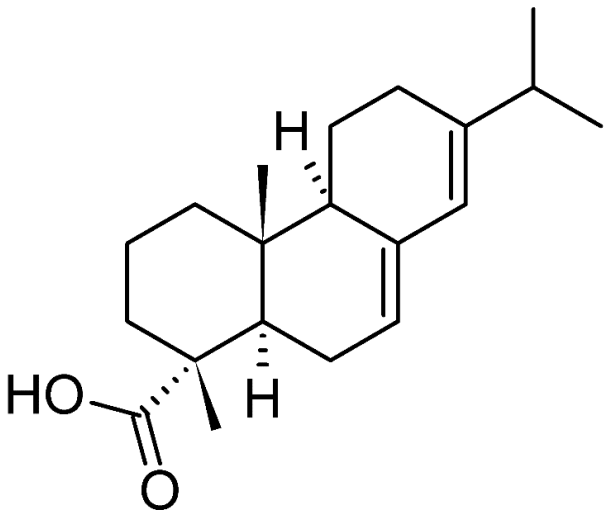

Fig. 4.2 Abietic acid

2. Materials and Methods

2.1 *In vitro* sensitivity of clinical canine *S. pseudintermedius* isolates to DIBI

DIBI, a novel water-soluble hydroxypyridinone-containing iron chelating polymer, developed by Chelation Partners Inc. (Canada), was tested against 5 *S. pseudintermedius* strains isolated from dogs suffering from otitis externa. As reported in chapter 1, the identification of *S. pseudintermedius* strains was performed by MALDI-TOF MS and confirmed by single PCR for the species-specific *nuc* and *hlb* genes. Among these strains, one was a methicillin-resistant *S. pseudintermedius* (MRSP) strain and four were methicillin-susceptible *S. pseudintermedius* (MSSP) strains, as confirmed by *mec*A gene PCR, reported in chapter 2. Moreover, these strains showed resistance profiles to antibiotics that are frequently prescribed to dogs for MRSP or MSSP infections (Table 4.1).

The antimicrobial DIBI activity was tested in collaboration with Dr. Maria Del Carmen Parquet and Dr. Bruce Holbein, Chelation Partners Inc., 1411 Oxford St., Halifax, NS, B3H 3Z1 Canada.

2.1.1 Bacterial cultivation

S. pseudintermedius strains were routinely cultured from glycerol frozen stocks (-80 °C) and maintained on Trypticase Soy Agar (TSA). To test DIBI activity, liquid cultures were grown in Roswell Park Memorial Institute Medium 1640 (RPMI, Sigma-Aldrich) supplemented with 2% (w/v) glucose, buffered with 0.165 M 3-(N-

morpholino)-propanesulfonic acid (MOPS) for 18-24 h at 37 °C with shaking. RPMI medium was chosen for this study because it contains a relatively low but yet sufficient iron content (approx 0.1 µM Fe) and this therefore better emulate *in vivo* iron availability conditions. RPMI grown cultures were used to prepare inocula for MIC determinations.

2.1.2 Susceptibility testing

DIBI stocks were prepared in RPMI (200 mg/mL). All stocks solutions were filter-sterilized (0.2 µm filter) before use. Differently from antibiotics, DIBI interrupts microbial iron supply and therefore it affects many iron-dependent targets at the level of DNA, protein and lipid synthesis as well as for energy production and defense.

MIC determinations were determined using the broth microdiluition method (EUCAST, 2017), in 96-well round-bottomed plates (Life Sciences). As a general rule, RPMI was the assay medium and diluent for testing *S. pseudintermedius* strains.

The MIC for DIBI was determined and for this, DIBI was diluted in RPMI with serial 1/2 dilutions made covering the ranges 0.06-128 µg/mL. To prepare the serially diluted 96 well plates, RPMI was added first to each well. Next, a similar volume of DIBI at a concentration equal to twice the highest concentration (2X) desired was added to the well. Then, the contents of this wells were mixed and serially diluted across the row to achieve the dilution series.

For inoculum preparation, RPMI overnight cultures were diluted in their respective fresh media to an optical density (OD 600 nm) of 0.1 and MIC plates were inoculated to a final dilution of 1/200. Negative and positive controls were included in parallel. All MIC plates were incubated at 35°C for 24-72 h and then read as to MIC. MIC value was defined as the lowest concentration of chelator required to inhibit visible growth at 24 h incubation.

