UNIVERSITA' DEGLI STUDI DI MILANO

FACOLTA' DI MEDICINA VETERINARIA
Ph.D. Course in Veterinary and Animal Science
Class XXXII

Molecular characterization of *Staphylococcus pseudintermedius*: research on virulence factors, antibiotic-resistance and quorum-sensing

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Academic Year 2018-2019

To my family

Always in my thoughts

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Abstract

Staphylococcus pseudintermedius (*S. pseudintermedius*) is one of the most important pathogens in small animal veterinary medicine. In the dog, it is a commensal bacterium able to cause severe diseases (mainly dermatological), which rapidly evolve to persistent and chronic infections. This pathogen is also considered a public health problem due to the rapid evolution and spread of antibiotic-resistant clones, in particular, the worldwide dissemination of Multi-Drug Resistant (MDR) strains and methicillin-resistant *S. pseudintermedius* (MRSP). Nowadays, its zoonotic potential is under constant investigations.

The first chapter of the thesis centers the attention on the typing techniques used to study a population of *S. pseudintermedius* isolated from the skin of dogs and raw milk of Holstein-Fresian dairy cattle. The aims of this study were the investigation of the genetic relatedness of different *S. pseudintermedius* strains using three molecular typing techniques; the Ribosomal Spacers Amplification PCR (RSA-PCR), the Random Amplification of Polymorphic DNA PCR (RAPD-PCR) and the Restriction Fragment Length Polymorphism PCR (RAPD-PCR). Moreover, the dissemination of species-specific genes coding for exfoliative toxins (*siet*, *expA,* and *expB*), enterotoxin (seC_{canine}) and a bicomponent leukocidin (*lukS-F*) was studied to understand the virulence of the strains.

The study was carried out on a final population of 40 *S. pseudintermedius* strains isolated from 42 clinical cases of deep canine pyoderma and from 5 samples of raw milk from Holstein-Fresian dairy cattle with intramammary infection. After the microbiological examination and phenotypic typing, 47 *S. pseudintermedius* strains were collected and genetically analyzed. RSA-PCR revealed the presence of a unique biotype. The dendrogram obtained from RAPD-PCR disclosed two important results: i) the staphylococcal strains isolated from dogs and cows are genetically similar underlying the ability of this pathogen to colonize also dairy cattle; ii) the presence of a single cluster grouping all the *S.*

pseudintermedius strains. Based on the presence of a specific restriction site in the phosphotransacetylase (*pta*) gene, RFLP-PCR confirmed the presence of 40 *S. pseudintermedius* and 7 non-*S. pseudintermedius* strains. The sequencing unveiled the effective presence of one strain of *S. aureus* and one of *E. faecalis*, 5 strains of *S. schleiferi* and 40 of *S. pseudintermedius*. All the *S. pseudintermedius* isolates were positive for *siet* gene, while *expA* and *expB* were amplified by, respectively, 10% and 17,5%. Specific enterotoxin seC_{canine} and leukotoxin *luk-S-F* were found with a prevalence of 35% and 92,5%, respectively. Therefore, the combined use of molecular typing techniques and virulence factors screening allows to better characterize *S. pseudintermedius* and to understand its zoonotic potential.

The second chapter points out the attention to the problem of antibiotic resistance. Frequently, in Veterinary and Human medicine too, wide-spectrum antibiotics are prescribed to face with bacteria-based diseases without the microbiological examination and relative antibiotic-resistance screening. This could lead to the selection and spread of antibiotic-resistant strains.

The aim of this work was to study the antibiotic-resistance profile of a large population of *S. pseudintermedius* strains collected in two Italian veterinary teaching hospitals, the first in Milan (Università degli Studi di Milano) and the second in Naples (Università degli Studi di Napoli "Federico II")

One-hundred sixteen and one-hundred twenty-six strains were collected in Milan and Naples, respectively. A panel of 10 different antibiotic molecules was tested with the Kirby-Bauer assay, qualitative PCR was used to describe the dissemination of *mecA* and *tetM/K* genes. The results showed a high rate of resistance for amoxicillin+clavulanate, clindamycin, tobramycin and tetracycline also confirmed by statistical significance. The prevalence of methicillin-resistant strains (MRSP) was 30% in Milan and18% in Naples also confirmed by the amplification of *mecA* gene. The cohort of methicillin-sensible strains in Naples (N= 103)

was significantly more resistant compared to that from Milan (N= 81). In relation to the MDR population, all the MRSP in Milan and 91% in Naples are considered resistant to three or more pharmaceutical categories. These results totally agree with the recent report about the "*Surveillance of antimicrobial resistance in Europe, 2017"* and could be a starting point to set out a clearer use and prescription of antibiotics.

The third chapter describes *quorum-sensing* and biofilm. It is known that bacteria are able to communicate via small peptides (Gram-positive) or lipids (Gram-negative) using a system called *quorum-sensing.* This complex behavior acts in response to external stimuli (e.g. cell density) and controls gene expression. Biofilm is one of the products of this communication, defined as a complex ecosystem of bacteria (sometimes also fungi and algae) enclosed in a self-produced matrix and adherent to a biotic or abiotic surface. This structure is frequently resistant to commonly used antibiotics and causes severe and more complex re-infections.

The aim of the work was to describe the biofilm-forming ability of MDR and non-MDR *S. pseudintermedius* strains investigating the dissemination of virulence factors and antibiotic-resistance genes. A panel of 22 antibiotics was used to screen the antibiotic-resistance profiles of 73 *S. pseudintermedius* strains isolated from deep canine pyoderma. The *biofilm*-forming ability was investigated using a microtiter assay. The results clearly highlight a correlation between antibiotic-resistance and the ability to produce *biofilm*. All the MDR strains (N=42) are *biofilm* producers while 27 out of 31 non-MDR strains were able to produce biofilm.

The last chapter of the thesis focused on an alternative strategy for the treatment of bacterial infections caused by Gram-positive and Gram-negative bacteria. For centuries, silver was used for its antimicrobial properties. Recently, the nanobiotechnology research area aimed to synthesize small particles (less than 100 nm) of silver metal to use as an alternative to traditional antibiotic-based therapies. The aim of this work was to synthesize silver

nanoparticles using i) a *Curcuma longa* infusion and ii) the supernatant of *E. coli*. Furthermore, the antibacterial properties of synthesized nanoparticles were determined against 10 *S. pseudintermedius* strains and 10 *Pseudomonas aeruginosa* isolates by Kirby-Bauer disk diffusion assay and via Minimum Inhibitory Concentration (MIC). The anti-*biofilm* capacities were also investigated using an *in vitro* model of *biofilm* growth. Results showed that silver nanoparticles exert strong antibacterial activity compared to antibiotic alone and these properties are magnified when antibiotic was adjuvated with silver. Nanoparticles are also able to disrupt mature *biofilm* by directly linking to the cell wall of bacteria causing bacterial lysis. The use of silver nanoparticles obtained with green technology (e.g plant or organism derived molecules) is an eco-friendly alternative to classical antibiotic-based therapies and can be used to fight against both Gram-positive and Gram-negative infections.

1. Introduction

1.1. Canine pyoderma

Canine pyoderma is an inflammation of the skin (pyoderma= pus in the skin). It is the second most frequent cause of presentation to opinion in veterinary practices and frequently leads to the prescription of systemic antimicrobial agents (Hill *et al*., 2006; Hughes *et al*., 2012). Like mastitis, it is considered a disease caused by the interaction of three factors: the host (genetics), the pathogen and the environment.

Despite the (relatively) high prevalence of identification, canine pyoderma is often misdiagnosed leading to inappropriate treatment (Loeffler and Lloyd, 2018). Taking into account that peculiar skin lesions and their distribution are essential parameters (secondary to a careful inspection of the skin) for the correct diagnosis of this disease, a classification based on the depth of infection allows to range and discriminate between i) surface, ii) superficial and iii) deep pyoderma.

Surface pyoderma is the less understood type of pyoderma, being characterized by pyotraumatic dermatitis (hot spots), intertrigo (fold pyoderma, most related to some specific breeds [e.g. shar pei]) and the "bacterial overgrowth syndrome". This last condition presents an excessive multiplication of bacteria on the skin surface and is considered a minor determinant in the pathogenesis of the disease, that could arise by a dominant inflammatory cause (Pin *et al*., 2006). Erythema is the only clinical sign that makes suspect this condition; cytological examination demonstrates large numbers of bacteria on the inflamed skin.

Superficial pyoderma is recognized as the most frequent type of pyoderma and requires the invasion of the epidermis by bacteria, that infect follicles and extend to epidermal tissue via the follicular ostium. Papules, pustules and epidermal collarettes associated with areas of alopecia and varying degrees of pruritus are the most important clinical signs that characterize this type of pyoderma.

Deep pyoderma is more serious (but less common), because the bacterial infiltration into the dermis and the underlying blood vessels increase the risk of bacteriaemia. Typical lesions include: draining sinuses, *fistulae*, hemorrhagic crusts, nodules and varying degrees of erythema. Based on the presence of neutrophils and intracellular cocci, cytology of superficial lesions has a diagnostic sensitivity of 93% (Udenberg *et al*., 2014).

1.2. Aetiology and pathogenesis

The aetiological origin of canine pyoderma has to be found in cutaneous bacterial dysbiosis. In a recent study on the skin microbiota of dogs, the number of different species isolated from the dorsal nose varies from a minimum of 115 up to 490, identifying a total of 17 *Phyla* such as Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes and Cyanobacteria (Hoffmann *et al*., 2014). Coagulase-positive *Staphylococci* (CoPS) have been recognized as having a major role in pyoderma (Loeffler and Lloyd, 2018) and before the refinement in microbiological diagnostic techniques, *S. intermedius* was described as its main causative agent. Nowadays, the predominant role of the causative agent of the superficial pyoderma is reserved to *S. pseudintermedius*, but other contagious staphylococci, including *S. aureus*, *S. schleiferi,* and *S. hycus* may be involved in the pathogenesis of pyoderma even if with a low prevalence (1%) (Loeffler and Lloyd, 2018). Among environmental bacteria, the genus *Pseudomonas* remains the second most frequently isolated especially in auricular pinna and ear. Because of its peculiar metabolism, these opportunistic pathogens are able to grow even in the presence of a low amount of oxygen making the auricular environment the best growing area to colonize.

Staphylococcus pseudintermedius belongs to the genus *Staphylococcus*, from the family of *Staphylococcaceae* belonging to Bacillales Order and Firmicutes *Phylum.* The word "*staphylococcus*" comes from the Greek: σταφυλή, staphylē, "grape" and κόκκος, kókkos, "granule", which means a bunch of grapes. All staphylococci are Gram-positive cocci, with a diameter of 0.5-1 μm, facultative anaerobes (which allows these bacteria to grow both aerobically or anaerobically). On blood agar plates, *S. pseudintermedius* grows with large, round, flat and typically white colonies, with total or partial hemolysis. It is a canine commensal and opportunistic pathogen and can be considered analogous to *S. aureus* in human beings. As being a commensal bacterium, it should not represent a clinical problem unless the skin barrier

is broken (due to trauma, surgery, etc.) or the animal is immunosuppressed. In these circumstances, *S. pseudintermedius* acts like a pathogen, being able to cause severe infections to ears, urinary tract and the surgical site. Contrary to *S. aureus*, the pathophysiological mechanisms involved in its virulence are incompletely understood (Maali *et al*., 2018).

The history of *S. pseudintermedius* should be used as a gold standard to describe what the evolution in microbiological diagnostic techniques means. Until 2005, year in which Devriese described the new species *Staphylococcus pseudintermedius* (Devriese *et al*., 2005), *S. intermedius,* described by Hajek in 1976, has been considered the agent causing skin and soft tissue infections in dogs. The name "pseudintermedius" literally means false intermedius and reflects the close genetic relatedness (99% similar) to *S. intermedius* highlighting the inefficient discriminative power of phenotypic tests used. Only the progress in technology and the development of new molecular techniques allowed the differentiation of three diverse staphylococcal species within *S. intermedius*: *S. intermedius*, *S. pseudintermedius*, and *S. delphini*. The term *Staphylococcus intermedius Group* (SIG) refers to the three previously cited staphylococci (*S. intermedius*, *S. pseudintermedius,* and *S. delphini*) as a group (Bannoehr *et al*., 2007b; Sasaki *et al*., 2007a). Only the whole genome analysis and the DNA-DNA hybridization assay, for species delineation and species differentiation respectively, were able to determine that most canine isolates phenotypically identified as *S. intermedius*, were *S. pseudintermedius* (Bannoehr *et al*., 2007b; Sasaki *et al*., 2007a). Based on these results, the scientific community proposed that all canine isolates belonging to SIG should have been considered as *S. pseudintermedius* unless proven the contrary by genetic methods. The presence of a single restriction site for the endonuclease MboI in the gene coding for phosphotransacetylase (*pta*) in *S. pseudintermedius* only, allowed to elaborate a molecular protocol for the diagnostic identification of this pathogen (Bannoehr *et al*., 2009).

1.3. Typing of Staphylococcus pseudintermedius

The typing and characterization of *S. pseudintermedius* have a primary role in the spread of infection and in the determination of the genetic structure of the staphylococcal population. A utopic typing technique should be simple, reproducible in different laboratories, highly discriminatory, easily available and, as much as possible, inexpensive. The typing techniques most frequently used in the study of genetic population of *S. pseudintermedius* are based on the genetic variability among the isolates. During the last decades, different molecular techniques were developed in order to better discriminate between isolates that are epidemiologically related to those that are not. The molecular techniques nowadays available are grouped in the following categories: i) enzymatic restriction, ii) DNA amplification, iii) DNA amplification and enzymatic restriction and iv) gene sequencing.

In the group of enzymatic restriction, *Pulsed Field Gel Electrophoresis* (PFGE) was considered as the gold standard for *S. aureus* typing until the last two decades. This technique allows discriminating different isolates based on the enzymatic restriction of total DNA in agarose in which was applied a pulsed electrical field that causes the generation of different strain profiles; resulting in the comparison of large genomic DNA fragments. Despite the great potential exhibited by this technique, nowadays is considered not suitable for long-term or retrospective studies because point mutations change the sites recognized by enzymes that will lead to loss of band pattern among similar isolates from a close population (Mehndiratta *et al*., 2012). Another used technique is the *Restriction Fragment Length Polymorphism* (RFLP) based on the polymorphisms in genomic fragments derived from the enzymatic restriction of total DNA.

Based on the amplification of DNA, the *Random Amplification of Polymorphic DNA* (RAPD), belonging to amplification-based typing techniques, uses a combination of universal primers (of approximately 10 nucleotides) to amplify DNA fragments of different length that

will be processed through a statistical program able to identify homologous regions between isolates and build a dendrogram showing the percentage of identity in a root-like structure. This technique can be used to screen mini-hospital associated outbreaks of Methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) (Lin *et al*., 2014). *Multiple-Locus Variable-Number Tandem-Repeat Analysis* (MLVA) is a method based on the analysis of the variation in the number of repeats in different genes. Originally developed in 2003 to study *S. aureus* within 7 different genes (*sspA*, *spa*, *sdrC*, *sdrD*, *sdrE*, *clfA* and *clfB*), this technique was soon applied to *S. pseudintermedius* because its discriminative power was similar to PFGE but more cheaper (Pei *et al*., 2008; Volpe Sperry *et al*., 2008).

RFLP-PCR is considered an evolution of RFLP in which, the amplification of a gene is followed by the enzymatic restriction of the amplicons. This technique was used by Bannoehr to set up the first molecular diagnostic protocol for the identification of *S. pseudintermedius* (Bannoehr *et al*., 2009).

Multi Locus Sequence Typing (MLST) belongs to the last group of previously cited typing techniques (gene sequencing). This method focuses on the variations occurring in slowly mutating genes with high discriminative power. It compares DNA sequences of 7 different housekeeping genes; generating an allelic profile in which the differences found in each gene were compared and assigned to a sequence type (ST) for each isolate. The first MLST used to type *S. pseudintermedius* was adapted from that of *S. aureus* (Bannoehr *et al*., 2007a), which targeted 4 genes (*cpn60*, *tuf*, *pta,* and *agrD*). Recently, a more robust 7 genes-based MLST was set up by Solyman (*cpn60*, *tuf*, *pta, purA, fdh, ack,* and *sar*) (Solyman *et al*., 2013).

Staphylococcal Cassette Chromosome (SCCmec) Typing (*SCCmec* Typing) analyses the nucleotidic differences of *SCCmec* to epidemiologically study MRSP or MRSA clones and identify the common ancestor that originates clonal expansion. From 1999 (first *SCCmec* discovered) until now, eleven *SCCmec* types have been classified (Shore *et al*., 2011). The

typing of *SCCmec* can be made using three methods based on: i) enzymatic restriction, ii) PCR and/or Multiplex PCR and iii) Real-Time PCR (qPCR). Starting from the original work by Sasaki, in which *SCCmec* elements of *S. pseudintermedius* had been classified as *SCCmec* III, Descloux *et al*. reported two other *SCCmec* elements, which were named *SCCmec* II-III and VII (Sasaki *et al*., 2007a; Descloux *et al*., 2008). Finally, in 2012, Moon *et al*. reported *SCCmec* V as the last member of the *SCCmec* element of *S. pseudintermedius* (Moon *et al*., 2012).

The studies targeting the analysis of hypervariable regions have been extensively used in the last years to group bacterial genomes. The *agr* locus is a conserved and highly polymorphic region in the staphylococcal genome, responsible for the control of 70 different genes involved in bacterial homeostasis and virulence factors (George and Muir, 2007). In recent studies, this region was genetically analyzed and *S. pseudintermedius* clones can be classified in following *agr* groups: I, II, III, IV.

1.4. Antibiotic-resistance

Bacteria are, literally, the first and oldest living organisms able to colonize our planet. They originated almost 4 billion years ago and, through the (archeo)genetic screening of antibiotic biosynthetic pathways it was possible to clarify that antibiotics are, at least, hundreds of millions of year old; making the fight for life the most oldest war almost in progress on our planet (Dcosta *et al*., 2011; Wright and Poinar, 2012).

The core of the great majority of therapeutic approaches against infectious diseases resides in the use of antibiotics. The enormous over-use of these chemicals during the past decades has caused the natural selection and rapid spread of antibiotic-resistant (also called mutants) bacteria. The natural development of antibiotic-resistance represents one of the most critical issues able to hazard the treatment of infectious diseases and other therapeutic-related procedures (Martínez, 2012).

Also in *S. pseudintermedius*, the antibiotic-resistance is of great concern due to the continuous increase of methicillin resistance among SIG members combined with multiple resistance (Schwarz *et al*., 2008). Until 2006, *S. (pseud)intermedius* isolates were generally less resistant to penicillinase stable β-lactam antibiotics and the MRSP-related infections were totally under control due to their poor prevalence (van Duijkeren *et al*., 2011); now MRSP has emerged as a significant animal health problem in Veterinary Medicine and Human Medicine. The *mecA* gene, naturally present on mobile elements in the staphylococcal genome, called Staphylococcal Chromosome Cassette (SCCmec) is the genetic cause of methicillin resistance. Several studies have clarified the ability of genetic exchange of SCCmec elements between different staphylococcal species (Wielders *et al*., 2001). In the study of Robinson and Enright, the authors proposed that the *mecA* gene currently found in MRSA may have originally been carried by coagulase-negative staphylococci and later exchanged to *S. aureus* (Robinson and Enright, 2003). Moreover, Kadlec and Schwarz stated, in their study, the identification of most

antibiotic-resistance genes in common between *S. pseudintermedius* genome and other staphylococcal species as well as in a few other Gram-positive bacteria (Kadlec and Schwarz, 2012).