2.2 Antimicrobial properties of abietic acid against clinical canine *S. pseudintermedius* strains

Abietic acid (75%) used in this study was purchased commercially (Alfa Aesar, Ward Hill, Massachusetts, USA) and there was no need of a further purification for its use. Abietic acid was only dissolved in ethanol and diluted in TSB broth to obtain a stock solution.

Abietic acid was tested against 2 clinical canine otitis *S. pseudintermedius* isolates. As described in chapter 1, the identification of *S. pseudintermedius* strains was performed by MALDI-TOF MS and confirmed by single PCR for the species-specific *nuc* and *hlb* genes. Furthermore, *mec*A gene was detected by PCR (chapter 2) revealing one methicillin-resistant *S. pseudintermedius* (MRSP) strain and one methicillin-susceptible *S. pseudintermedius* (MSSP) strain. These strains showed also interesting resistance profiles (Table 4.2).

The antimicrobial abietic acid activity was evaluated in collaboration with the Department of Molecular Medicine and Medical Biotechnology and the Department of Pharmacy, University of Naples "Federico II", Italy.

2.2.1 *S. pseudintermedius* cultivation and susceptibility testing

In order to evaluate acid abietic antimicrobial activity, two *S. pseudintermedius* isolated strains were plated again on blood agar base supplemented with 5% sheep blood and on Mannitol Salt agar (MSA), and incubated aerobically at 37 °C for 24–48 h.

Minimal inhibitory concentrations (MIC) of abietic acid was determined in TSB medium by the broth microdilution assay, according to the European Committee on Antimicrobial Susceptibility Testing (2017). Bacterial suspensions were diluted to yield an OD around 0.5 at 595 nm and further diluted to a final concentration of 1×10^6 CFU/mL. The compounds were added to bacterial suspension in each well yielding a final cell concentration of 5×10^5 CFU/mL and a final compound concentration ranging from 2 to 128 µg/mL. Negative control wells were set to contain bacteria in TSB plus the amount of ethanol used to dilute each compound. Positive controls included oxacillin (2 µg/mL) and vancomycin (2 µg/mL). Medium turbidity was measured by a microtiter plate reader (Tecan, Milan, Italy) at 595 nm. Absorbance was proportional to bacterial growth. Minimum bactericidal concentration (MBC) was defined as the concentration that caused $\geq 3\log_{10}$ reduction in colony count from the starting inoculum plated on TSA, incubated for 24 h at 37 °C.

2.2.2 Killing rate and checkerboard method

Time kill assay was carried out as previously described in Olajuyigbe *et al.* (2012) with minor modifications. Bacterial suspension (10^5 CFU/mL) was added to microplates along with abietic acid at the MIC

concentrations (8 µg/mL and 32 µg/mL). Plates were incubated at 37 °C on an orbital shaker at 120 rpm. Viability assessments were performed at 0, 2, 4, 6, and 24 h by plating 0.1 mL undiluted and 10-fold serially diluted samples onto LB plates in triplicate. After the overnight incubation at 37 °C, bacterial colonies were counted and compared with counts from control cultures.

The interaction between abietic acid and oxacillin against MRSP was evaluated by the checkerboard method in 96-well microtiter plates containing TSB. Briefly, abietic acid and oxacillin were serially diluted along the y and x axes, respectively. The final antimicrobial substance concentrations (after two-fold dilution) ranged from 1/16 to 1 times the MIC for oxacillin and from 1/64 to 1 times the MIC for abietic acid. The checkerboard plates were inoculated with bacteria at an approximate concentration of 10^5 × CFU/mL and incubated at 37°C for 24 h, following which bacterial growth was assessed visually and the turbidity measured by microplate reader at 595 nm. To evaluate the effect of the combination treatment, the fractional inhibitory concentration (FIC) index for each combination was calculated as follows: FIC index = FIC of abietic acid + FIC of oxacillin, where FIC of abietic acid (or oxacillin) was defined as the ratio of MIC of abietic acid (or oxacillin) in combination and MIC of abietic acid (or oxacillin) alone. The FIC index values were interpreted as follows: ≤0.5, synergistic; >0.5 to ≤1.0, additive; >1.0 to ≤2.0, indifferent; and >2.0, antagonistic effects (Pillai *et al*., 2005).