S. pseudintermedius, like *S. aureus* and *S. haemolyticus*, displays multi-resistance against tetracyclines (Schwarz *et al*., 1998), macrolides, lincosamides and streptogramins (Boerlin *et al*., 2001), aminoglycosides and aminocyclitols (Boerlin *et al*., 2001), fluoroquinolones (Intorre *et al*., 2007) and methicillin (Sasaki *et al*., 2007b; Zakour *et al*., 2012). In the *S. pseudintermedius* ED99 genome, (the first strain sequenced in 2011 (Zakour *et al*., 2011)) four transposons containing one or more genes coding for antibiotic-resistance were identified: Tn*552*, Tn*554*-like, Tn*5405* and Tn*5801*. Tn*552* and Tn*554*-like encode for the *bla* operon, responsible for beta-lactam resistance, and were also found in *S. aureus*, *S. epidermidis,* and *S. haemolyticus*. Tn*5405* encodes the aminoglycoside-streptothricin resistance (gene *aad6-sat4-aphA-3)* and is found to be associated with the *ermB* gene, coding for macrolide-lincosamide-streptogramin resistance. Tn*5801* is a conjugative transposon carrying *tetK* gene and coding for tetracyclines resistance (Zakour *et al*., 2012).

The colonization and infections of MRSP have been described in different animal species such as dogs, cats, horses, and birds with higher prevalence in dogs than in cats (Hanselman *et al*., 2009; Weese and Duijkeren, 2009; Ruscher *et al*., 2010). In dogs with pyoderma staphylococcal strains isolated on the skin as the one collected from the lesions are phenotypically indistinguishable and they can act as a reservoir for MRSP infections (Beck *et al*., 2012). In different studies, the prevalence of MRSP colonization was 7% in dogs with skin diseases and 4% in healthy cats, whereas no MRSP was found in cats with inflammatory skin disorders (Pin *et al*., 2006; Hanselman *et al*., 2009; Moon *et al*., 2012). In a recent study, Perreten *et al*. reported the resistance to 11 antibiotics by 103 epidemiologically not related MRSP clones isolated from a dog in Europe (Italy, Germany, France, Denmark, Sweden,

Switzerland, and the Netherlands) and North America (Perreten *et al*., 2010). From a genetical point of view (MLST analysis), the major clonal lineage commonly isolated in Europe is the ST 71 carrying the SCCmec II-III, whereas in America is the ST 68 carrying the SCCmec V (van Duijkeren *et al*., 2011).

1.5. Virulence factors of S. pseudintermedius

The genome of *S. pseudintermedius* is composed of 2,5 Mb and an average G+C content of 37.6% (the average of other staphylococci is 32%). From the analysis of the chromosome, Zakour *et al*. defined that the genetic core of *S. pseudintermedius* is composed of 1214 genes, these are shared between *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, *S. carnosus*, *S. delphini*, and *S. intermedius* (Zakour *et al*., 2011). The genetic core harbors housekeeping genes that are associated with metabolism and homeostasis. Also species-specific genes (e.g. surface binding proteins, adhesion factors, and exoenzymes) are classified as core genome because in most the cases these virulence determinants are essential for pathogenicity and exploitation of pivotal cellular functions. All the other genes are formerly constituent of the accessory genome. This class of genes is considered the most variable because frequently subjected to selective pressure and environmental selection that acts in terms of positive selection (maintenance of genetic function) or negative (loss of non-functional genetic determinants). Horizontal transfer genes (or plasmids), pathogenicity islands transposons and chromosomal cassettes are all examples of accessory genes that act in synergy with the core genome and can be lost or acquired by different bacteria. It is already known that SCCmec of *S. aureus* was derived from coagulase-negative staphylococci and then acquired and clonally shared by coagulase-positive staphylococci (Robinson and Enright, 2003).

Bacteria live in strict connection to the host and the surrounding environment and are absolutely dependant on a precise modulation of virulence determinants expression (Dhanawade *et al*., 2010; Iyori *et al*., 2010a; Cue *et al*., 2012). For this reason, the evolution has selected well-characterized regulatory elements: i) the locus *sar* or staphylococcal accessory regulator and ii) the locus *agr* or accessory gene regulator. In *S. pseudintermedius* genome the following SarA proteins have been identified: *sarA*, *sarR,* and *sarZ* (Bannoehr *et*

al., 2011; Zakour *et al*., 2012; McCarthy *et al*., 2014). They depend on *agr* locus and regulate the synthesis of enzymes with protease activity (Ji *et al*., 2005; Little *et al*., 2019).

Belonging to the two-component regulatory systems family, *agr* locus is devoted to the global communication system called *quorum-sensing* (QS) able to control over 70 different genes (Little *et al*., 2019). This locus consists of 4 genes (agrA, D, C and B) that are co-expressed. The AgrD pro-peptide is the precursor of the autoinducing peptide (AIP) that is the main responsible for QS activities. After the export by AgrB, the AIP molecules are tracked by the intermembrane complex AgrC/AgrA. The activation of AgrA protein induces positive feedback on *agr* locus leading in the upregulation of proteases and toxins and the downregulation of surface proteins (Koenig *et al*., 2004; George and Muir, 2007; McCarthy *et al*., 2014). The presence of different types of AIP strictly define the species-specific activity exerted by the autoinducers; in particular, four AIP variants (RIPTSTGFF [variant I], RIPISTGFF [variant II], KIPTSTGFF [variant III], and KYPTSTGFF [variant IV]) are specific for *S. pseudintermedius* (Sung *et al*., 2006; Bannoehr *et al*., 2007b).

Similar to *S. aureus*, *S. pseudintermedius* expresses a huge panel of virulence factors, including surface proteins (e.g. clumping factor and protein A), enzymes (e.g. coagulase, thermonuclease, and proteases) and toxins (e.g. cytotoxins, exfoliative toxins, and enterotoxins).

The initial adherence of micro-organisms to host epithelial tissue remains the fundamental step without which bacteria can not infect and disseminate through the host's structures. Of pivotal importance to exert this essential function are the Cell Wall Anchored proteins (CWA proteins) which are peptides physically connected to the underlying peptidoglycan layers (Iyori *et al*., 2010b; Crawford *et al*., 2016). Based on structure-function analysis, a classification of CWA proteins was proposed by Foster *et al*., creating four distinct groups: i) Microbial Surface Components Recognizing Adhesive Matrix Molecules

(MSCRAMMs), ii) Near Iron Transporter (NEAT), iii) three-helical bundle motif proteins and iv) G5-E repeat family (Foster *et al*., 2014).

S. pseudintermedius shares with *S. aureus* several host binding-proteins, including those that link to the extracellular matrix (ECM), fibrinogen, fibronectin, and cytokeratin (Bannoehr *et al*., 2011; McCarthy *et al*., 2014). The genome of *S. pseudintermedius* contains 18 cell wall-associated (CWA) proteins classified as *Staphylococcus pseudintermedius* surface proteins (Sps proteins, followed by the capital letter A to R). Two of them (SpsD and SpsL) have been demonstrated to mediate binding to host proteins (e.g. fibrinogen, fibronectin, and cytokeratin-10) (Richards *et al*., 2018). *In vitro* studies have proven evidence about the invasive abilities of both SpsD and SpsL to penetrate canine progenitor epidermal keratinocytes in a fibronectin-dependent manner (Pietrocola *et al*., 2013, 2015). The immunoglobulin G (Ig-G)-binding protein A (spA) was used as a target for molecular typing in *S. aureus*. In *S. pseudintermedius* two Sps proteins (SpsP and SpsQ) were found to be orthologues to spA protein (Bannoehr and Guardabassi, 2012; Balachandran *et al*., 2018).

S. pseudintermedius is able to express different types of exoproteins including: i) enzymes (e.g. lipases, thermonuclease, aureolysin), ii) non-enzymatic activators (e.g. staphylocoagulase), and iii) exotoxins (e.g. cytolytic toxins, leukocidins, exfoliative toxins, and enterotoxins).

In the first group, are encased different virulence factors important for the maintenance of bacteria inside the host's tissues. Triacylglycerol lipase (*lip*) and lipase (*geh*) are considered the most abundant surfactant produced by bacteria, they reduce the surface tension of the medium in which they are secreted, avoiding the bacterial clearance exerted by amphiphilic molecules produced by host's tissues (e.g. fatty acids and lipid molecules), in vitro they impair the function of different immune cells like macrophages or platelets (Jaeger *et al*., 1994). Following the classification proposed by Gautam and Tyagi, the major classes of biosurfactants

include: glycolipids, lipopeptide, lipoprotein, fatty acids, phospholipids, neutral lipids, polymeric biosurfactants, particulate biosurfactant (Gautam and Tyagi, 2006).

Proteases are able to degrade a large variety of pivotal host's proteins. One of the most important proteases in the *Staphylococcus* genus is the aureolysin, a member of the thermolysin family of zinc-metalloprotease. In *S. aureus*, this protein was shown to have a role in staphylococcal innate immune escape through the cleavage of peptide LL-37 (Laarman *et al*., 2011). Another important family of proteases, directly linked to virulence and survival under stress conditions (Frees *et al*., 2003), is ClpP (caseinolytic protease) family. This protease is associated with two different ATPase proteins: ClpA or ClpX. If ClpP is not linked with ATPases, it can only degrade small peptides(Woo *et al*., 1989), but when associated with either ClpA or ClpX it can degrade specific substrates (Frees *et al*., 2003). The *htraA* gene encodes for a serine protease that was found in all the genomes of *S. pseudintermedius* analyzed by McCarthy in 2015. This exoenzyme confers the ability to adhere to fibronectin through the degradation of the fibronectin-binding protein.

One of the most used tests to classify staphylococci is based on the detection of coagulase activity. Currently, seven species of coagulase-positive staphylococci (CoPS) have been identified: *S. aureus*, *S. intermedius*, *S. delphini*, *S. pseudintermedius S. schleiferi* subsp. *coagulans*, *S. hyicus*, and *S. lutrae*, (Sasaki *et al*., 2010). The coagulase test is based on the ability of this enzyme to bind to prothrombin and trigger the conversion of fibrinogen to fibrin (Vanassche *et al*., 2010).

Exotoxins produced by *S. pseudintermedius* have the ability to lyse numerous cell lines because their molecular targets reside on the cytoplasmatic membrane.

Among cytotoxins, *S. pseudintermedius* produces two of the four predicted hemolysins of *S. aureus*, α-hemolysin, and β-hemolysin and is able to cause hemolysis of rabbit erythrocytes and hot-cold hemolysis of sheep erythrocytes (Bannoehr and Guardabassi, 2012).

As happen for *S. aureus*, β-hemolysin has a peculiar activity against sphingomyelin (Dziewanowska *et al*., 1996) this characteristic explains why the erythrocytes of ungulates (naturally with a high content of sphingomyelin) are more susceptible to this cytotoxin.

Leukocidins have to be taken into account when describing the virulence of staphylococcal strains. *S. pseudintermedius* produces a bi-component leukocidin, Luk-I. Its characterization and the analysis of the sequence have shown that similar to PVL, Luk-I is encoded by two co-transcribed genes, lukS and lukF, in the lukI operon (Keiko Futagawa-Saito *et al*., 2004). Luk-I has a specific cytotoxic activity for polymorphonuclear cells but is only slight hemolytic for rabbit erythrocytes (Prevost *et al*., 1995).

Being the first causative agent of dermatological-related infections, the ability of *S. pseudintermedius* to produce different exfoliative toxins is not a novelty. It is known, today, that this pathogen is able to express three distinct exfoliative toxins: SIET, ExpA, and ExpB. The first that has been described, in 2006, is the *Staphylococcus pseudintermedius* exfoliative toxin (SIET) (Lautz *et al*., 2006). This toxin has a specific effect on epithelial cells. Through numerous *in vitro* tests, in which animal models (e.g old chickens, hamsters, and dogs) were exposed by subcutaneous injections to this cytotoxin, the exfoliative effect was observed 1-day post-inoculation (Terauchi *et al*., 2003b, 2003a). In particular, dogs injected with purified SIET shown clinical signs (e.g. erythema, exfoliation, etc) similar to symptoms seen in pyoderma (Bannoehr and Guardabassi, 2012). The second exfoliative toxin, originally EXI and subsequently ExpA, was identified in three canine isolates by PCR with primers designed for the amplification of exfoliative toxins of *S. aureus* and *S. hyicus* (Futagawa-Saito *et al*., 2009). The sequence homology of ExpA with exfoliative toxins of *S. aureus* and *S. hyicus* was about 43-68%. The third and last toxin, ExpB, was discovered in 2010 (Iyori *et al*., 2010a). It is able to cause intraepithelial splitting targeting desmoglein-1 (Dsg-1) only in dogs.

The last family of virulence determinant analyzed are those able to cause an unnatural immune response and commonly known as superantigens, in particular, enterotoxins. Also, *S. pseudintermedius* is able to produce two different enterotoxins. A distinct variant of *S. aureus* SEC was the fist described, being found in dogs the new superantigen was named SEC_{canine} (Edwards *et al*., 1997). This protein share with all the other component of the enterotoxin family, quite the same sequence and exactly the same function, being able to cause T- cells proliferation. The second enterotoxin that has been discovered was se-int in 2004 (K. Futagawa-Saito *et al*., 2004).

1.6. Quorum-sensing and biofilm

Biofilm was supposed to be discovered by Antoni Van Leeuwenhoek, the father of modern microscopy, during his studies on tooth surfaces that lead to the observation of the first microbial biofilms even if not knowing about its existence. In 1943 Zobell experimentally demonstrated that the number of bacteria adherent to a surface in seawater was substantially higher than in the seawater itself (Zobell, 1943). A few years later, Costerton presented a theory of biofilm that focused on the mechanisms by which bacteria adhere to living and non-living materials (Costerton, 1978). In its study, Costerton, observed that bacterial aquatic communities, adherent on a surface, were encased in a glycocalyx matrix that was shown to mediate adhesion, suggesting that biofilm formation could be assumed as an evolutionary mechanism that allows bacteria also to survive in competitive environments, supporting the selection of pathogens protected from the external ecosystem (Costerton, 1978).

The most complete and accepted definition of biofilm was given by Donald and Casterton and assume that "[biofilm] *is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription*" (Donlan and Costerton, 2002). From this definition can be derived that biofilm-forming bacteria have a unique gene expression pattern that results different to the planktonic counterpart; experimentally it was observed that various genes, varying from 1 to 38% of the total genome, can be up- or down-regulated during this process (Sauer, 2003). The biofilm is a complex and dynamic architecture coordinated by the *quorum-sensing* communication system and based on five steps involving: *i*) attachment, *ii*) aggregation, *iii*) accumulation, *iv*) maturation and *v*) detachment (Otto, 2009).

Staphylococcal attachment is the first and most important moment in biofilm formation, without the commitment between the cell surface and the host, the colonization and infection could not easily happen. The expression of proteins devoted to bacterial adhesion to the host tissues remains fundamental. The family of Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMs) together with wall teichoic acid (WTA) and adhesins/autolysins (*AltE* and *Aea*) play a crucial role in the initial attachment (Arciola *et al*., 2005; Kong *et al*., 2006; Otto, 2009; Cue *et al*., 2012). The presence of covalent bonds is ensured by a family of enzymes (sortase) that link the bacterial peptidoglycan to the conserved motif expressed in MSCRAMMs (Mazmanian *et al*., 1999).

Subsequently, during the aggregation and accumulation phases, the production of Polysaccharide Intercellular Adhesin (PIA)/poly-N-acetylglucosamine (PNAG) ensures the cell-to-cell adhesion process. The synthesis of PIA is controlled by the *ica*ADBC locus which gives the ability to produce PNAG in *ica*-dependent biofilm producers (Cue *et al*., 2012; Casagrande Proietti *et al*., 2015). The extracellular matrix is mainly composed of exopolysaccharides (PNAG), proteins (e.g. *Bap*, *Aap*, fibronectin-binding proteins *FnbPs*) and extracellular DNA (eDNA) (Arciola *et al*., 2005, 2012). Staphylococci are also able to produce biofilm through an *ica*-independent pathway mainly controlled by biofilm-associated protein (*Bap*) and Accumulation Associated Protein (Aap) (Kirmusaoglu, 2016).

A mature biofilm has a specific three-dimensional structure which has been defined as “tower” or “mushroom” (Costerton *et al*., 1999). In between these structures, there are water channels that are believed to have a crucial function in the physiology of the biofilm itself because are devoted to the delivery of nutrients to cells in deeper layers(Götz, 2002; Otto, 2004). A progressive decrease in oxygen levels and nutrient delivery was observed in the deeper biofilm layers; at this level, bacterial cells have a particular gene expression pattern and a quiescent metabolism (Dhanawade *et al*., 2010; Singh *et al*., 2013; Stefanetti *et al*., 2017).

Finally, in the detachment phase, bacteria detach from the mature matrix and disperse throughout the host tissues. Several factors may drive the detachment: i) mechanical forces, ii) suspension of exopolysaccharide production, iii) detachment factors (e.g. enzymes). Via *quorum-sensing*, bacteria communicate with each other to detach from the surface (Shopsin *et al*., 2003; O'Gara, 2007). An *in vivo* study focused on *S. epidermidis* showed how this step depends on a well-characterized *quorum-sensing* system, using *agr* mutant bacteria in a rabbit model for medical device-related infection (Vuong *et al*., 2004). This locus directly controls some crucial pathways up-regulating acute virulence factors and down-regulating surface proteins involved in bacterial adhesion (Otto, 2004, 2009).

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Aim

The introduction section reviewed current knowledge concerning *Staphylococcus pseudintermedius* (SP). Deep bibliographic research was fundamental to describe its molecular typing approaches and provide strong data about the genetic structure of the circulating clones. The antibiotic-resistance screening of dog-derived strains remains a basic element to support Veterinary clinicians in the prescription of the most effective antibiotic in a one-health vision of both human and animal Medicine. The plastic genome of all staphylococcal species helps bacteria to exchange genetic information, resulting in the acquisition of new genetical traits that undergo natural selection. Quite all the knowledge about the virulence determinants properly possessed by *S. pseudintermedius* is resumed in the relative chapter, making sometimes comparisons with *S. aureus*. In the last decades, biofilm has raised as a new and powerful enemy to fight against, especially due to its natural antibiotic resistance and the rapid spread, between the bacteria encased inside the slime structure, of antibiotic-resistance genes.

To address these issues, the first aim of the thesis was to describe the genetic structure of a population of SP, isolated from canine pyoderma and Holstein-Friesian's milk, using molecular typing techniques and determine the dissemination of specific virulence determinants (Chapter 1).

The description of antibiotic-resistance was the second aim of the thesis. Two large populations of SP, isolated in two Italian veterinary teaching hospitals, were compared in terms of antibiotic-resistance to a common panel of veterinary prescribed antibiotics. We coupled the phenotypic analysis with the genetic amplification of antibiotic-resistance genes (Chapter 2).

The disease is the result of the interaction between host and microorganisms. The unbalance of one lead in the victory of the second. For this reason, the third aim of this thesis was the description of the quorum-sensing pathway and the determination of the ability to form biofilm using both MDR and non-MDR SP strains (Chapter 3).

Thereafter, the last chapter is based on the use of silver nanoparticles as an alternative strategy to fight against infectious pathogens.

Chapter 1

Molecular characterization of *Staphylococcus pseudintermedius* strains isolated from deep pyoderma in dogs and cows

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Conflicts of interest

The authors declare that they have no conflict of interest

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors

Article in preparation

Abstract

Background – *Staphylococcus pseudintermedius* is considered one of the main causative agents of canine pyoderma and regarded as a potential zoonotic pathogen.

Hypothesis/Objective – To investigate the genetical relatedness of *S. pseudintermedius* strains isolated from dogs and cows

Animal – Samples were collected from 42 dogs with clinical deep pyoderma and 5 cows with intramammary infection (IMI).

Methods and materials – The Restriction Fragment Length Polymorphism PCR (RFLP-PCR), the Ribosomal Spacers Amplification PCR (RSA-PCR) and the Random Amplification of Polymorphic DNA (RAPD-PCR) were coupled with the sequencing of *sodA*, *pheS,* and *rpoA* genes. The detection of specific exfoliative toxins genes (*siet*, *expA,* and *expB*), enterotoxin seC_{canine} and bicomponent leukotoxin *lukS-I/lukF-I* was used to screen the pathogenicity potential of the strains.

Results – The sequencing revealed that 40 out of 47 original strains were *S. pseudintermedius* the remaining 7 were not the expected species. This result was in accordance with all the typing techniques used. RSA-PCR revealed the presence of a single biotype for all the interested strains while RAPD-PCR showed a clear genetic relatedness also between canine and the 5 bovine staphylococcal strains. All the isolates were positive for the *siet* gene, while *expA* and *expB* were amplified by, respectively, 10% and 17,5%. Specific genes for enterotoxin seC_{canine} and leukotoxin luk-I were respectively found in 35% and 92,5% of staphylococci.

Conclusions and clinical importance – The results confirm at the genetic level the relatedness between strains from both dogs and bovine, underlying the potential transmission to humans too.

Introduction

Staphylococcus pseudintermedius is a normal component of the skin and mucosae of dogs and cats and can be isolated from nares, mouth, pharynx, and anus (Devriese *et al*., 2005). It is also a commensal pathogen regarded as the leading cause of skin (pyoderma), ear and post-surgical infections in dogs (van Duijkeren *et al*., 2011; Bannoehr and Guardabassi, 2012; Tabatabaei *et al*., 2019). This pathogen is one of the last added members in the *Staphylococcus* genus, being described in 2005 in dogs and pigeons (Devriese *et al*., 2005), firstly isolated in human in 2006 (Van Hoovels *et al*., 2006) and with a molecular identification protocol available from 2009 (Bannoehr *et al*., 2009). In recent years, the description of *S. pseudintermedius* as a human pathogen is being increasingly reported in particular for the global spread of methicillin-resistant clones (MRSP) (Little *et al*., 2019). The incidence of MRSP strains has increased significantly in the past few years and has emerged as an important problem because of multidrug resistance-associated to oxacillin resistance and the limited number of antibiotics choices remaining to face with infections caused by this microorganism (Kang *et al*., 2014; Stefanetti *et al*., 2017).

As a member of the *Staphylococcus* genus, *S. pseudintermedius* has an extensive panel of virulence factors, some of them are closely related to those expressed by *S. aureus* (van Duijkeren *et al*., 2011). The majority of these are firstly involved in the colonization of host tissues and in the dissemination in the colonized district. In particular, exfoliative toxins (*siet*, *expA,* and *expB*) are virulence factors involved in canine pyoderma, mainly found among staphylococcal strains isolated from skin infections (Futagawa-Saito *et al*., 2009; Iyori *et al*., 2010; Yoon *et al*., 2010 a,b). The cytotoxic potential of these proteins was studied in dogs in 2003 by Terauchi revealing that dogs underwent local injection of purified exfoliative toxins

develop pathognomonic signs of clinical pyoderma such as erythema, exfoliation, and crusting (Terauchi *et al*., 2003).

In this study, 40 *S. pseudintermedius* strains out 47 isolates from clinical cases of deep pyoderma were analyzed with three commonly used typing techniques (RFLP-PCR, RSA-PCR, and RAPD-PCR) to determine their genetic relation. Moreover, the presence of gene coding for staphylococcal exfoliative toxins (*siet*, *expA,* and *expB*), bicomponent leukotoxin (*lukS-I* and *lukF-I*) and enterotoxin seC_{canine} by previously reported PCR.

Materials and methods

Bacterial isolation and phenotypic identification

A total of 47 samples were included in this study: forty-two were obtained from clinical cases of deep canine pyoderma (cutaneous swabs), and five from the milk of Holstein-Fresian bovines with intramammary infection. Bacteria were isolated in the Microbiology Laboratory of the Department of Veterinary Medicine (University of Milan) while the molecular characterization was performed at the molecular microbiology laboratory of the Institute of Sciences of Food Production (Milan) - National Research Council (ISPA-CNR).

Cutaneous swabs were plated on TSA+5% sheep blood (Microbiol, Uta, Italy) and incubated aerobically at 37°C for 24 hr. Following the morphological characteristics of Staphylococcus genus (colonies whit white appearance, regular shape, and total or partial hemolysis), the suspected staphylococcal colonies were sub-cultured on Mannitol Salt Agar (MSA, Microbiol, Italy) and incubating at 37°C for 24 hours. A single isolated colony from MSA was grown in 10 ml of Brain Heart Infusion broth (BHI, Scharlau, Barcelona, Spain) and incubated at 37°C for 24 hr. The purity of the isolates was checked by streaking repeatedly on Homofermentative-Heterofermentative Differential (HHD) (Biolife, Milan, Italy) agar and sub-

culturing using the same isolation media and temperatures. After purification, the strains were also examined using standard techniques: Gram stain, catalase and coagulase test.

For the five samples from bovine, milk sample analysis was carried out following the National Mastitis Council guidelines (Adkins *et al*., 2017). The isolates were cultured in 10 ml of BHI broth and, after incubation at 37°C for 24 hr, stored at +4°C for further analysis. The strains were also stored in duplicate; in sterile milk at -20°C and in 25% of glycerol at -20°C.

DNA extraction

The DNA was extracted starting from 1 ml of overnight bacterial culture containing approximately $2x10^9$ CFU/ml (spectrophotometrically measured at 550 nm) (LabSystem Multiskan Plus, Thermofisher Scientific) using the commercial kit DNA Isolation System (Clonit, Milan, Italy) according to the manufacturer's instructions. After the extraction, the amount of DNA was checked for its quantity and quality using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). Approximately an average of 82 ng/μL was obtained from 10^9 CFU/ml.

Target gene		Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
16S-23S	F	GAAGTCGTAACAAGG	*	(Jensen *et al*., 1993)
	R	CAAGGCATCCACCGT		
pta	F	AAAGACAAACTTTCAGGTAA	320	(Bannoehr *et al*., 2009)
	R	GCATAAACAAGCATTGTACCG		
siet	F	ATGGAAAATTTAGCGGCATCTGG	359	(Lautz *et al*., 2006)
	R	CCATTACTTTTCGCTTGTTGTGC		
expA	F	GTKTTAATTGGWAAAAATACA	413	(Futagawa-Saito *et al*., 2009)
	R	ATNCCWGAKCCTGAATTWCC		
expB	F	GGGCATGCACATATGATGAAGCC	843	(Iyori *et al*., 2010)
	R	CCAGATCTATCTTCTGATTCAGC		
rpoA	F	ATGATYGARTTTGAAAAACC	820	(Naser *et al*., 2005)
	R	ACHGTRTTRATDCCDGCRCG		
pheS	F	CAYCCNGCHCGYGAYATGC	488	
	R	CCWARVCCRAARGCAAARCC		
sodA	F	CATAACACTTATGTTACTAAATTAAAA	233	(Heikens *et al*., 2005)
	R	ATCTAAAGAACCCCATTGTTC		
seC$_{canine}$	F	GGCGGCAATATTGGCGCTCG	271	(Jang Won Yoon *et al*., 2010)
	R	TTACTGTCAATGCTCTGACC		
lukS-I	F	TGTAAGCAGCAGAAAATGGGG	503	
	R	GCCCGATAGGACTTCTTACAA		(Futagawa-Saito *et*
lukF-I	F	CCTGTCTATGCCGCTAATCCA	572	*al*., 2004)
	R	AGGTCATGGAAGCTATCTCGA		
M13		GAGGGTGGCGGTTCT	*	(Hassan *et al*., nd)
OPA10		GTGATCGCAG	*	(Andrighetto *et al*., 2001)

* amplicon size depends on the bacterial species

Table 1

Primers used in this study

Ribosomal Spacers Amplification PCR (RSA-PCR)

The RSA-PCR was firstly described by Jensen *et al* (1993) as a test that "facilitates genus and species identification of different bacteria without the requirement of identification" (Jensen, 1993). According to this author, amplification reactions were carried out in a final volume of 25 µL containing 2 µL of DNA extract, 12,5 µL of PCR Master Mix 2X (Thermo Fisher Scientific, Massachusetts, USA), 20 pmol each forward and reverse primer (see Table 1) and 10,1 µL of DNase free water. PCR reactions were ended in a Mastercycler ep Gradient S (Eppendorf) with initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 7 min, extension at 72°C for 2 min followed by a final extension at 72°C for 7 min. The amplified PCR products were analyzed on 2,5% (w/v) gel agarose (Gel Phor, Euroclone, Milan, Italy) in 1X TAE buffer stained with Atlas Clear Sight DNA Stain (Bioatlas, Tartu, Estonia) at 100V for 2,5 hours.

Random Amplification of Polymorphic DNA (RAPD-PCR)

RAPD-PCR was performed with two universal primers taken from literature: M13 and OPA 10. The amplification reaction consisted of 1 µL of DNA extract in a total volume of 25 µL composed of 2,5 µL MgCl 10 X Buffer (Finnzymes, *Espoo*, Finland), 5mM each dNTPs (Finnzymes, *Espoo*, Finland), 125 pmol of primer and 2 U DyNazyme *II* DNA Polymerase (Finnzymes). PCR reactions were carried out in a Mastercycler ep Gradient S (Eppendorf) following the conditions described below: (i) M13: 35 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 20 seconds, extension at 72°C for 2 min followed by a final extension step at 72°C for 7 min. (ii) OPA 10: 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1min, extension at 72°C for 1,5 min followed by a final extension at 72°C for 7 min. DNA fragments were analysed by electrophoresis on 1,5% (w/v)agarose gel (GelPhor) in 1X TAE at 100V for 1hr.The RAPD-PCR profiles analysis was done with Gel Compar vers. 5.0

software (Applied Maths, Sint-Martens-Latem, Belgium), using the Pearson product-moment correlation coefficient and UPGMA cluster analysis.

Sequencing

In order to be sure about the phenotypic identification, the sequencing of all the strains has been performed. The following genes were chosen from literature and amplified using the same conditions described by authors: *sodA*, *rpoA,* and *pheS* (Naser *et al*., 2005). After amplification, the PCR products were analyzed on 1% (w/v) agarose gel (GelPhor) and then purified using the commercial kit Wizard® SV Gel and PCR Clean-up System (Promega, Wisconsin, USA) according to manufacturer's instructions. The purified PCR products were sent to Macrogen Europe (Macrogen, Amsterdam, Netherlands) for sequencing. The deduced nucleotide sequences were analyzed with the BLAST programs (National Center for Biotechnology Information, NCBI) for the homology search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Restriction Fragment Length Polymorphism PCR (RFLP-PCR)

As previously described by Bannoehr in 2009, this assay is based on the amplification of the *pta* gene (coding for phosphotransacetylase) followed by the enzymatic digestion with MboI. For the enzymatic restriction, performed in a final volume of 20 µL, 1 µL of Anza 55 *MboI* (Thermo Fisher Scientific), 8 µL of PCR products and 2 µL of 10X Red Anza Buffer (ThermoFisher Scientific) were used. After digestion at 37°C for 15 min, was performed the electrophoretic analysis on 2% (w/v) agarose gel at 100V for 1 h. The gel was photographed under ultraviolet light.

Virulence factors

As a member of the *Staphylococcus* genus, also *S. pseudintermedius* is able to produce a panel of different virulence factors. The specific genes are reported in Table 1. Predicted amplicon size and primers specificity were defined using BLAST search available through the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and coupled with BioEdit freeware software (https://omictools.com/bioedit-tool). Either the amplification condition and amplicon size were available by the references indicated in table 1. The amplification for each gene was carried out with PCR Master Mixer (2X) (ThermoScientific, Italy) following the manufacturer's instructions in a final volume of 25 µL.

Results

RSA-PCR

The amplification of 16S-23S spacers created a set of bands specific for the characterization of different species of staphylococci. Fig. 1 showed the pattern of bands (biotypes) for some strains of interest. It appeared clear, on the 47 strains analyzed, the presence of seven patterns that were different from the majority. Each one represented a bacterium belonging to the staphylococcal genus but not the interesting species. In particular, 5 of these profiles were the same, suggesting the presence of five strains belonging to the same species but still different from the interested one. More in detail, the pattern of *S. pseudintermedius* consisted of three bands between 450 bp and 650 bp. Focusing on this profile and according to some previous studies made on *S. aureus* (Cremonesi *et al*., 2005; Bardiau *et al*., 2016; Graber, 2016), *S. pseudintermedius* strains here analyzed didn't have different biotypes supporting the hypothesis of lack of interspecies variation.

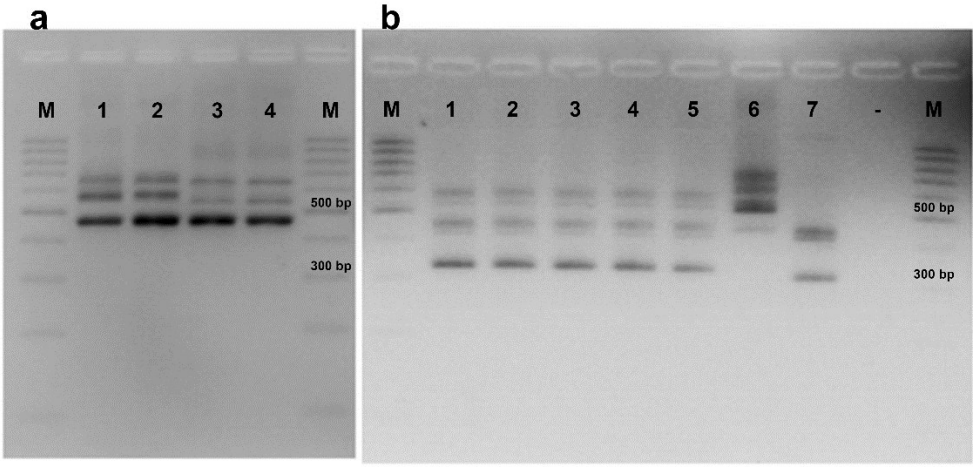

Figure 1

RSA-PCR profiles of some analyzed strains. (A) lanes 1-4 *S. pseudintermedius* profiles, M: 100 bp molecular marker. (B) profiles of the 7 non-*S. pseudintermedius* strains. Lanes 1-5 *S. schleiferi*, lane 6 *S. aureus*, lane 7 *E. faecalis*; M: 100 bp molecular marker.

RAPD-PCR

The presence of species different from *S. pseudintermedius* appeared also clear by the RAPD-PCR analysis with the primers M13 and OPA 10 (Fig. 7). At 90% of similarity two clusters were shown. The first one grouped 40 strains, 35 (87,5%) came from dogs and 5 (12,5%) from cows. In this case, could be noted the genetic similarity between the canine isolates and the five of bovine. The second was composed of 7 different strains in concordance with the RSA-PCR profiles findings, in fact, 5 out of 7 strains showed a high degree of genetic similarity.

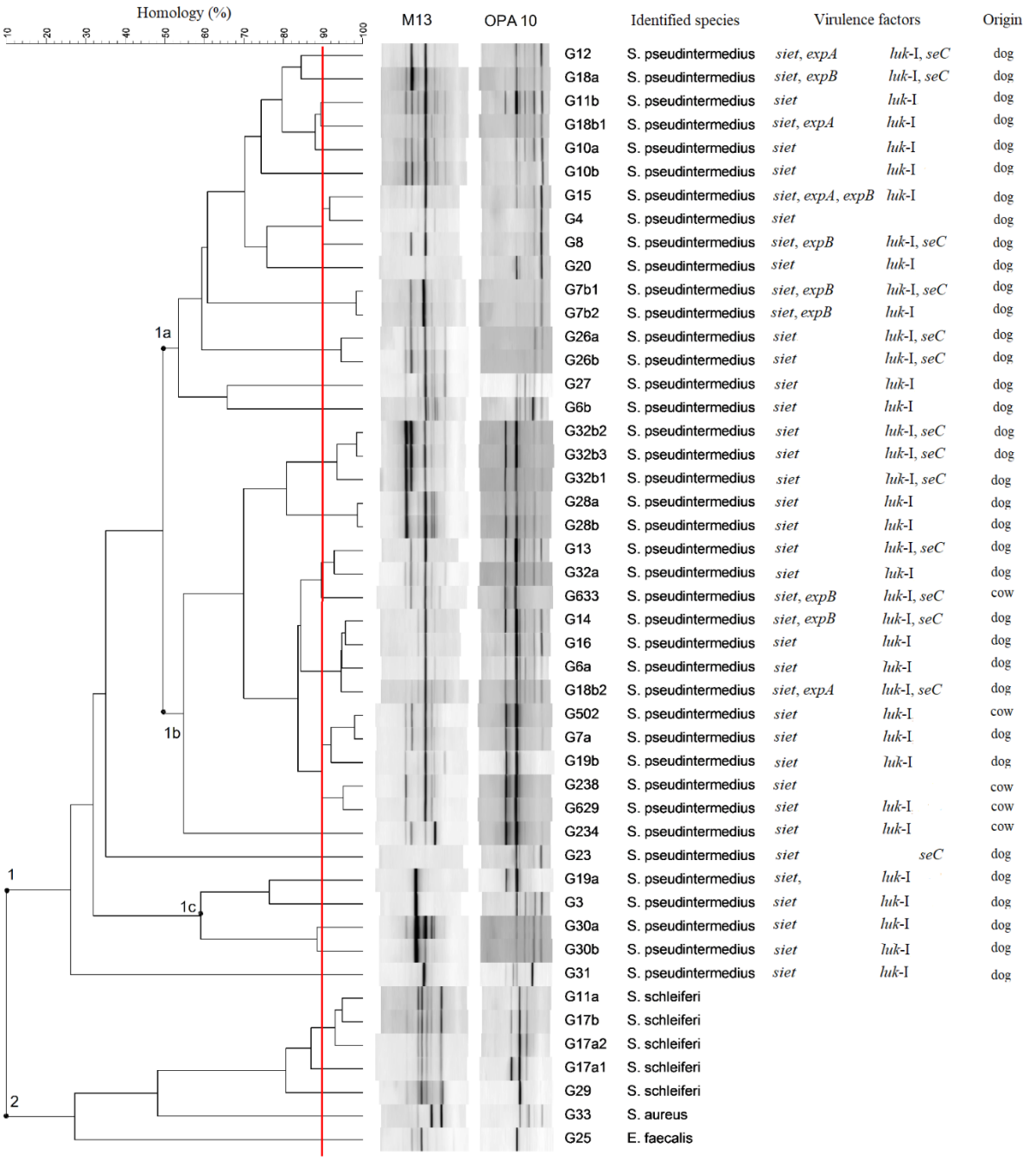

Figure 2

Dendrogram derived RAPD-PCR profiles generated with primers M13 and OPA10. The amplification of specific virulence factors is reported near each strain.