3. Results

3.1 Susceptibility of *S. pseudintermedius* isolates to DIBI

All *S. pseudintermedius* strains displayed high sensitivities to DIBI with a MIC of 2 µg/mL at 24 h (Table 4.1). Furthermore, DIBI sensitivity was not linked to antibiotic sensitivity or resistance of the strains.

Tab. 4.1 Antibiotic resistance, methicillin resistance profiles, DIBI antimicrobial activity in 5 clinical canine otitis S. pseudintermedius isolates.

Sample n.	Key antibiotic resistance	*mec*A gene detection	*DIBI MIC µg/mL*
3	AMC, AMP, CD E, K, P, S, SXT, TE	-	2
4	AMC, AMP, FOX, KF, CRO, CD, E, K, OX, P, S, SXT, TE	+	2
7	AMC, AMP, E, CN, K, P, S, SXT, TE, TOB	-	2
8	AMC, AMP, P, SXT, TE	-	2
18	AMC, AMP, E, K, IMI, P, S, TE	-	2

3.2 *In vitro* abietic acid antimicrobial activity

The antimicrobial activity of abietic acid against MRSP and MSSP strains was evaluated. Antibiotic resistance patterns of both strains are reported in Table 4.2, whereas MIC values are shown in table 4.3. Abietic acid exhibited a significant MIC_{90} value at 32 µg/mL (MRPS) and 8 µg/mL (MSSP), while MBC values were 64 µg/mL (2X MIC) and 32 µg/mL (4X MIC) against MRSP and MSSP strains,

respectively. The MBC/MIC ratio or MIC_{index} was also calculated. When the ratio of MBC/MIC was ≤ 2.0, the extract was considered bactericidal or otherwise bacteriostatic. The results in Table 4.3 showed a bacteriostatic activity against MSSP (MIC_{index} 4), while the result about the MRSP has been further investigated. The time-kill assay results, presented in terms of the changes in the log_{10} CFU/mL of viable colonies, confirmed the bacteriostatic activity of abietic acid (Table 4.4) against both *S. pseudintermedius* strains. Abietic acid was able to inhibit bacterial growth already at 1 h, with a percentage of CFU reduction of 60% and 30% for MSSP and MRSP, respectively, compared to untreated bacteria. The highest inhibition activity of abietic acid was observed after 6 h exposure with a percentage reduction of 90% and 75% for MSSP and MRSP, respectively. However, after 24 h a cell growth resumption was observed in abietic acid-treated strains, even though not comparable to untreated cells (<3log_{10}). Thus, the bacteriostatic effect of abietic acid on both *S. pseudintermedius* strains was confirmed.

Tab. 4.2 Antibiotic resistance and methicillin resistance profile in 2 clinical canine otitis S. pseudintermedius strains

Sample n.	Key antibiotic resistance	*mec*A gene detection
21	AMC, AMP, IMI, P, TE	-
22	AMC, AMP, FOX, KF, CRO, ENR, E, CN, K, IMI, OX, P, S, SXT, TE	+

Tab. 4.3 MIC_{90}, MBC, MIC_{index}, FIC_{index} values of abietic acid on selected S. pseudintermedius strains

Strains	MIC (μg/mL)	MBC (μg/mL)	Oxacillin (μg/mL)	Vancomycin (μg/mL)	MIC_{index}	FIC_{index}
MSSP	8	32	<0,25	2	4	n.d.*
MRSP	32	64	10	2	2	0,375

* *N.d.= not determined. Each experiment is the result of three independent experiments performed in triplicate.*

Tab. 4.4 Time-kill assay of abietic acid against MSSP and MRSP

Strains	Log_{10}CFU/mL					
	0h	**1h**	**2h**	**4h**	**6h**	**24h**
MSSP untreated	5,60	5,84	5,90	5,97	7,30	9,77
MSSP + abietic acid	5,50	3,20	2,60	2	1,60	6,97
MRSP untreated	5,30	5,47	5,74	5,81	6,91	8,36
MRSP + abietic acid	5,40	3,77	3,38	2,30	1,77	5,86