Sequencing

As described above, the sequencing involved all the strains subject of this study. Despite Sasaki *et al* (2007) suggestions, *sodA* wasn't the most discriminative gene for the identification of *S. pseudintermedius*; in fact, after aligning the sequences of *sodA*, *pheS,* and *rpoA* in BLAST the percentage of correct identification were 83% (25/30), 93% (13/14) and 70% (7/10) respectively. In concordance with the results obtained with the previous techniques, the sequencing clarified the presence of strains belonging to the *Staphylococcus* genus but not at the interesting species. on the 47 strains analyzed in this study, 40 were identified as *S. pseudintermedius* the remaining 7 were different species. in particular, 5 strains of *S. schleiferi*, 1 of *S. aureus* and 1 of *Enterococcus faecalis*. The last one was thought to be a contaminant, from the literature it could be able to grow on MSA and misidentified as *S. pseudintermedius*. For this reason, the research for exfoliative toxins was conducted over the *S. pseudintermedius* strains only.

RFLP-PCR

As supposed, the enzymatic digestion produced two restriction fragments: the first of 213 bp and the second of 107 bp in all *S. pseudintermedius* strains Fig. 3. According to Bannoehr *et al*., (2009) also *S. aureus* contained a single *MboI* restriction site that produced two fragments of 156 bp and 164 bp. Unfortunately, the electrophoretic analysis on 2% (w/v) agarose gel wasn't able to separate these two fragments, resulting in the visualization of a single band. All the other 6 strains of different species were positive for *pta* detection but didn't contain any MboI restriction site resulting in the lack of fragments generation.

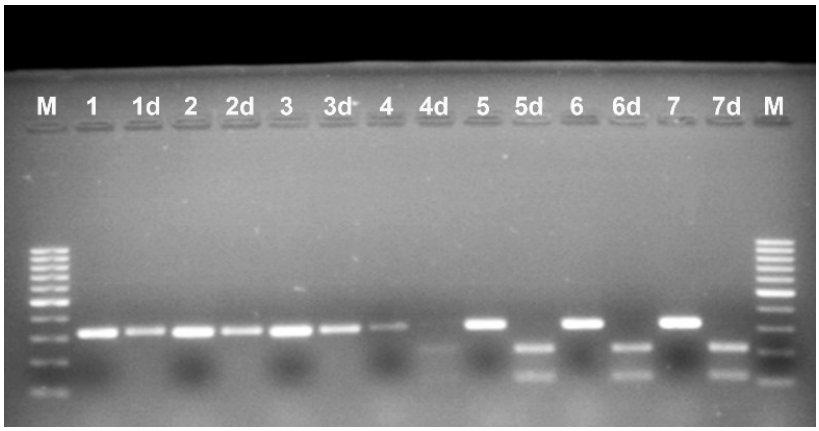

Figure 3

RFLP-PCR of some strains. Lane 1 *pta* from *S. aureus*, lane 2 *pta* from *S. schleiferi*, lane 3 *pta* from *E. faecalis*. Lanes 4-7 *pta* from *S. pseudintermedius*. The lanes with final "d" contain cleaved *pta* amplicons. Only *pta* amplicons of *S. pseudintermedius* strains present two fragments.

Virulence factors

The prevalence of the virulence factors was calculated only on the 40 *S. pseudintermedius* strains and reported in Fig. 4. Fig. 2 illustrates the dissemination of the virulence determinants among each strain. The PCR results demonstrated that all the strains carried the *siet* gene, while *expA* and *expB* were found in 10% and 17,5% respectively. Only one strain carried these three genes. Thirty-seven strains out 40 were positive for the leukocidin gene while the 35% carried seC_{canine}.

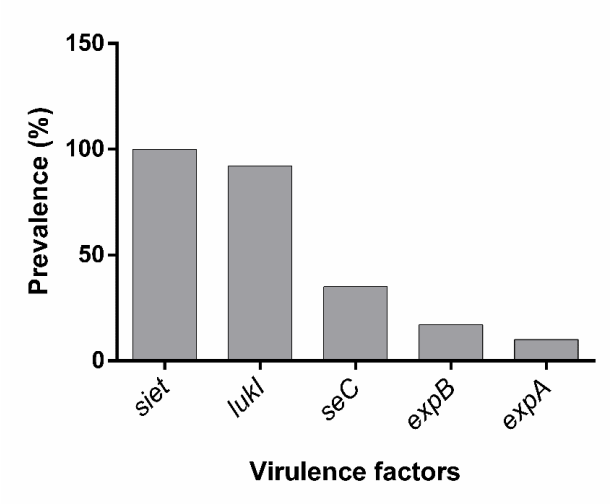

Figure 4

Distribution (%) of the virulence determinants among the 40 *S. pseudintermedius* strains

Discussion

The genetic similarity of the 40 staphylococcal strains was clear by RAPD-PCR and, coupled with RSA profiles, allows the identification of three distinct sub-groups inside the 40 *S. pseudintermedius* strains (1a, 1b, 1c). From literature, it was not so common to isolate *S. pseudintermedius* from bovine because traditionally it was related to cutaneous disease in dogs, in addition in this study, this pathogen was found in milk (Bannoehr and Guardabassi, 2012). It has to be taken in account that in the majority of bovine farms the presence of guardian dogs is a common finding, so we hypothesize that the original reservoir of *S. pseudintermedius* has to be found in guardian dogs that could have infected bovine leading in the intramammary infection development (Igbinosa *et al*., 2016).

Among the virulence determinants analyzed in this study, *siet* and the gene coding for the bicomponent leukocidin were the two most representative with the highest prevalence respectively about 100% and 92,5% among the 40 strains. One of the most interesting observations is that 14/40 (35%) staphylococcal strains carried both *siet* and seC_{canine} genes

while in the study of Yoon (2010 a) the authors found a lower prevalence about 24%. The second most interesting finding is that 13/40 (32.5%) isolates simultaneously carry *siet*, seC_{canine} and *lukI* genes. Staphylococcal exfoliative toxins act like "molecular blades" while bacteria penetrate the skin by cleaving cell-to-cell adhesion proteins. In that sense staphylococci that carry at the same times genes coding for exfoliative toxins and leukocidins may better penetrate and reverse the immune system. It is already known, for *S. aureus* only, a clear correlation between RSA profile and the presence of specific virulence factors (genotype B [most frequently isolated from IMI] is associated with *sed*, *sej*, *sea* and *lukE*; genotype C is linked to *seg*, *sec*, *tst* and *lukE*) (Fournier *et al*., 2008; Graber *et al*., 2009); this correlation is absent for *S. pseudintermedius*. The amplification of polymorphic regions between 16S and 23S sequences has played a crucial role in past typing techniques.

In conclusion, it is clear that strains from dogs and cows share genetic relatedness. The presence of a single biotype may be a good finding in terms of genetic variation but the presence of different virulence factors highlights a potential zoonotic risk.

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Chapter 2

Original article

Prevalence and antimicrobial susceptibility patterns of canine *Staphylococcus pseudintermedius* strains isolated from two different Italian university veterinary hospitals.

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Article in preparation

Abstract

Staphylococcus pseudintermedius is one of the last added members in the *Staphylococcus* genus, and is considered one of the most frequently isolated bacteria on the skin of dogs and, recently, recognized as a zoonotic pathogen able to cause severe diseases also in humans.

This study aimed to identify methicillin-resistant and -susceptible *Staphylococcus pseudintermedius* (MRSP and MSSP) strains isolated from canine skin disorders and to determine their antimicrobial resistance patterns. The study was carried out during 2015-2017 at veterinary microbiology laboratory of two different Italian veterinary teaching hospitals, Milan and Naples.

The statistical comparison of the results from the two different locations revealed significant differences of MRSP prevalence, displaying values of 30% (35/116) and 18% (23/126) in Milan and Naples, respectively (*p-value*= 0,0351).

Furthermore, all the MRSP strains in Milan and 91% in Naples resulted as multidrug-resistant (MDR). Among the MSSP strains, 7% and 34%, respectively in Milan and Naples, resulted MDR showing statistical differences (*p-value*= <0,0001).

These results confirmed the differences in MRSP and MSSP prevalence and highlight the antimicrobial resistance patterns in the two different geographical areas. However, the high prevalence of multidrug resistance in canine *S. pseudintermedius* strains, either MRSP and MSSP, stands out the need for further investigation considering its impact and importance on animal and human health.

Introduction

Skin and ear infections in dogs 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, *S. pseudintermedius* is an opportunistic pathogen and a leading cause of skin, ear and post-operative wound infections in dogs and marginally in cats (Ross Fitzgerald, 2009; van Duijkeren *et al*., 2011)

Canine otitis externa and pyoderma are featured consistently as the major diseases affecting canine skin system. Bacterial pyoderma is usually triggered by an overgrowth/over colonization of normal resident or transient flora. Since *S. pseudintermedius* is a normal commensal of dog, it is the most commonly responsible for canine pyoderma, in particular, superficial pyoderma. Precisely, *S. pseudintermedius* can be isolated from nares, oral mucosa, pharynx, forehead, groin, and anus of healthy dogs (Garbacz *et al*., 2013).

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 the canine population. However, already since 2006 methicillin-resistant *S. pseudintermedius* (MRSP) strains have been isolated in Europe, becoming a relevant problem in veterinary medicine.

MRSP has been reported with an increasing frequency (Loeffler *et al*., 2007; Duijkeren *et al*., 2011; Kasai *et al*., 2016) and they show often multidrug resistance profiles worldwide, including resistance to several classes of antimicrobial drugs (Perreten *et al*., 2010). This behavior limits the treatment options and represents a relevant threat to small animal therapy in veterinary medicine, challenging infection control measures (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 Duijkeren, 2009).

The *S. pseudintermedius* virulence potential and its zoonotic transmission should not be underestimated, even though it is not often reported. In fact, *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; R. Somayaji *et al*., 2016; Ranjani Somayaji *et al*., 2016; Lozano *et al*., 2017; Robb *et al*., 2017). As a member of the *Staphylococcus* genus, *S. pseudintermedius* has an extensive panel of virulence factors, some of them are closely related to those expressed by *S. aureus* (Duijkeren *et al*., 2011). The majority of these are firstly involved in the colonization of host tissues and in the dissemination in the colonized district. In particular, exfoliative toxins (*siet*, *expA,* and *expB*) are virulence factors involved in canine pyoderma, mainly found among *Staphylococcal* strains isolated from skin infections (Futagawa-Saito *et al*., 2009; Iyori *et al*., 2010; Yoon *et al*., 2010). Staphylococcal exfoliative toxins act like "molecular blades" during the penetration of the bacteria through the skin by cleaving cell-to-cell adhesion proteins. In that sense staphylococci that carry at the same time, genes coding for exfoliative toxins and leukocidins may better penetrate and reverse the immune system.

For the best knowledge of all the authors, this is the first study focused on a comparison in Italy for the description of antibiotic-resistance of *S. pseudintermedius*. This study aims to describe the phenotypic/genotypic features and the antibiotic resistance patterns of both MRSP and MSSP strains, isolated from dogs suffering from skin disorders in two different Italian veterinary teaching hospitals.

Materials and Methods

Samples collection

The present study was designed to investigate the isolation of *S. pseudintermedius* strains that had been collected from dogs in the years 2015-2017 from two Italian university veterinary hospitals, located in Milan and Naples, during routinary microbiological examinations. *S. pseudintermedius* strains were isolated from dogs with skin disorders (e.g. pyoderma, otitis externa). A collection of 242 *S. pseudintermedius* isolates were included in this study; more in details 116 strains were isolated in Milan and 126 in Naples.

Isolation and phenotypic identification of S. pseudintermedius

Upon arrival at the laboratories, specimens were cultured and streaked on Columbia CNA agar (Liofilchem, Italy) and incubated aerobically at 37°C for 24. Suspected staphylococcal colonies were firstly identified by standard, rapid screening techniques: colony morphology, β-hemolysis, cellular morphology (after Gram's staining), catalase test and then sub-cultured on Mannitol Salt Agar (MSA, Liofilchem, Italy). Each mannitol salt negative colony was also subjected to staphylocoagulase (tube coagulase) test (Oxoid, Ltd, UK) to confirm their capacity to produce coagulase enzyme.

Proteomic profile was assessed by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) analysis (Bruker Daltonics, Germany), using fresh colonies. Briefly, a bacterial colony was loaded in the plate for mass spectrometry, and then 1 µl of the organic matrix (usually cinnamic acid) was added to the sample. Afterward, the plate was placed in the equipment for MALDI-TOF MS analysis and the identification was based on the score value released by the equipment's instructions. Values from 2.3 to 1.9 indicated the best identification of genus and species of staphylococci (Santos *et al*., 2013, Nisa

et al., 2019). All the strains were stored in 25% glycerol (Carlo Erba, Italy) at -20°C until use. Before use, samples were thawed at room temperature and 10 µL were plated on Tryptic Soy Agar + 1% sheep blood (Microbiol, Italy) and incubated aerobically at 37°C for 24 h. Three or four isolated colonies were picked up and used for the analyses.

Genetic identification of S. pseudintermedius

For the molecular identification of the selected strains, DNA was extracted from fresh cultures of each *S. pseudintermedius* isolates. The bacterial DNA extraction was carried out by two different protocols: in Milan, the boiling method was used (Adwan *et al*., 2014), while the commercial kit Isolate II Genomic DNA Kit (Bioline, London, UK) was used in Naples.

Referring to the first method, 1 to 5 colonies were picked up from the pure cultures of the isolated strain and then resuspended in 100 µL of Tris-EDTA 1X (TE 1X) fresh solution and then denatured at 100°C for 10 minutes. After centrifugation, the supernatant was used for genomic techniques and stored at -20°C.

The quantity and quality of DNA were determined by a spectrophotometric reading of A260/A280 ratio (Eppendorf BioPhotometer 6131)

Molecular identification, using species-specific *nuc* gene (Sasaki *et al*., 2010) was performed by single PCR to confirm the identification of *S. pseudintermedius* strains. DNA from *S. pseudintermedius* ATCC® 49444TM was used as a positive control. The oligonucleotide primer sequences and PCR programs are summarized in Table 1.

Gene		Primer sequence 5'-3'	Amplicon size (bp)	Amplification program	Reference
nuc	F	TRGGCAGTAGGATTCGTTAA	926	94°C 5 min; 94°C 30 s, 58°C 60s, 72°C 90s, for 30 cycles; 72°C 5 min.	(Sasaki *et al*., 2010)
	R	CTTTTGTGCTYCMTTTTGG			
mecA	F	AAAATCGATGGTAAAGGTTGGC	532	95°C 5 min; 95°C 1 min, 58°C 1 min s, 72°C 1 min, for 30 cycles; 72°C 7 min.	(Zubeir *et al*., 2007)
	R	AGTTCTGCAGTACCGGATTTGC			
*tet*K	F	GTAGCGACAATAGGTAATAGT	360	94°C 15s; 94°C 1min, 52°C 1min, 72°C 90s, for 30 cycles; 72°C 5 min	(Strommenger *et al*., 2003)
	R	GTAGTGACAATAAACCTCCTA			
*tet*M	F	AGTGGAGCGATTACAGAA	158		
	R	CATATGTCCTGGCGTGTCTA			

Table 1

Primers sequences, amplicon sizes and amplification programs

Antimicrobial susceptibility testing

The antimicrobial susceptibility testing was performed on 242 canine *S. pseudintermedius* isolates (116 strains in Milan and 126 strains in Naples) by using Kirby-Bauer disk diffusion method according to CLSI (Clinical Laboratory and Standards Institute) guidelines (CLSI, 2017). The interpretation of susceptibility patterns was recognized as sensitive, intermediate and resistant by comparison of the zone of inhibition as indicated by the same Guidelines.

The antibiotics and the relative concentration are reported in Table 2.

Antibiotic name	Concentration (µg)	Group

1	Amoxicillin + Clavulanic acid	AMC	30 (20/10)	Penicillins (β-lactams)
2	Oxacillin	OX	1	Penicillins (β-lactams)
3	Ceftriaxone	CRO	30	Cephalosporins (β-lactams)
4	Clindamycin	DA	2	Lincosamides
5	Enrofloxacin	ENR	5	Fluoroquinolones
6	Erythromycin	E	15	Macrolides
7	Gentamicin	CN	10	Aminoglycosides
8	Kanamycin	K	30	Aminoglycosides
9	Tobramycin	TOB	10	Aminoglycosides
10	Tetracycline	TE	30	Tetracyclines

Table 2

List of all antibiotics tested

Amplification of antibiotic-resistance genes (ARg)

In order to study the dissemination of genes coding for antimicrobial resistance, the amplification of the following genes was performed by single PCRs: *mecA*, and the tetracyclines family genes (*tetK*, *tetL*, *tetM,* and *tetO*). For each reaction, the final volume was set to 25 µL including 12,5 µL of 2X Master Mix (ThermoFisher Scientific, Italy), 20 pmol of each primer pairs and 10,1 µL of nuclease-free water. The cycling conditions and the oligonucleotide sequences were either reported in Table 1.

Statistical analysis

Data derived from experiments were stored in Microsoft Excel. Samples were grouped in MRSP and MSSP, according to the resistant profile against oxacillin and the presence of *mec* gene. The antibiotics were statistically studied with the *Chi*-squared test to verify if the dissemination of resistant strains was accidental or not. Cohen's kappa coefficient (k) for dichotomous data was used to verify the agreement between phenotypic analyses and PCR detection of resistance genes. The strength of the accordance was interpreted according to Landis and Koch (Landis and Koch, 1977), who classified agreement in the following categories: 0-0.2 as poor; 0.21-0.4 as fair; 0.41-0.6 as moderate, 0.61-0.8 as good; 0.81-1 as very good. C*hi*-squared test and Cohen's kappa coefficient were performed with GraphPad Prism v6 (GraphPad Software®, USA) and Microsoft Excel, respectively.

Results

Fig. 1 and Supplementary Table 1 report the frequency of resistance to the antibiotic molecules tested among the strains from Milan (N=116) and Naples (N=126) and the confidence intervals (95%) calculated for the prevalence of resistance to each single antibiotic molecules. Despite the similarity in the frequencies of resistance among the two hospitals, four molecules (AMC, DA, TOB, and TE) resulted in statistical significance between Milan and Naples. More in detail, the strains from Milan showed higher resistance to clindamycin (DA, for the abbreviations, see Table 2) and tobramycin (TOB); on the contrary, the resistance against AMC and TE was higher in Neapolitan isolates.

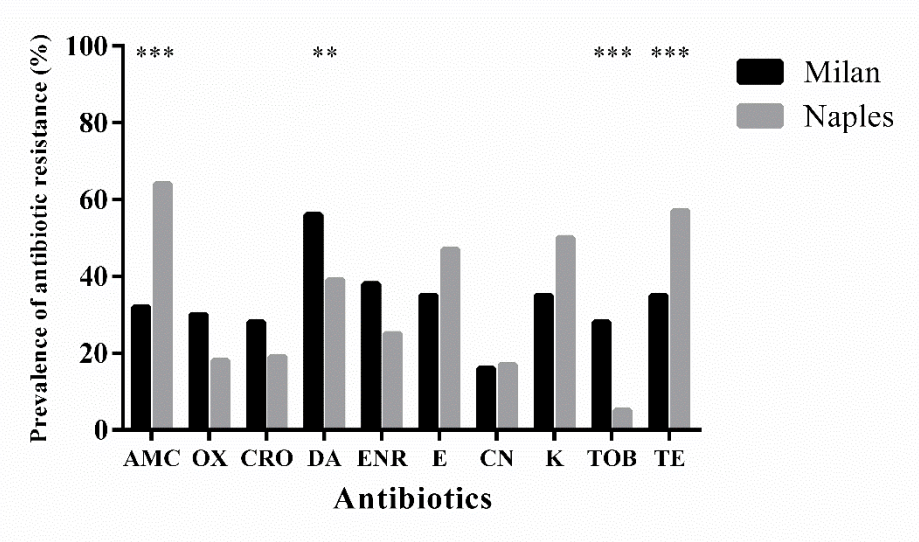

Fig.1

Overall prevalence of antibiotic resistance among isolates

The prevalence of methicillin/oxacillin-resistant strains (MRSP) was 30% (35/116) and 18% (23/126) in Milan and Naples, respectively; these data resulted in statistical significance (χ^2 *p*-value=0.0351). At a genetic level, the amplification of the *mecA* gene occurred in all the MRSP strains derived from the Kirby-Bauer assay, confirming the phenotypic findings and resulting in a total concordance (*k*=0.9) by Cohen's kappa coefficient. Looking with more detail to the MRSP and MSSP populations, the resistance frequencies and statistical differences were totally different (Fig. 2).

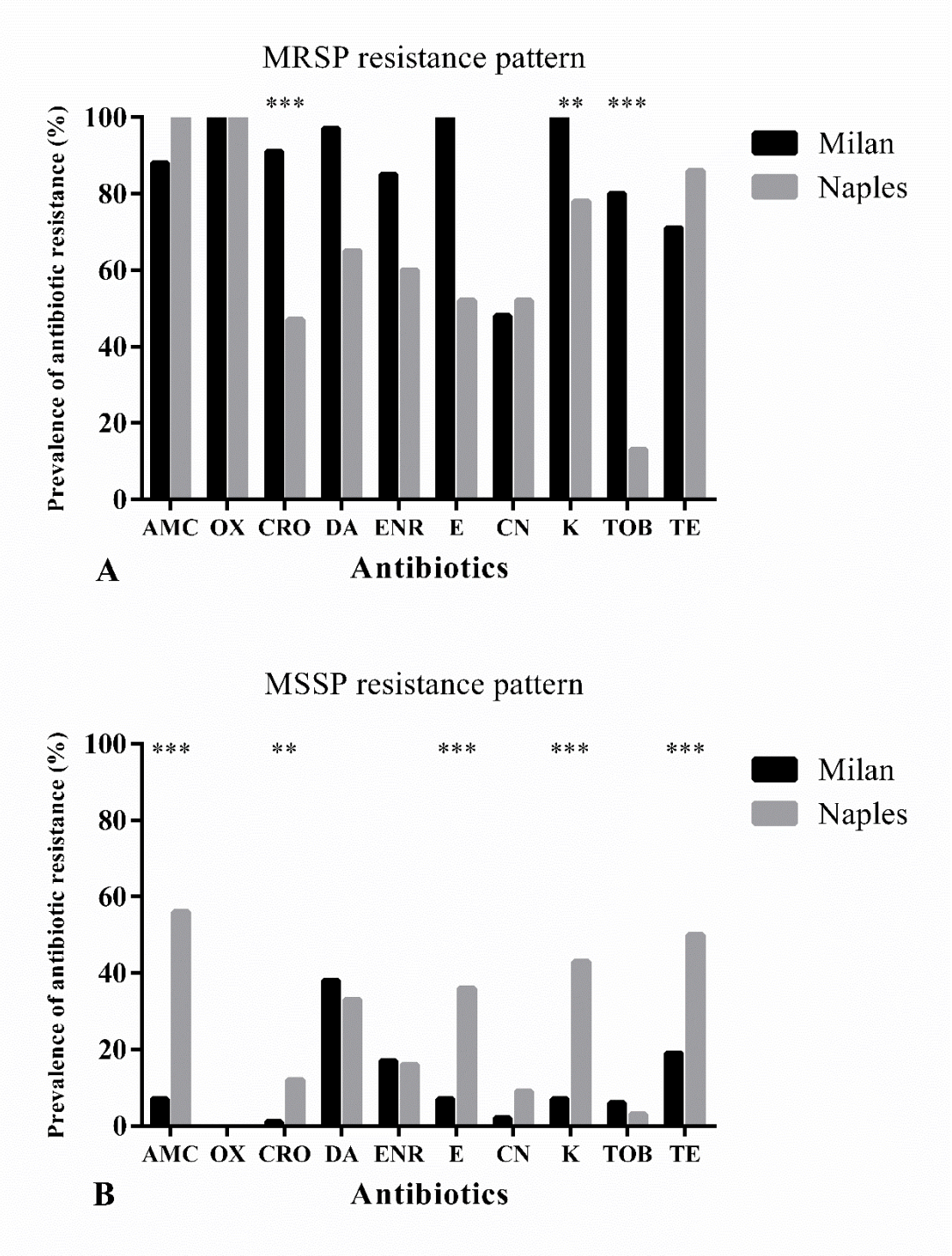

Fig. 2

Distribution of antimicrobial resistance among MRSP (A) and MSSP (B) strains in Milan and Naples.

Among the strains from Milan and those from Naples, a clear difference in terms of Multi-Drug Resistant (MDR) strains was found; the 35 MRSP strains were also MDR while 21/23 (91%) of the strains from the second hospital was MDR. The situation in the MSSP populations was very different from the one previously described. Strains from Milan exhibited a lower resistance compared to those from Naples and resulting in a more divergent MDR dissemination: 6/81 (7%) and 35/103 (34%), from Milan and Naples, respectively, resulted as MDR.

Fig. 3 shows the dissemination of the *tet* family genes between MRSP and MSSP populations. No statistical differences were found among the MRSP group, only the amplification of *tetK* gene in MSSP group showed statistical differences between Milan and Naples.

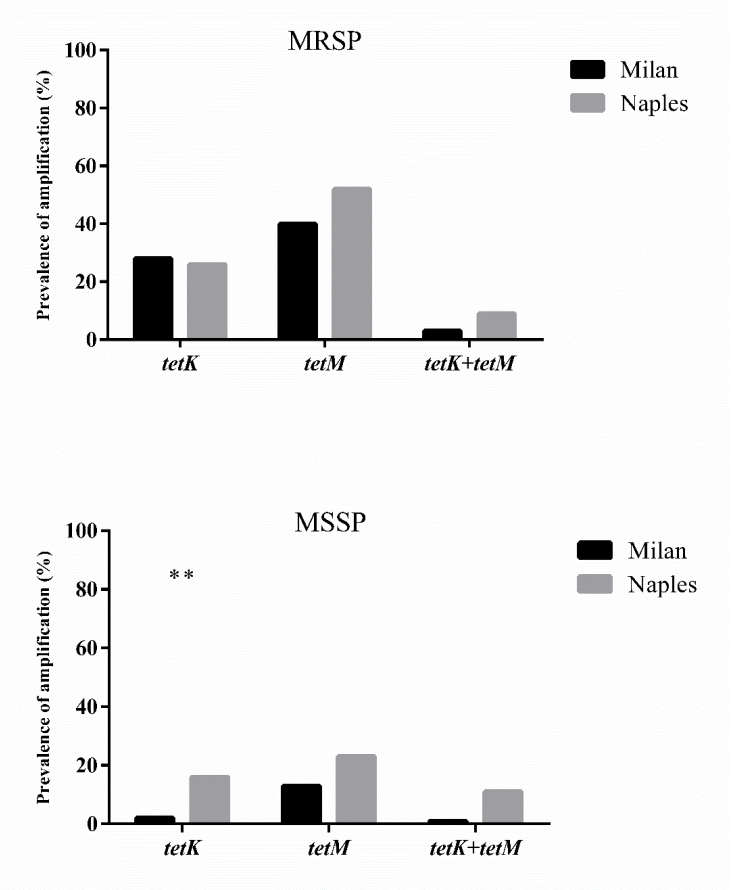

Fig. 3

Prevalence of *tet* genes amplification among MRSP (up) and MSSP (bottom)

Discussion

Microbial resistance is a normal behavior for bacteria that respond to environmental stimuli and, as a consequence, develop resistance as a strategy for surviving (Rodríguez-Rojas *et al*., 2013). Methicillin/oxacillin resistance, in the member of *Staphylococcus* genus (both coagulase-positive and negative), is mediated by the synthesis of a novel penicillin-binding protein, the penicillin-binding protein 2a (PBP2a), that confers resistance to all β-lactams and is mediated by *mecA* gene (Fuda *et al*., 2004). The phenotypic resistance to methicillin/oxacillin has to be validated by the amplification of the *mecA* gene to classify staphylococci as methicillin-resistant. Our data provide evidence of a high incidence of MRSP strains in two Italian veterinary teaching hospitals about 30% and 18% in Milan and Naples, respectively. These results are in partial concordance with the available Italian literature in which was reported, in 2017, a prevalence of MRSP of 59% (30/51) (Stefanetti *et al*., 2017). The situation is completely different if compared to Finland (Grönthal *et al*., 2015) and Germany (Nienhoff *et al*., 2011), in which the dissemination of MRSP was about 3% and 704%, respectively.

Furthermore, MDR bacteria are defined as resistant to at least three different pharmaceutical categories (Magiorakos *et al*., 2012). In our study, a large proportion of *S. pseudintermedius* strains resulted in MDR and exhibited multiple resistances in particular to amoxicillin, clindamycin, erythromycin, kanamycin, and tobramycin, reflecting the over-use of these antibiotics as first aid in Veterinary Medicine. Also in this case, our results are in accordance with the literature (Casagrande Proietti *et al*., 2015; Stefanetti *et al*., 2017).

Conclusions

All these findings, once again, should advise a revision in the current over-use of antibiotics. The last edition of the Surveillance of antimicrobial resistance in Europe (2018) stated that "*high levels of resistance remain in the EU/EEA for several bacterial species–*

antimicrobial group combinations" (ECDC, 2018). In Veterinary Medicine, also, national and international guidelines for the correct use of antibiotics focus the attention of all the EU members to reduce the amount of antibiotics prescribed (Division, 2018; Tacconelli *et al*., 2018). One of the most interesting findings, in this study, is the high prevalence of MRSP strains. Further studies have to be planned in particular to study the relation between antibiotic-resistance and biofilm formation.

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Appendix A: Supplementary materials

Supplementary Table 1

Prevalence of antibiotic resistance among *S. pseudintermedius* strains from Milan and Naples

Antibiotic	Milan (N= 116)		Naples (N=126)	
	Prev (%)	(95% CI) (%)	Prev (%)	(95% CI) (%)
AMC	**32** (***)	23.5-40.4	**64** (***)	55.6-72.3
OX	30	21.6-38.3	18	11.2-24.7
CRO	28	19.8-36.1	19	12.1-25.8
DA	**56** (**)	46.9-65	**39** (**)	30.4-47.5
ENR	38	29.1-46.8	25	17.4-32.5
E	35	26.3-43.6	47	38.2-55.7
CN	16	9.3-22.6	17	10.4-23.5
K	35	26.3-43.6	50	41.2-58.7
TOB	**33** (***)	24.4-41.5	**5.5** (***)	1.5-9.4
TE	**35** (***)	26.3-43.6	**57** (***)	48.3-65.6

N= number of strains. χ^2: * $0.05<p<0.01$; ** $p< 0.01$; *** $p< 0.001$

Chapter 3

Article

Investigation on Antibiotic-Resistance, Biofilm Formation and Virulence Factors in Multi Drug Resistant and Non Multi Drug Resistant *Staphylococcus pseudintermedius*

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The data reported in this chapter refer to the already published paper: **Meroni, G., Filipe, J.F.S., Drago, L., Martino, P.A.** (2019). Investigation on antibiotic-resistance, biofilm formation and virulence factors in multi drug resistant and non multi drug resistant *Staphylococcus pseudintermedius*. *Microorganisms,* 7 (12) 702-713. doi:10.3390/microorganisms7120702

Abstract

Staphylococcus pseudintermedius is a commensal bacterium frequently isolated from canine skin and recognized as a zoonotic agent especially for dog-owners. This study focused on (a) the antibiotic-resistance phenotypes; (b) the ability to produce biofilm (slime); and (c) the dissemination of virulence factors in *S. pseudintermedius* strains. Seventy-three *S. pseudintermedius* strains were screened for antibiotic-resistance against 22 different molecules by means of Kirby-Bauer assay. The ability to produce biofilm was investigated using the microtiter plate assay (MtP) and the amplification of *icaA* and *icaD* genes. Virulence factors such as cytotoxins (*lukI*), enterotoxins (*seC*), and exfoliative toxins (*siet*, *expA*, and *expB*) were evaluated. The antibiotic-resistance profiles revealed 42/73 (57%) multi-drug resistant (MDR) strains and 31/73 (43%) not-MDR. All the MDR strains and 8/31 (27%) of not-MDR resulted in biofilm producers. Leukotoxin LukI was found in 70/73 (96%) of the isolates. Moreover, the enterotoxin gene *seC* was detected in 47/73 (64%) of the strains. All the isolates carried the *siet* gene, whereas *expA* and *expB* were found in 3/73 (4%) and 5/73 (7%), respectively. In conclusion, *S. pseudintermedius* should be considered a potential zoonotic and human agent able to carry different virulence determinants and capable of producing biofilm which facilitates horizontal gene transfer.

Introduction

Staphylococcus pseudintermedius (SP) is one of the youngest members of the *Staphylococcus* genus, being described and recognized only in 2005 (Devriese *et al*., 2005), with the first reported case in humans in 2006 (Van Hoovels *et al*., 2006) and the first molecular identification protocol in 2009 (Bannoehr *et al*., 2009). It is an opportunistic pathogen also known as one of the leading cause of skin, ear, and post-surgical infections in domestic animals, especially in dogs (Guardabassi *et al*., 2004; Bartlett *et al*., 2011; Bannoehr and Guardabassi, 2012; Little *et al*., 2019). Even if infections in humans are less common than those reported in pets, the description of *S. pseudintermedius* as a human pathogen is being increasingly reported (Starlander *et al*., 2014; R. Somayaji *et al*., 2016; Little *et al*., 2019), however little is known about its pathogenesis and distribution, and in human medicine is still misdiagnosed as *S. aureus* (Börjesson *et al*., 2015). A progressive expansion in resistance to commonly prescribed antimicrobial agents has been observed in the past years, in particular with the emergence and the global spread of multi drug resistant (MDR) bacteria with particular regards for the Methicillin Resistant *S. pseudintermedius* (MRSP) clones that dramatically complicate the treatment of these infections (Stefanetti *et al*., 2017; Little *et al*., 2019).

The ability to form biofilm is one of the major virulence determinants studied nowadays in bacteria because it facilitates the adherence to biotic and/or abiotic surfaces (Arciola *et al*., 2005). Biofilm-related infections are fastidiously faced because sessile bacteria are generally much more tolerant to antibiotics compared to the equivalent planktonic forms and can easily resist to host immune responses (Resch *et al*., 2005). To form a biofilm, bacteria require at least

two features: a) Adherence to a surface, and b) the possibility to accumulate it in order to form a multi-layered complex structure. The ability to form a biofilm resides in the formation of an extracellular matrix known as polysaccharide intercellular adhesion molecule (PIA), that is encoded by the *ica* operon, including four distinct genes (A, B, C, and D) (Arciola *et al*., 2005; Casagrande Proietti *et al*., 2015; Little *et al*., 2019). The biofilm forming ability of *S. pseudintermedius* has been reported by different authors but nowadays is still not fully understood; in a recent study Stefanetti *et al*., (2017), up to 96% of canine SP strains was able to produce biofilm (Singh *et al*., 2013; Stefanetti *et al*., 2017). Moreover, *ica*-independent biofilm formation has been reported in staphylococci (Hennig *et al*., 2007; O'Gara, 2007; Panda and Singh, 2018).

The aim of the present study is to analyze the possible correlation between antibiotic-resistance, ability to form biofilm, and dissemination of virulence determinant in different isolated strains of SP.

Materials and Methods

Bacterial Isolation and Identification

The 73 *S. pseudintermedius* strains included in this study were collected between 2016 and 2018 at the Microbiology Laboratory of the Department of Veterinary Medicine (Università degli Studi di Milano). Clinical samples were cultivated on Trypticase Soy Agar (TSA) + 5% defibrinated sheep blood agar (Microbiol, Uta (CA), Sardinia, Italy) and incubated aerobically at 37 °C for 24 h. Following morphological analysis, the suspected staphylococcal colonies

were sub-cultured on Mannitol Salt Agar (MSA; Microbiol, Uta (CA), Sardinia, Italy) for genus identification and incubated at 37 °C for 24 h. To confirm the isolation of *S. pseudintermedius*, standard phenotypic techniques were used such as Gram stain, catalase test, and coagulase test. Finally, the amplification of the *nuc* gene (Table 1) described by Sasaki in 2010, was used to genetically confirm the isolates at species level (Sasaki *et al*., 2010). All the strains were stored at −20 °C in 25% glycerol.

DNA Extraction

Pure cultures stocks in glycerol were thawed at room temperature and grown on blood agar plates at 37 °C for 24 h. A single colony was picked up and grown in Brain Heart Infusion Broth (BHI, Scharlau, Spain) at 37 °C for 8 h. One mL aliquot was used for DNA extraction using the previously described boiling method (Adwan, 2014). DNA was quantified and checked for its purity using the NanoDropTM 2000 Spectrophotometer (Thermo Fisher Scientific, Monza (MB), Lombardy, Italy).

Molecular Typing

In order to better characterize the *S. pseudintermedius* strains, two commonly used typing techniques were used: MultiLocus Sequence Typing (MLST) and SCCmec Typing.

Genetic diversity of the strains was determined by MLST of seven genes (*tuf*, *cpn60*, *pta*, *purA*, *fdh*, *ack*, *sar*); the primers used as well as the amplification conditions were the same as those previously described by Solyman *et al*. (2013) (Solyman *et al*., 2013). MLST sequences were aligned with sequences present in the NCBI nucleotide database in order to set out the

allele number. Sequence types (STs) were assigned according to the literature (Bannoehr *et al*., 2007) and using *Staphylococcus pseudintermedius* MLST database (https://pubmlst.org/spseudintermedius/).

SCCmec types I–VI were assigned, among MRSP strains only, using a specific set of multiplex PCR assays as reported by different authors, using the same set of primers and amplification conditions previously described (Kadlec *et al*., 2010; Perreten *et al*., 2010).

Table 1. Primers, amplicon size, and amplification conditions.