* *Each experiment is the result of three independent experiments performed in triplicate.*

3.3 Synergistic assay of abietic acid with oxacillin against MRSP

The synergism between abietic acid and oxacillin was determined using the checkerboard technique. Oxacillin and abietic acid were tested alone and in combination against MRSP planktonic cells. The drugs concentrations decreased along the rows and column from 32 µg/mL to 0 µg/mL (abietic acid) and 10 µg/mL to 0 µg/mL (oxacillin). The highest synergistic interaction against MRSP was obtained in the wells with the best combination values of 4 µg/mL abietic acid (1/8 MIC) and 2,5 µg/mL oxacillin (1/4 MIC). The FIC index, equal to 0.375, confirmed the synergistic effect of abietic acid and oxacillin (Table 4.3).

4. Discussion and conclusions

Antibiotic resistance is one of the most urgent threats to the public's health and the increasingly limiting therapeutic options both in human and veterinary medicine underline the need of alternative treatments. Since the lack of effective antibiotics in veterinary field and the increase of zoonotic transmission are becoming a looming scenery, the antimicrobial properties of DIBI (a proprietary iron chelator of Chelation Partners Inc.) and abietic acid were here investigated.

In this study, DIBI resulted to be effective against MRSP and MSSP strains, inhibiting their growth at a MIC value of 2 µg/mL. This investigation has also shown that DIBI had stand-alone as an alternative anti-infective potential as a "non-antibiotic" anti-infective for treating otitis caused by *S. pseudintermedius* strains. The obtained results provided evidence that the new, highly water-soluble, low molecular weight co-polymer DIBI, depriving microorganisms of bioavailable iron, may represent a promising strategy for new anti-infective agents. In fact, it was proved that the novel chelator DIBI could restrict growth and also enhance the activity of antimicrobial agents against clinical bacterial and fungal isolates (Savage *et al*., 2018). An iron chelator, such as DIBI, offers potential broad utility as an alternative anti-infective for treating staphylococcal infections in man and other animals. Recently, Del Carmen Parquet *et al*. (2018) demonstrated that DIBI was found to be strongly inhibitory to a diverse group of *S. aureus* isolates regardless of their animal origin (human, cattle or dogs) and irrespective of their antibiotic resistance

characteristics. Thus, DIBI has potential as a non-antibiotic alternative therapy.

Abietic acid and its derived compounds are known for their antibacterial activity (Helfenstein *et al*., 2017). In this study, abietic acid strongly reduced both MSSP and MRSP growth (MIC_{90}) showing a bacteriostatic activity in the first hours of treatment. Bacterial growth was inhibited already after 1 h, reaching the peak after 6 h of exposure. Interesting, the MIC values here reported were lower than those observed in a study on *S. aureus* by Helfenstein *et al*. (2017).

At the MBC concentration (32 and 64 µg/mL for MSSP and MRSP, respectively) bacterial cell death was observed. The abietic acid activity here observed might be attributable to the carboxylic group, which interacts with the lipid component of the bacterial cellular membrane and allows the penetration of the molecule inside the membrane, altering its functions (Aranda *et al*., 1997; Cuzzucoli Crucitti *et al*., 2018). Worthy of note, abietic acid presented good biocompatibility as assessed by hemolytic assay, demonstrating a negligible lytic activity, and it was no toxic against human normal fibroblast (Sadashiva *et al*., 2015; Cuzzucoli Crucitti *et al*., 2018). The synergistic interaction between abietic acid and oxacillin highlighted a relevant result, since abietic acid was able to increase oxacillin sensibility of MRSP.

Taking everything into account, there is currently no certain indication that MRSP is more virulent than MSSP, since both MRSP and MSSP show an increased resistance to most of all antibiotics licensed for use in pets. The results reported above, suggest that DIBI and abietic acid

may be considered potential therapeutic agents to be used alone or in combinatorial antibiotic therapy, particularly useful to treat infections caused by multidrug-resistant strains, that are becoming more and more difficult to manage. Awareness, current research and comprehensive management of infections are required by veterinarians not only to support treat infected companion animals but also to limit the spread and prevent the establishment of this highly drug-resistant and zoonotic pathogen in veterinary facilities and in the community.