	Genes		Sequence (5'-3')	Amplicon Size (bp)	PCR Conditions	References
Antibiotic-resistance genes	*mecA*	F	AAAATCGATGGTAAAGGTTGGC	532	95 °C × 4 min, 30 x (95 °C x 1 min, 58 °C x 1 min, 72 °C × 1 min) 72 °C × 7 min, 4 °C	(Kang *et al*., 2014)
		R	AGTTCTGCAGTACCGGATTTGC			
	blaZ	F	TGACCACTTTTATCAGCAACC	750		
		R	GCCATTTCAACACCTTCTTTC			
	tetK	F	GTAGCGACAATAGGTAATAGT	360	94 °C × 3 min, 30 × (94 °C × 30 s, 55 °C × 30 s, 72 °C × 30 s) 72 °C × 7 min, 4 °C	(Strommenger *et al*., 2003)
		R	GTAGTGACAATAAACCTCCTA			
	tetM	F	AGTGGAGCGATTACAGAA	158		
		R	CATATGTCCTGGCGTGTCTA			
	aacA-aphD	F	TAATCCAAGAGCAATAAGGGC	227		
		R	GCCACACTATCATAACCACTA			
Biofilm genes	*icaA*	F	ACTGTTTCGGGGACAAGCAT	134	94 °C × 3 min, 35 × (94 °C × 15 s, 60 °C × 20 s, 72 °C × 20 s) 72 °C × 7 min, 4 °C	(Resch *et al*., 2005)
		R	ATTGAGGCTGTAGGGCGTTG			
	icaD	F	CGTTAATGCCTTCTTTCTTATTGCG	166	94 °C × 3 min, 35 × (94 °C × 15 s, 56 °C × 20 s, 72 °C × 20 s) 72 °C × 7 min, 4 °C	
		R	ATTAGCGCACATTCGGTGTT			
Quorum-sensing genes	*pan-agr*	F	ATGCACATGGTGCACATGC		94 °C × 3 min, 35 × (94 °C × 15 s, 56 °C × 20 s, 72 °C × 20 s) 72 °C × 7 min, 4 °C	(Shopsin *et al*., 2003)
	agrI	R	GTCACAAGTACTATAAGCTGCGAT			
	agrII	R	GTATTACTAATTGAAAAGTGCCATAGC			
	agrIII	R	CTGTTGAAAAAGTCAACTAAAAGCTC			
	agrIV	R	CGATAATGCCGTAATACCCG			
Virulence factors	*luk-F*	F	CCTGTCTATGCCGCTAATCCA	572	94 °C × 3 min, 35 × (94 °C × 1 min, 57 °C × 1 min, 72 °C × 1 min) 72 °C × 7 min, 4 °C	(Futagawa-Saito *et al*., 2004)
		R	AGGTCATGGAAGCTATCTCGA			
	luk-S	F	TGTAAGCAGCAGAAAATGGGG	503		
		R	GCCCGATAGGACTTCTTACAA			
	seC	F	GGCGGCAATATTGGCGCTCG	271	95 °C × 2 min, 30 × (95 °C × 1 min, 55 °C × 1 min, 72 °C × 2 min) 72 °C × 5 min, 4 °C	(Yoon *et al*., 2010)
		R	TTACTGTCAATGCTCTGACC			
	nuc	F	TRGGCAGTAGGATTCGTTAA	926	95 °C × 2 min, 30 × (95 °C × 30 s, 52 °C × 30 s, 72 °C × 30 s) 72 °C × 2 min, 4 °C	(Sasaki *et al*., 2010)
		R	CTTTTGTGCTYCMTTTTGG			
	siet	F	ATGGAAAATTTAGCGGCATCTGG	359	94 °C × 3 min, 30 × (94 °C × 30 s, 56 °C × 30 s, 72 °C × 1 min) 72 °C × 5 min, 4 °C	(Lautz *et al*., 2006)
		R	CCATTACTTTTCGCTTGTTGTGC			
	expA	F	GTKTTAATTGGWAAAAATACA	413	94 °C × 3 min, 30 × (94 °C × 1 min, 42 °C × 1 min, 72 °C × 1 min) 72 °C × 4 min, 4°C	(Futagawa-Saito *et al*., 2009)
		R	ATNCCWGAKCCTGAATTWCC			
	expB	F	GGGCATGCACATATGATGAAGCC	820	95 °C × 3 min, 30 × (95 °C × 1 min, 53 °C × 1 min, 72 °C × 1 min) 72 °C × 4 min, 4 °C	(Iyori *et al*., 2010)
		R	CCAGATCTATCTTCTGATTCAGC			

Determination of Antibiotic-Resistance Profile

Kirby-Bauer Disk Diffusion Method

Susceptibility to a panel of 22 antimicrobial agents was determined by the Kirby-Bauer disk diffusion test according to the Guidelines of the Clinical Laboratory and Standards Institute (CLSI, 2015). Disks of 22 different antibiotics were used as reported below (in brackets the concentration in μg): Oxacillin (OX, 5), Amoxicillin + Clavulanic acid (AMX, 30 [20/10]), Amoxicillin (AML, 30), Carbenicillin (CAR, 100), Cephalexin (CL, 30), Cefovecin (CVN, 30), Ceftiofur (EFT, 30), Ceftriaxone (CRO, 30), Clindamycin (DA, 10), Lincomycin + Spectinomycin (MY, 15 [5/10]), Doxycycline (DO, 5), Enrofloxacin (ENR, 5), Marbofloxacin (MAR, 5), Pradofloxacin (5, 5), Amikacin (AK, 30), Gentamicin (CN, 30), Neomycin (N, 30), Tobramycin (TOB, 10), Kanamycin (K, 30), Rifampicin (RD, 30), Azithromycin (AZM, 15), Erythromycin (E, 30). The results were recorded as susceptible, intermediate, or resistant by the measurement of the inhibition halo diameter.

Amplification of Antibiotic-Resistance Genes (ARg)

The primers used for the detection of five different antibiotic-resistance genes (ARg) were taken from the literature (Strommenger *et al*., 2003; Zubeir *et al*., 2007; Kang *et al*., 2014), synthesized by Eurofins Genomics, and listed in Table 1. Predicted amplicon size and primers specificity were defined using BLAST search available through the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) and coupled with BioEdit freeware software. To check specificity on DNA from genotypically defined isolates, single PCRs for each primer pair were performed before the Multiplex PCR assay.

For *tetK, tetM,* and *aacA-aphD* genes, the protocol described by Strommenger in 2003 was followed (Strommenger *et al*., 2003). Multiplex PCR amplifications were carried out with AccuPrime^{TM} Taq DNA Polymerase system (Invitrogen, Italy) following the manufacturer's

instruction in a 25 µL volume comprising approximately 40 ng of DNA, 10 pmol of each of 6 primers, 2.5 µL of 10× AccuPrime™ PCR Buffer II, 4 nM of $MgCl_2$ (final concentration), 0.3 U of AccuPrime™ Taq DNA Polymerase, and nuclease-free water (NFW) to reach the final volume.

For *mecA* and *blaZ* genes, the protocol used was described by Kang in 2014 (Kang *et al*., 2014). Multiplex amplifications were carried out using the same kit previously described. The PCR products were resolved on a 1.5% agarose gel (GellyPhore^{LE}, EuroClone, Italy).

Biofilm Analysis

Identification of Biofilm-Forming Strains

The identification of biofilm-forming strains was carried out by the microtiter plate (MtP) assay, as previously described (Stepanovic *et al*., 2000; Stephanovic *et al*., 2007). Briefly, after growing in BHI broth at 37 °C for 24 h, pure staphylococcal cultures were 1:100 diluted in fresh Trypticase broth (Oxoid, Italy) + 1% glucose (TSBg) and seeded in 96 well-plates (Corning, USA). After 24 h of incubation at 37 °C, planktonic bacteria were washed out and biofilm was stained with Crystal violet (Carlo Erba, Italy). Negative controls consist of TSBg only. Each strain was analyzed in triplicate on the same plate and three independent plates were used. The absorbance (570 nm) of negative controls was used to set the optical density cut-off (ODc) as three standard deviations above the mean OD of the negative control. Strains were classified as follows: Not adherent $OD \leq ODc$; weakly adherent $ODc < OD \leq 2 \times ODc$; moderately adherent $ODc < OD \leq 4 \times ODc$; strongly adherent $OD > 4 \times ODc$.

Amplification of Biofilm-Associated Genes and Agr-Typing

To confirm data from MtP assay, the detection of two pivotal genes of *ica locus* (*icaA* and *icaD*) was performed by conventional qualitative PCR (see Table 1) using primer according

to literature (Casagrande Proietti *et al*., 2015). Taking into account that the ability to produce biofilm could be associated with a specific antibiotic-resistance profile (Melchior *et al*., 2009; Little *et al*., 2019), the *agr locus* was analyzed with two duplex PCRs for the determination of *agr* type (I-IV), as previously reported (Shopsin *et al*., 2003). For the two duplex PCRs, the already described AccuPrime™ Taq DNA Polymerase system (Thermo Fisher Scientific, Monza (MB), Lombardy, Italy) was used.

Virulence Factors Carriage

To establish the pathogenicity of the isolated strains, specific virulence factors (*lukS-F*, *seC*, *siet*, *expA* and *expB*) were searched by qualitative PCR. Their specific thermal cycling conditions and primer pairs are listed in Table 1.

Statistical Analysis

Statistical significance was determined by GraphPad Prism v6 (GraphPad Software®, La Jolla California, USA) using Fisher′s exact test. Data were analyzed by contingency tables (2-by-2 layout). A *p-value* < 0.05 was considered significant. The agreement between the MtP assay and PCR detection of *icaA* and *icaD* genes was calculated using Cohen's Kappa values for dichotomous data in Microsoft Excel. The strength of the accordance was interpreted according to Landis and Koch (1977), who classified agreement in the following categories: 0–0.2 as poor; 0.21–0.4 as fair; 0.41–0.6 as moderate, 0.61–0.8 as good; 0.81–1 as very good (Landis and Koch, 1977).

Results

Molecular Identification of S. pseudintermedius, MLST, and SCCmec Typing

All the isolates were from dogs with clinical deep pyoderma, a single 926 bp fragment was derived from the amplification of themonuclease gene (*nuc*) confirming, at species level, the phenotypic isolation of 73 *S. pseudintermedius* strains.

The 73 isolates were assigned to three different STs with the following prevalences: ST 71 56/73 (77%), ST 258 12/73 (16.4%), and ST 106 5/73 (6.6%).

SCCmec types were assigned to 35/76 (48%) strains that resulted positive to *mecA* gene detection and classified as methicillin-resistant *S. pseudintermedius* (MRSP), resulting in two different types, as reported in Table 2.

Table 2. Molecular characterization of the 35 methicillin-resistant *S. pseudintermedius* (MRSP) isolates.

MLST	SCCmec Types	No. of Isolates (%)
ST 71	II-III	24 (68.5%)
ST 258	IV	9 (25.7%)
ST 106	IV	2 (5.7%)

Overall Antibiotic-Resistance

The antibiotic-resistance of all 73 *S. pseudintermedius* isolates is shown in Figure 1. Kirby-Bauer assay demonstrated that 42/73 (57.5%) of SP isolates were MDR, exhibiting resistance against macrolides (97%), fluoroquinolones (86%), and β-lactams (72%). The most in-vitro effective molecules were amikacin (100% susceptible), rifampicin (93% susceptible), and partially gentamicin (45% susceptible). The remaining 31 not-MDR strains had a low prevalence of resistance for all the antibiotics with the exception of clindamycin which had a

rate of resistance of 35% (11/31). Table 3 shows the prevalence of ARg by using Multiplex PCRs.

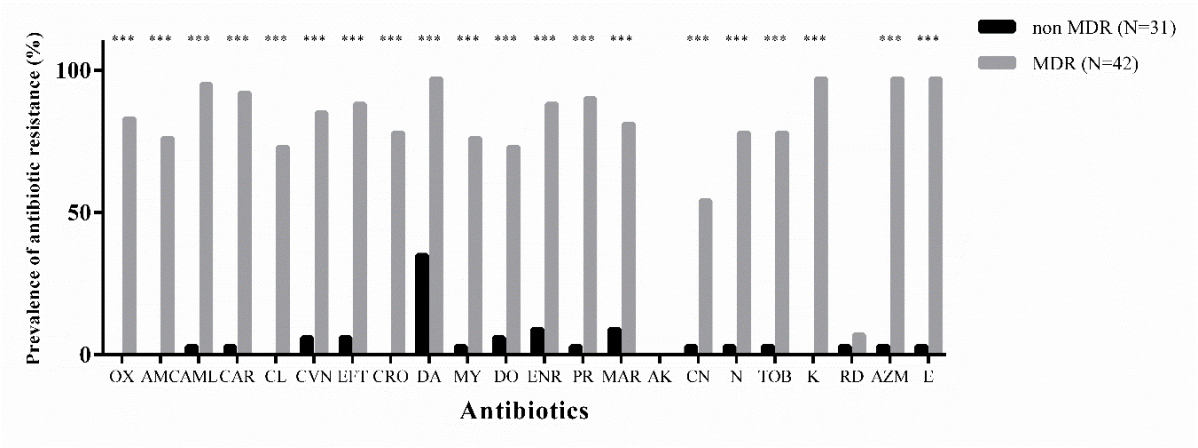

Figure 1. Overall prevalence of antibiotic-resistance among multi-drug resistant (MDR) and not-MDR isolates. The *Chi*-squared test showed statistical differences among the two groups for the majority of antibiotic molecules tested except for amikacin and rifampicin. (*: *p-value* between 0.05 and 0.01; **: *p-value* < 0.01; ***: *p-value* < 0.001).

Table 3. Distribution of antibiotic-resistance and virulence factors genes.

	Genes	not MDR Strains	MDR Strains	*p-Value*
Antibiotic-resistance genes	*mecA*	0	35/42 (83%)	**<0.0001**
	blaZ	7/31 (23%)	42/42 (100%)	**<0.0001**
	tetK	2/31 (6.4%)	13/42 (31%)	**0.0171**
	tetM	0	22/42 (50%)	**<0.0001**
	aacA-aphD	5/31 (16.6%)	32/42 (76%)	**<0.0001**
Biofilm genes	*icaA*	30/31 (97%)	29/42 (69%)	**0.0026**
	icaD	30/31 (97%)	41/42 (97%)	>0.05
Virulence factors	*luk-I*	30/31 (97%)	40/42 (95%)	>0.05
	seC	14/31 (45%)	31/42 (74%)	**0.016**
	nuc	31/31 (100%)	42/42 (100%)	>0.05
	siet	31/31 (100%)	42/42 (100%)	>0.05
	expA	0	3/42 (7%)	>0.05
	expB	0	5/42 (12%)	>0.05

Biofilm Formation Assay

Figure 2A shows the difference in terms of number of non-biofilm producing strains among not-MDR and MDR strains. Between these two groups, there is a clear difference in terms of the ability to produce biofilm (Figure 2B). The MDR bacteria were all able to produce

exopolysaccharide, resulting in 20/43 (46.5%) strong biofilm producers, 20/43 (46.5%) moderate, and 2/43 (4.6%) weak producers. In the not-MDR group, only 1/31 (3.22%) strains was strong producer, 9/31 (29%) strains were categorized as moderate producers, and 17/31 (55%) resulted in weak biofilm producers. The remaining 4/31 (13%) did not show slime production. At the molecular level, the presence of *icaA* and *icaD* genes was demonstrated by the amplification of the corresponding amplicons. Both the *icaA* and *icaD* genes were detected in 30/31 (97%) not-MDR strains, while 28/42 (66.6%) of MDR bacteria had both the targeted genes. Among the remaining 14 strains, one was negative for both these genes (but still able to produce biofilm) and the remaining 13 were all positive for *icaD* only. Neither one of these genes was detected in 2/73 (2.7%) isolates (details are reported in Supplementary Table S1). The agreement between the microplate assay and the amplification of *ica locus* genes was not significant ($k < 0.01$). The determination of *agr*-typing showed that all the 73 SP strains belong to the *agr* type *I*.

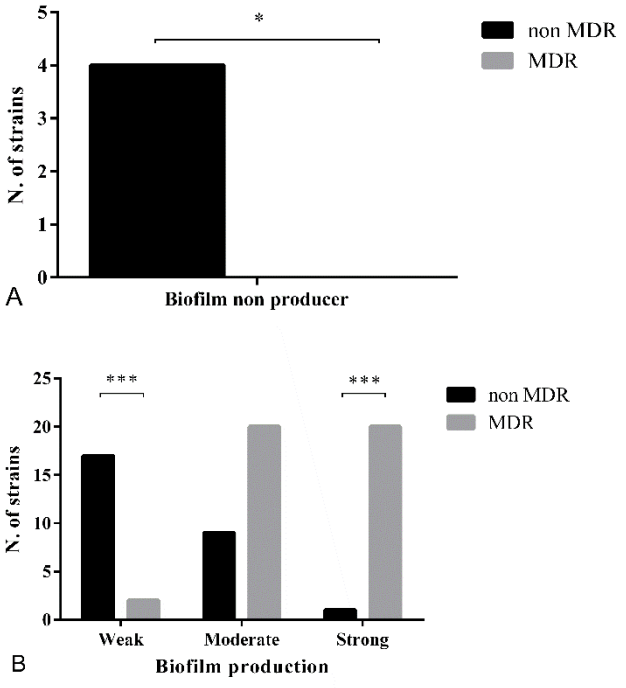

Figure 2. Biofilm forming ability in not-MDR and MDR *Staphylococcus pseudintermedius* (SP) strains. (**a**) All the MDR strains are able to produce biofilm and these

data are statistically significant compared to the not MDR group in which 4 strains resulted in non-biofilm producers. (**b**) The majority of MDR strains were strongly biofilm producers (20/42), whereas not-MDR strains were mostly categorized as weak slime producers. (*: *p-value* between 0.05 and 0.01; **: *p-value* < 0.01; ***: *p-value* < 0.001).

Virulence Factors

Table 2 shows the distribution of all the genes analyzed in this study. Among the virulence factors, *Chi*-squared test showed differences in *icaA* and *seC* genes between MDR and not-MDR bacteria, for all the other virulence determinants, no differences were found (Figure 3).

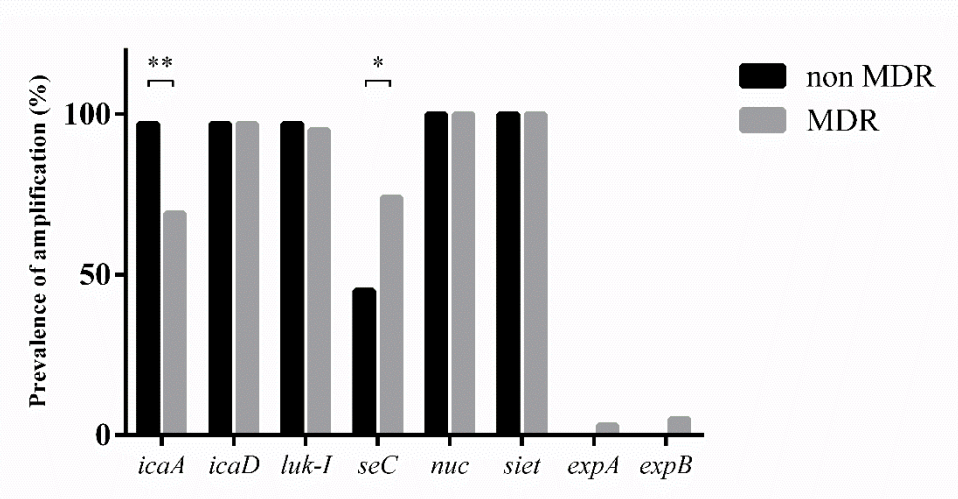

Figure 3. Dissemination of virulence factors among MDR and not-MDR strains. (*: *p-*value between 0.05 and 0.01; **: *p-value* < 0.01; ***: *p-value* < 0.001).

Discussion

Antibiotic-resistance remains one of the most important problems to face within treatment and control of *S. pseudintermedius* related infection in human and in veterinary medicine. SP is nowadays considered a potential zoonotic agent able to colonize also humans and is regarded as one of the increasing skin and soft tissue-related pathogens (R. Somayaji *et*

al., 2016; Ranjani Somayaji *et al*., 2016; Kmieciak and Szewczyk, 2018). MDR strains are defined when "resistant to at least 1 agent in 3 or more antimicrobial categories"(Magiorakos *et al*., 2012). In this study, 57.5% (42/73) of the strains had MDR phenotype, also confirmed by the detection of AR genes. The majority of the resistant strains 35/42 (83%) resulted positive for *mecA* gene, associated with oxacillin resistance; this result is in concordance with a previous study which revealed a 100% of *mecA* gene amplification in MRSP strains (Moyaert *et al*., 2019). A large portion of isolates showed a pattern of resistance against β-lactams, macrolides, and fluoroquinolones.

A total of three STs (ST 71, ST 258 and ST 106) were detected among the 73 SP strains. This result is not totally concordant with the literature in which different authors found a higher genetic diversity in the analyzed population (in terms of STs assigned) (Damborg *et al*., 2016; Bergot *et al*., 2018). ST 71 is predominant and was assigned to 56/73 (77%) strains; this particular sequence type remains the most abundant in Europe, while ST 68 is predominant in United States, and ST 45/ST 112 in Asia (Solyman *et al*., 2013; Dos Santos *et al*., 2016). In Europe, between 2012–2013 and 2015–2016, the prevalence of ST 71 rapidly decreased from 65.3% to 55.2%, whereas the emergence of ST 258, originally derived from Northern Europe, was described (from 1.1% to 5.78%) (Bergot *et al*., 2018). ST 71 was highly resistant to antibiotics, showing multiple resistances against commonly veterinary-licensed antibiotics (e.g., tetracyclines). On the other hand, ST 258 was reported to be more frequently susceptible to antibiotics (e.g., enrofloxacin, gentamicin) (Dos Santos *et al*., 2016; Bergot *et al*., 2018).

In this study, all the MRSP strains (35/73; 48%) resulted in MDR, and the majority (68.5%) were classified as ST 71. SCCmec typing showed two chromosomal cassettes types: II-III present in all ST 71, and IV detected in the other MRSP strains belonging to STs 258/106 (Table 2).

A clear correlation was found between antibiotic-resistance and the ability to produce biofilm, suggesting that MDR staphylococci are more prone to produce large quantities of slime. This particular result is in accordance with other studies and could be reasonably explained by the presence of transposons which are the mobile element that easily can be exchanged between strains (Arciola *et al*., 2005; Casagrande Proietti *et al*., 2015; Stefanetti *et al*., 2017).

The majority (94.5%) of the 73 isolates were biofilm producers, and this result is in agreement with the current literature on biofilm in *Staphylococcus* clinical isolates (Rinsky *et al*., 2013; Casagrande Proietti *et al*., 2015). Moreover, a genetic approach revealed the concomitant presence of either *icaA* or *icaD* in 79.5% (58/73) of the strains studied, while 18% (13/73) of the isolates presented the amplification of *icaD* and only two strains were negative for both these two genes but still able to produce biofilm. Authors suggest that the presence of *icaD* only (found in 42/43 MDR strains) is sufficient to produce exopolysaccharide, as demonstrated for *S. epidermidis* (Arciola *et al*., 2005). These findings are discordant with a previous study (Singh *et al*., 2013) in which the author suggested that biofilm formation occurred only when both *icaA* and *icaD* are expressed, but however concordant with another study which reported the absence of correlation between the presence of both *ica* genes and biofilm formation (*ica*-independent biofilm producers strains). These results suggest the importance of combined, phenotypic, and genetic methods for checking biofilm formation in *S. pseudintermedius*.

The prevalence of *agr* groups in our clinical isolates is very different to that described by Little *et al*. (Little *et al*., 2019), with *agrI* being the unique group found in our collection, while any of the strains were assigned to one of the other three (*agr II*, *III* and *IV*). Following the suggestions of the author, in this contest, it is not possible to correlate the ability to produce biofilm with the corresponding *agr* group. Little *et al*. (2019) stated that strains harboring *agrI*

were more prone to produce biofilm and to be MDR. Our findings indicate a major ability for MDR bacteria to be strong biofilm producers.

Among the virulence determinants analyzed in this study, only the prevalence of specific enterotoxin *seC* was found statistically significant between MDR and not-MDR strains (*p-value*: 0.016). The prevalence of individual genes was similar to the literature (Futagawa-Saito *et al*., 2004; Yoon *et al*., 2010).

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1 Table S1: Phenotypic and genetic determination of biofilm-forming ability.

Author Contributions: Conceptualization, G.M. and P.A.M.; Data Curation, G.M. and J.F.S.F.; Writing-Original Draft Preparation, G.M.; Writing-Review & Editing, G.M. and L.D.; Supervision, P.A.M. and L.D.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest

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Appendix A: Supplementary materials

Supplementary Table 1

Phenotypic and genetic determination of biofilm-forming ability

non MDR strains			MDR strains		
ID	PCR *icaA*/*icaD*	MtP	ID	PCR *icaA*/*icaD*	MtP
3	+/+	Weak	4	+/+	Moderate
6A	+/+	Weak	12	+/+	Strong
7B1	+/+	Moderate	14	+/+	Strong
8	+/+	Moderate	27	+/+	Weak
10A	+/+	Weak	28A	+/+	Strong
11B	+/+	Weak	28B	+/+	Strong
13	+/+	Weak	238	+/+	Moderate
16	+/+	Moderate	34	+/+	Moderate
18A	+/+	Weak	35	+/+	Strong
19A	+/+	Moderate	36	+/+	Strong
20	+/+	Weak	37	+/+	Strong
23	+/+	Weak	39	+/+	Strong
26A	+/+	Absent	41	+/+	Strong
30B	+/+	Moderate	42	+/+	Strong
31	-/-	Moderate	45	+/+	Strong
48	+/+	Weak	46	-/+	Strong
51	+/+	Weak	54	+/+	Strong
53	+/+	Weak	56	+/+	Strong
55	+/+	Weak	58	+/+	Moderate
59	+/+	Weak	60	+/+	Moderate
61	+/+	Weak	62	+/+	Strong
70	+/+	Absent	63	-/+	Moderate
74	+/+	Weak	64	-/+	Strong
79	+/+	Moderate	66	-/+	Moderate
84	+/+	Moderate	67	+/+	Moderate

85	+/+	Weak	71	+/+	Moderate
91	+/+	Moderate	72	-/+	Strong
96	+/+	Absent	76	+/+	Moderate
98	+/+	Strong	77	-/+	Moderate
234	+/+	Weak	78	-/+	Moderate
502	+/+	Absent	81	-/+	Moderate
			82	+/+	Moderate
			87	-/+	Moderate
			89	-/+	Moderate
			93	+/+	Weak
			94	-/-	Strong
			99	-/+	Strong
			102	-/+	Moderate
			104	-/+	Moderate
			105	+/+	Moderate
			107	+/+	Moderate
			117	+/+	Strong

Chapter 4

In vitro **antibacterial activity of biological-derived silver nanoparticles: preliminary data**

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Article submitted to *Veterinary Sciences, Special Issue: Italian Society of the Veterinary Sciences SISVet 2019*

Abstract

Silver nanoparticles (AgNPs) are promising alternatives to antibiotics. The aims of this study were the production of AgNPs using two biological methods and the determination of their antibacterial activity against P. aeruginosa and *S. pseudintermedius*.

AgNPs were biosynthesized from an infusion of Curcuma longa and the culture supernatant of E. coli. Characterization was achieved by ultraviolet-visible spectroscopy and by Transmission Electron Microscopy (TEM). The antibacterial properties of NPs from C. longa (ClAgNPs) and E. coli (EcAgNPs), alone and in combination with carbenicillin and ampicillin, were investigated through the Kirby-Bauer disk diffusion assay and the minimum inhibitory concentration (MIC).

Dimensions of NPs ranged from 11.107±2.705 nm (ClAgNPs) to 27.282±2.68 nm (EcAgNPs). Kirby-Bauer and MIC assays showed great antibacterial abilities for both NPs alone and in combination with antibiotics. EcAgNPs alone showed the most powerful antibacterial activities, resulting in MIC values ranging from 0.438±0.18 µM (P. aeruginosa) to 3.75±3.65 µM (*S. pseudintermedius*) compared to those of ClAgNPs: 71.8±0 µM (P. aeruginosa) and 143.7±0 µM (*S. pseudintermedius*). The antibiofilm abilities were strain dependent but no statistical differences were found between the two NPs.

These results suggest the antibacterial potential of AgNPs for the treatment of infectious diseases.

Introduction

The introduction of nanoscale materials in the health industry is an emerging area of nanotechnology. In Europe, nanomaterials are defined as "natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm -100 nm"(Commision, 2011).

The unique characteristics of silver nanoparticles are due to their surface-to-volume ratio that considerably changes physical, chemical and biological properties; for these reasons the nanoparticles have been used for various purposes (Sharma *et al*., 2009) from textiles, keyboards, wound dressings, and biomedical devices (Sondi and Salopek-Sondi, 2004; Li *et al*., 2010). Size, shape, and surface coatings of nanoparticles are important characteristics that directly determine their biocidal activities (Zhang *et al*., 2016). Smaller particles have a larger surface to volume ratio and, therefore, display greater toxic potential. A recent study has found that the biological properties of AgNPs strictly depend on the different surface charges of their coatings, which can affect the physical interaction of AgNPs with microorganisms (Powers *et al*., 2011).

Many methods have been used for the synthesis of silver nanoparticles, and nowadays are categorized as: a) chemical methods; b) physical methods and c) biological methods.

In the chemical methods three main components, such as metal precursors, reducing agents, and stabilizing/capping agents are usually employed to produce metal nanoparticles. The physical approaches use direct interaction of laser or high voltage gate with raw metal, resulting in immediate nanoparticles release. To overcome the use of hazardous chemicals and the high energy consumption required by previous described procedures, biological methods have emerged as viable alternatives. The synthesis of AgNPs using green, cost-effective, and biocompatible methods can be achieved by bacteria (Kalimuthu *et al*., 2008; Kalishwaralal *et*

al., 2010), fungi (Birla *et al*., 2013), and plant extracts (Shameli *et al*., 2012; Gurunathan, 2015; Das *et al*., 2017).

Biological methods offer numerous advantages compared to both chemical and physical ones: a) proteins and secondary metabolites are released by living organisms in the synthesis process; b) elimination of steps to prevent particles aggregation; c) eco-friendly and pollution-free characteristics of biological molecules are used (Zhang *et al*., 2016).

Silver (as silver ion) has been used as an antibacterial agent for centuries until the discovery of modern antibiotics. Today, the global spread of bacteria resistant to the most common antibiotics redirects the scientific research to alternative strategies able to fight also against multi-drug resistant (MDR) bacteria. Silver nanoparticles (AgNPs) have become a target for researchers due to their antimicrobial efficacy against bacteria, fungi, and viruses. In literature, the antibacterial properties of AgNPs against *S. aureus*, *E. coli*, *P. aeruginosa* and *S. typhi* are described by different authors (Liu *et al*., 2007; Sharma *et al*., 2009; Dakal *et al*., 2016; Das *et al*., 2017). The antibacterial actions exerted by AgNPs are linked to: a) adhesion to cell wall, b) penetration and damaging of cytoplasmatic organelles; c) induction of oxidative stress via reactive oxygen species (ROS) production; d) modulation of signal transduction pathways (e.g. stress response pathway) (Quinteros *et al*., 2016).

The specific aims of this study are: a) the set-up and optimization of protocols for the synthesis of silver nanoparticles using two biological methods and b) the analysis of their antibacterial and anti-biofilm properties against *P. aeruginosa* and *S. pseudintermedius* strains isolated from animals.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains used to study the antibacterial ability of AgNPs were randomly chosen from the bacterial collection of the Microbiology Laboratory of the Department of Veterinary Medicine of Università degli Studi di Milano. Ten strains of P. aeruginosa were isolated from eyes lavages of Chameleons (*Furcifer pardalis*); sheep blood agar and Cetrimide agar (Oxoid, Italy) were used to isolate and verify bacterial isolates belonging to Pseudomonas aeruginosa species. Ten Multi Drug Resistant (MDR) *S. pseudintermedius* strains were isolated from canine pyoderma and characterized phenotypically and genetically using, respectively, Mannitol Salt Agar (Microbiol, Italy) and the amplification of thermonuclease (*nuc*) gene as reported previously (Sasaki *et al*., 2010). The antibiotic-resistance profiles of each strain were assessed before this study with the Kirby-Bauer disk diffusion method; all the isolates were tested for amoxicillin + clavulanic acid (30 μg), amoxicillin (10 μg), cephalexin (30 μg), cefovecin (30 μg), ceftiofur (30 μg), ceftriaxone (30 μg), clindamycin (10 μg), lincomycin + spectinomycin (15 μg), doxycycline (30 μg), enrofloxacin (5 μg), marbofloxacin (5 μg), pradofloxacin (5 μg), amikacin (30 μg), gentamicin (30 μg), neomycin (30 μg), tobramycin (30 μg), kanamycin (30 μg), rifampicin (30 μg), azithromycin (15 μg), erythromycin (30 μg). P. aeruginosa strains were also tested for carbenicillin (100 μg). All the strains were stored in 25% glycerol (Carlo Erba, Italy) at -20°C until use. However, before use, samples were thawed at room temperature and 10 μL were plated on Tryptic Soy Agar + 1% sheep blood (Microbiol, Italy) and incubated aerobically at 37°C for 24 h. Three or four isolated colonies were picked up and used to assess the antibacterial activity of silver nanoparticles.

Biosynthesis of AgNPs using Curcuma longa extract

Preparation of C. longa extract

The *C. longa* powder was kindly provided by Dr. G. Graziani (Farmacia Graziani, Italy). The production of *C. longa* extract was assessed using a protocol found in literature (Shameli *et al*., 2010) with some modifications. Briefly, after dissolving 0.1 g of *Curcuma* powder in 20 mL of double distilled sterile water (ddH_2O) the solution was stirred for 4 h at room temperature in dark conditions and filtered (Whatman® Grade 42, Ashless Filter Paper, USA) to remove debris. This solution was used to synthesize the nanoparticles.

Synthesis and purification of silver nanoparticles

Forty millilitres of 1% $AgNO_3$ (Carlo Erba, Italy) were added to the fresh *Curcuma* extract and mixed (at 200 rpm) for 24 h in the dark (to avoid photochemical reactions) and at room temperature. The reduction of Ag^+ to Ag^0 was monitored by checking the change in colour of the solution: from a clear yellow to a progressive brown as found in the literature (Shameli *et al*., 2010; Gurunathan *et al*., 2014; Das *et al*., 2017). To remove Ag^+, the solution was centrifuged at 4000 rpm for 20 minutes (Sigma 4-16KS); the supernatant was discarded and replaced with the same volume of sterile ddH_2O. This step was repeated three times. The purified nanoparticles were stored at room temperature in dark conditions and sonicated (30 kHz; SONICS Vibra cell), immediately before the assays, three times for 15 sec on and 45 sec off. The nanoparticles derived from *C. longa* synthesis were referred to as ClAgNPs.Biosynthesis of AgNPs using a cell-free extract of E. coli

Biosynthesis of AgNPs using a cell-free extract of E. coli

Preparation of cell-free extract

The bacterial strain *E. coli* ATCC® 25922 was used to synthesize silver nanoparticles. For the preparation of cell-free extract, the same conditions described by Kushawa *et al* (2015) were used (Kushwaha *et al*., 2015). Briefly, *E. coli* ATCC® 25922 was cultured in Mueller-Hinton broth (Oxoid, Italy) and incubated aerobically at 37°C until reaching the logarithmic phase of growth (assessed by spectrophotometric reading at 550 nm). The biomass was removed from the supernatant by centrifugation at 4000 rpm for 20 minutes (Sigma 4-16KS); the liquid phase was filtered (0.22 µm syringe filter, Minisart® Sartorius, USA) to obtain the cell-free supernatant. To be sure about the absence of any residual bacteria in the supernatant, 10 µL were plated on Plate Count Agar (PCA, Condalab, Spain) and incubated aerobically at 37°C for 24 h

Synthesis and purification of bacterial silver nanoparticles

Synthesis of AgNPs was carried out according to a method described previously (Gurunathan *et al*., 2009; Kushwaha *et al*., 2015). A 1% AgNO3 (Carlo Erba, Italy) solution was added to the cell-free extract and incubated by shaking (200 rpm) for 24 h in a dark environment at 37°C. The synthesized nanoparticles were centrifuged at 4000 rpm for 20 minutes (Sigma 4-16KS) to remove the Ag+, and resuspended in ddH2O. This step was repeated three times. The nanoparticles derived from *E. coli* were referred to as EcAgNPs.

Characterization of AgNPs

The characterization of purified nanoparticles was carried out using two of the most used methods reported by different authors (Gurunathan *et al*., 2009, 2014; Sharma *et al*., 2009; Saeb *et al*., 2014; Das *et al*., 2017).

Ultraviolet-visible spectroscopy (UV-vis)

Bio-reduction of Ag+ to Ag0 was monitored by measuring the absorption spectra (UV-vis) with a spectrophotometer (SpectraMax 340 PC) collecting spectra over a range of 310 to 770 nm (with an optical path length of 50 nm). Readings were recorded twice within 15 minutes.

Transmission Electron Microscopy (TEM)

Size analysis of colloidal silver was characterized by EFTEM Leo 912ab (Zeiss) at a voltage of 100 kV. The samples were briefly sonicated (30 kHz, 15 sec on and 45 sec off) and immediately a drop of the aqueous suspension of AgNPs was mounted on a carbon grid, which was placed on a filter paper to absorb the excess of solvent. The morphological analysis (particle diameter and size distribution) was calculated with Java image tool software (ImageJ).

Antimicrobial properties

Kirby–Bauer disk diffusion assay

The antimicrobial activity of both ClAgNPs and EcAgNPs was tested against the 10 *P. aeruginosa* and 10 *S. pseudintermedius* strains and compared with antibiotics (carbenicillin and ampicillin, alone and in synergistic combination with silver nanoparticles) using the agar disk diffusion assay. Carbenicillin (Liofilchem, 100 µg) was used against *P. aeruginosa* and ampicillin (Oxoid, 10 µg) against *S. pseudintermedius*. Briefly, after plating each strain on a sterile Mueller-Hinton agar plate (Oxoid, Italy), antibiotics and virgin discs were placed on the

plates. In detail: two sterile discs were loaded with 20 μL of silver nanoparticles, two discs with antibiotics and two discs with antibiotic supplemented with 20 μL of silver nanoparticles, one disc was used as a negative control. All experiments were done in aseptic conditions in a laminar airflow cabinet and two replicates were done for each strain. The plates were incubated aerobically at 37°C for 24 h. Zones of inhibition for AgNPs, antibiotics, and antibiotics + nanoparticles were measured and expressed in millimetres (zone of inhibition ± SD).