5. References

Ang MTC, Gumbau-Brisa R, McDonald R., Ferguson MJ, Holbein BE, Bierenstiel M, 2018. DIBI, a 3-hydroxypyrin-4-one chelator iron-binding polymer with enhanced antimicrobial activity. Med Chem Comm **9**, 1206-1212.

Aranda FJ, Villalaín J, 1997. The interaction of abietic acid with phospholipid membranes. Biochim Biophys Acta **1327**, 171–180.

Caetano da Silva SD, Mendes de Souza MG, Oliveira Cardoso MJ, da Silva Moraes T, Ambrósio SR, Sola Veneziani RC, Martins CHG, 2014. Antibacterial activity of *Pinus elliottii* against anaerobic bacteria present in primary endodontic infections. Anaerobe **30**, 146-152.

Cuzzucoli Crucitti V, Migneco LM, Piozzi A, Taresco V, Garnett M, Argent RH, Francolini I, 2018. Intermolecular interaction and solidstate characterization of abietic acid/chitosan solid dispersions possessing antimicrobial and antioxidant properties. Eur J Pharm Biopharm **125**, 114-123.

Del Carmen Parquet M, Savage KA, Allan DS, Davison RJ, Holbein BE, 2018. Novel iron-chelator DIBI inhibits *Staphylococcus aureus* growth, suppresses experimental MRSA infection in mice and enhances the activities of diverse antibiotics *in vitro*. Front Microbiol **9**, doi: 10.3389/fmicb.2018.01811.

The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for Interpretation of MICs and zone diameters. Version 7.1, 2017. http://www.eucast.org

Gigante B, Santos C, Silva AM, Curto MJM, Nascimento MSJ, Pinto

E, Pedro M, Cerqueira F, Pinto MM, Duarte MP, Laires A, Rueff J, Gonçalves J, Pegado MI, Valdeira ML, 2003. Catechols from Abietic Acid: Synthesis and Evaluation as Bioactive Compounds. Bioorg Med Chem **11**, 1631-1638.

Gu, W., Wang, S., 2010. Synthesis and antimicrobial activities of novel 1H-dibenzo[a,c]-carbazoles from dehydroabietic acid. Eur J Med Chem **45**, 4692-4696.

Helfenstein A, Vahermo M, Nawrot DA, Demirci F, İşcan G, Krogerus S, Yli-Kauhaluoma J, Moreira VM, Tammela P, 2017. Antibacterial profiling of abietane-type diterpenoids. Bioorg Med Chem **25**, 132-137.

Kingfisher, 2017. Dose escalation tolerance study of DIBI-R12, an otic chelating agent, in Beagles. Test facility study number: KFI-091-SC-0817.

Leandro LF, Cardoso MJO, Silva SDC, Souza MGM, Veneziani RCS, Ambrosio SR, Martins CHG, 2014. Antibacterial activity of *Pinus elliottii* and its major compound, dehydroabietic acid, against multidrug-resistant strains. J Med Microbiol **63**, 1649-1653.

Olajuyigbe OO, Afolayan AJ, 2012. In vitro antibacterial and time- kill evaluation of the *Erythrina caffra Thunb*. extract against bacteria associated with diarrhea. Sci World J, 738314.

Pillai SK, Moellering RC, Eliopoulos GM, 2005. Antimicrobial Combinations. In: Lorian V, Ed., Antibiotics in Laboratory Medicine, 5th Edition, the Lippincott Williams & Wilkins Co., Philadelphia, 365-440.

Ruiz J, Castro I, Calabuig E, Salavert M, 2017. Non-antibiotic

treatment for infectious diseases. Rev Esp Quimioter **1**, 66-71.

San Feliciano A, Gordaliza M, Salinero MA, del Corral JM, 1993. Abietane acids: sources, biological activities, and therapeutic uses. J Planta Med **59**, 485-488.

Savage KA, del Carmen Parquet M, Allas DS, Davison PJ, Holbein BE, Lilly EA, Fidel PL Jr, 2018. Iron restriction to clinical isolates of *Candida albicans* by the novel chelator DIBI inhibits growth and increases sensitivity to azoles *in vitro* and *in vivo* in a murine model experimental vaginitis. Antimicrob Agents Chemotherap **6**, e:02576-17.