MIC

The minimum inhibitory concentration (MIC) was determined using the microdilution assay according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2017). Briefly, all the strains were grown on Brain Heart Infusion agar (BHI, Scharlau) and three or four colonies were suspended in fresh sterile saline solution to reach an initial concentration of 1.5 x 10^8 CFU/mL. One hundred microliters of the 1:100 diluted cell suspensions were dispensed into each well of a 96-well microtiter plate. The 10 strains of *P. aeruginosa* were exposed to twofold dilution series of NPs alone and in synergistic combination (1:1 ratio) with carbenicillin. The same was done for *S. pseudintermedius* strains except for ampicillin instead of carbenicillin. To rule out that the antibacterial activity may be exerted by $AgNO_3$ instead of AgNPs, MIC for $AgNO_3$ was also performed (data not shown).

After incubation for 24 h at 37 °C, the MICs were determined as the lowest dilution of nanoparticles able to inhibit visible bacterial growth.

Antibiofilm properties

Screening for the biofilm-forming ability

In a preliminary study (Figure S1), the biofilm-forming ability of the *P. aeruginosa and S. pseudintermedius* strains was studied using a microtiter plate assay (MtP) (Stepanovic *et al*.,

2000; Stephanovic *et al*., 2007). As a negative control, TSB + 1% glucose (TSBg) without bacteria was used. Each strain was tested in triplicate on three independent plates. Briefly, fresh overnight subcultures of the strains were 1:100 fold diluted in TSBg and 200 µL were plated. After 24 h at 37 °C, the supernatant was gently removed and the biofilm was washed with sterile phosphate-buffered saline (PBS), fixed with methanol and stained with 2% crystal violet. The absorbance (550 nm) of negative controls was used to set the optical density cut-off (ODc) as three standard deviations above the mean OD of the negative controls. Strains were classified as follows: non-adherent OD≤ODc; weakly adherent ODc<OD 97 ≤2 × ODc; moderately adherent ODc<OD ≤4 × ODc; strongly adherent OD> 4 × ODc. Only the biofilm-producer strains were used to study the anti-biofilm properties of silver nanoparticles

Interaction between AgNPs and mature biofilm

To determine the ability of both ClAgNPs and EcAgNPs to disrupt mature biofilm, an MtP assay using a volume of 190 µL of bacteria culture (after 1:100-fold final dilution), and 10 µL of AgNPs was carried out. Non-treated bacteria (NT) group was used to make a comparison with treated bacteria.

Using the above-described method (Stephanovic *et al*., 2007), biofilm was grown at 37°C for 24 h, then 10 µL of nanoparticles were added and the plates were further incubated for 24 hours. The disruptive ability of nanoparticles was determined by staining the biofilm biomass with crystal violet and measuring the absorbance at 550 nm (Labsystem Multiscan Plus). Each experiment was replicated three times per strain and three independent plates were used.

Statistical analysis

The statistical analysis, together with the graphical representation, were performed with GraphPad Prism v6 (GraphPad Software®, La Jolla California, USA). The results are presented

as follows: means ± SD for disc diffusion assay; µM ± SD for MIC; OD ± SD for anti-biofilm activity. All data were tested for normality and analysed using Student's t-test and a *p-value* less than 0.05 was considered statistically significant.

Results

Synthesis of AgNPs

The change in colour (darkish-brown) of the silver solutions was used to monitor the bio-reduction of silver ions to silver nanoparticles (Fig. 1). The intensity of brown increased during time as found in the literature. *C. longa* solution showed a more explicit darkish-brown appearance after the period of incubation (Fig. 1A) suggesting a stronger synthesis of NPs. On the contrary, the supernatant of *E. coli* turned immediately from yellow to yellow-white appearance without showing a brown aspect (Fig. 1B). The confirmation of NPs synthesis and stability was monitored by UV-vis spectroscopy, while the morphological analysis was performed with TEM.

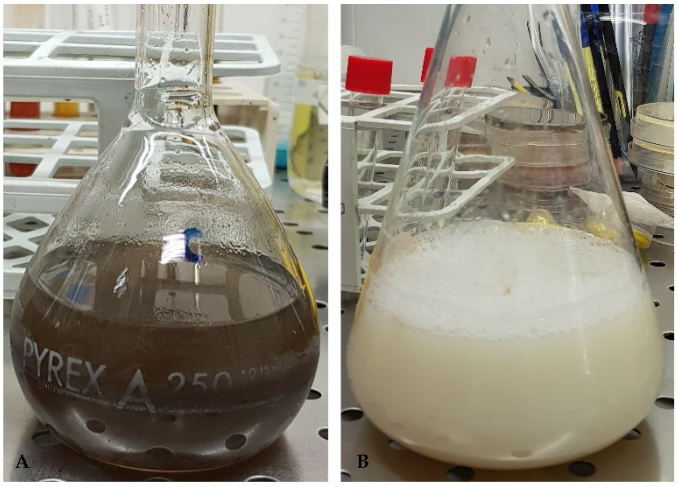

Figure 1. Biosynthesis of silver nanoparticles.

Plant-mediated method (a) after dissolving *C. longa* powder in ddH_2O the solution was filtered to remove the majorities of vegetal debris, finally, silver nitrate was added and the change in colour was monitored. Bacterial supernatant method (b). After growing *E. coli*

ATCC® 25922 in Mueller-Hinton broth, the supernatant was collected and silver nitrate was added to induce nanoparticle synthesis.

UV-vis spectroscopy

UV-vis spectroscopy is considered one of the most important tools for the evaluation of NPs synthesis (Gurunathan *et al*., 2014). The absorption spectra of the AgNPs are shown in Fig. 2. ClAgNPs showed a clear absorption peak at around 440 nm, confirming the production of colloidal silver nanoparticles. On the other hand, EcAgNPs showed a logarithmic-like curve which not clearly shows a specific absorption peak and suggest any NPs formation. To exclude an instrumental bias, serial dilution of EcAgNPs were loaded in the spectrophotometer with the same results in term of peak's absence.

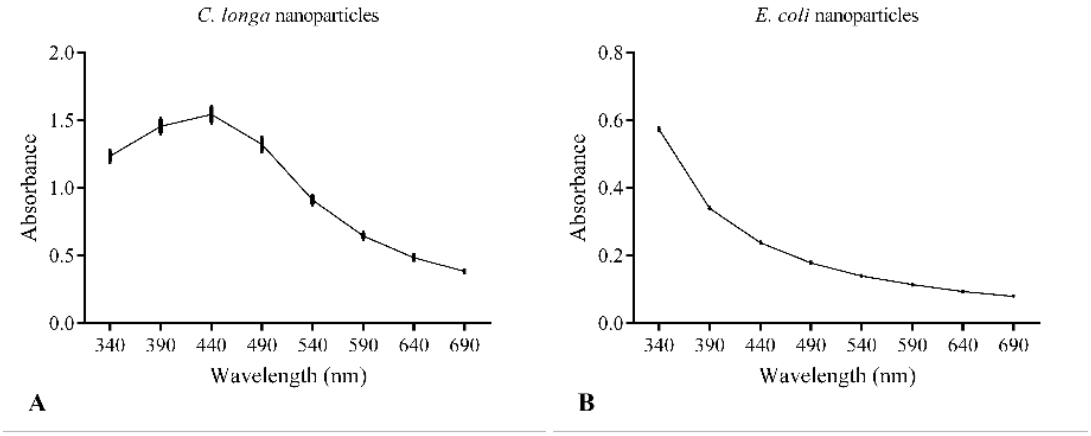

Figure 2 UV-vis spectroscopy of ClAgNPs and EcAgNPs.

Size and morphology analysis of silver nanoparticles

To understand the size of colloidal silver particles and provide more robust data about their morphology, TEM investigations were conducted with EFTEM Leo 912ab (Fig. 3 A-D) while morphological measurements were estimated with ImageJ software to build the frequency distribution of particle diameter (Fig. 3 C, E). It could be observed that NPs obtained with *Curcuma* showed a spherical symmetry and heterogeneous spatial dimensions ranging from 10

to 25 nm (11.1±2.75 nm). The presence of narrow particles was in accordance with the UV-vis results. Fig. 3A-B shows ClAgNPs enclosed in *Curcuma* extract.

the analysis of *E. coli* supernatant showed nanoparticles enclosed in a matrix that connected each particle to the other (Fig. 3B, C). These particles seemed to have a spherical symmetry and diameter ranging from 15 nm to 35 nm (27.28±2.68 nm). Maybe, this particular spatial configuration was responsible for the lack of a unique 440 nm peak investigated with UV-vis.

Overall, for both ClAgNPs and EcAgNPs the results were in accordance with the literature (Shameli *et al*., 2010; Zhang *et al*., 2016) confirming that biological methods for the synthesis of AgNPs produce nanoparticles lacking homogeneity.

After the morphological analysis, the determination of the molar concentration of silver nanospheres was determined as reported in the literature (Liu *et al*., 2007; Kalishwaralal *et al*., 2010)

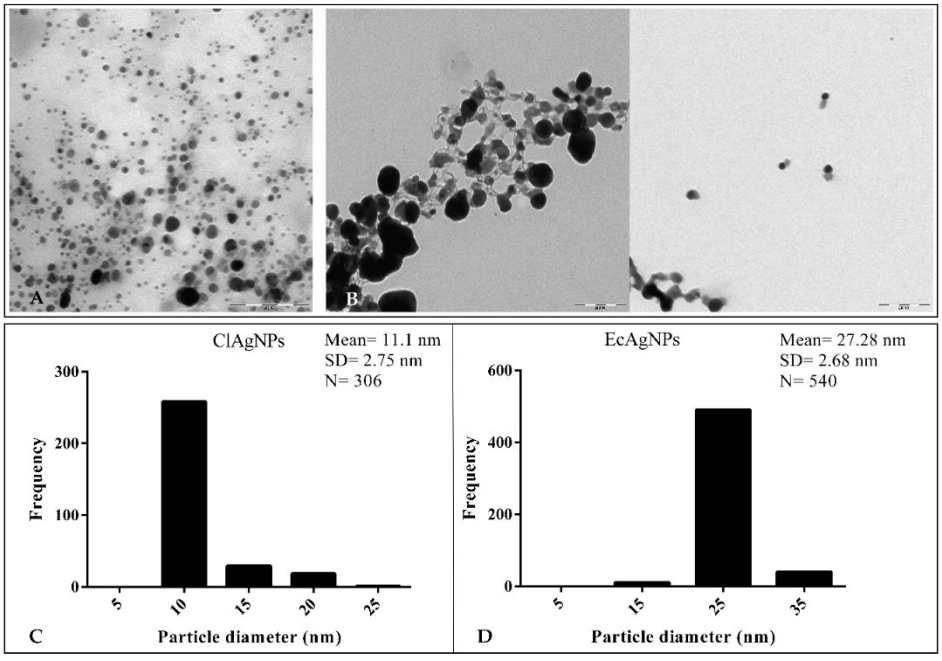

Figure 3 Morphological analysis by TEM.

(a,c) NPs derived from *C. longa* showed spherical symmetry and mean diameter of 11.1 nm. (b,d) EcAgNPs showed a highly-branched symmetry and dimension of 27.28 nm in diameter

Antibacterial activity

Kirby – Bauer disk diffusion assay

The potential synergistic effects of combining silver nanoparticles with antibiotics were evaluated with the disk diffusion assay (Fig. 4). All the *P. aeruginosa* and staphylococcal strains were, respectively, resistant to carbenicillin and ampicillin. The antibacterial activity of both ClAgNPs and EcAgNPs increased when in combination with antibiotics in all the tested strains showing significant differences ($p<0.05$) in comparison with silver nanoparticles alone (Fig. 4 A-D). For *P. aeruginosa* strains (Fig. 4 A, C) ClAgNPs and EcAgNPs alone showed a mean inhibition halo of 9.5 and 14.4 mm respectively resulting in statistical differences when compared to carbenicillin+ClAgNPs (14 mm), and carbenicillin+EcAgNPs (17.45 mm). For *S. pseudintermedius* strains (Fig. 4 B, D) statistically differences were found between ClAgNPs and EcAgNPs alone (12.8 mm, and 16.4 mm, respectively), and in combination with ampicillin (19.61 mm, and 21.38 mm, respectively). The interpretation of Kirby-Bauer assay, using CLSI breakpoints, showed that all the staphylococcal strains were considered resistant to ampicillin even after the synergistic combination with NPs (resistance for ampicillin is < 29 mm), while *P. aeruginosa* strains were resistant against carbenicillin alone, but when NPs were added the strains were susceptible.

The statistical differences between the two nanoparticles tested are shown in Fig. 5, giving reasons to hypothesize a better antibacterial ability of EcAgNPs, but only in staphylococcal strains. This result is in contrast with the literature in which Gram-negative

bacteria are reported as more susceptible to silver nanoparticles than Gram-positive depending on differences in their cell wall structure.

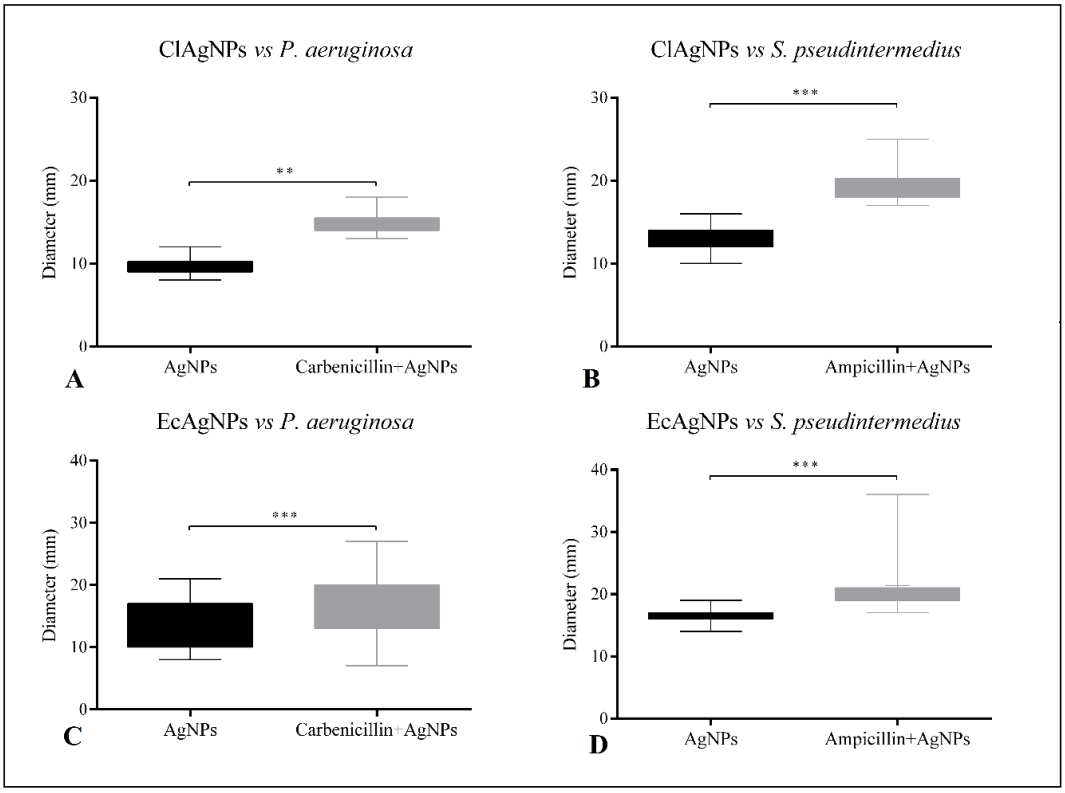

Fig. 4. Disk Diffusion Assay.

Effect of NPs used alone and in synergistic combination with carbenicillin against *P. aeruginosa* and ampicillin against *S. pseudintermedius*. Statistical analysis showed differences between inhibition halos of silver nanoparticles alone and those of silver in combination with carbenicillin (a,c) and ampicillin (b,d). Results are presented as mean±SD (*p value: 0,05-0,01; **p value: <0.01; ***p value: <0,001).

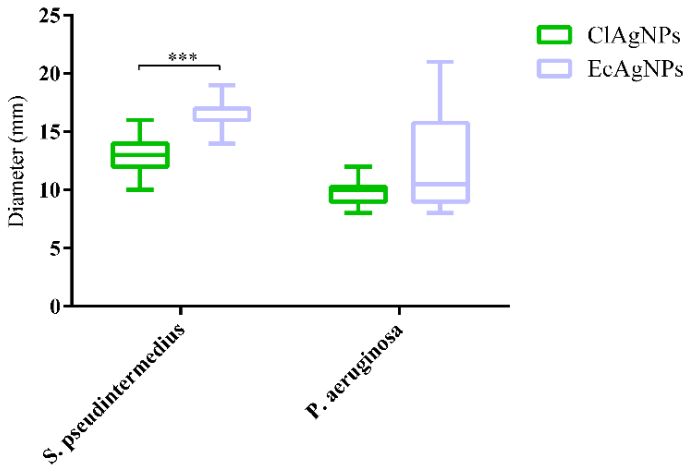

Fig. 5. Antibacterial ability of ClAgNPs and EcAgNPs

T-student's test revealed significant differences between ClAgNPs, and EcAgNPs in *S. pseudintermedius* strains (*p-value* ≤0.001).

MIC

MIC (Table 1 and Figure 6) of each tested strain was determined for both ClAgNPs, and EcAgNPs alone and in synergistic combination with carbenicillin (for *P. aeruginosa*) and ampicillin (for *S. pseudintermedius*). As found in Kirby-Bauer assay, *P. aeruginosa* and *S. pseudintermedius* were resistant to carbenicillin (512 µg/mL) and ampicillin (56±40.63 µg/mL). The results of NPs showed that MIC were different between Gram-negative and Gram-positive bacteria. When the antibiotics were adjuvated (1:1 ratio) with ClAgNPs and EcAgNPs, the MIC were significantly lower resulting in sensible profiles (according to CLSI breakpoints). For both Gram-positive and Gram-negative bacteria, significant differences were found comparing the same antibiotic adjuvated with ClAgNPs and EcAgNPs and showing a lower MIC (better antimicrobial activity) for nanoparticles synthesized from *E. coli* supernatant.

	P. aeruginosa	*S. pseudintermedius*
Atb (μg/mL)	512±0	56±40,63
ClAgNPs (μM)	71,8±0	143,7±0
Atb+ClAgNPs (μg/mL)	6,4±4,2	6,2±2,4
EcAgNPs (μM)	0,438±0,18	3,75±3,65
Atb+EcAgNPs (μg/mL)	0,48±0,37	0,5±0,27

Table 1. Determination of MIC values for ClAgNPs and EcAgNPs alone and in combination with antibiotics. (Atb: antibiotic; carbenicillin 512-1 μg/mL for *P. aeruginosa*; ampicillin 256-0.5 μg/mL for *S. pseudintermedius*).

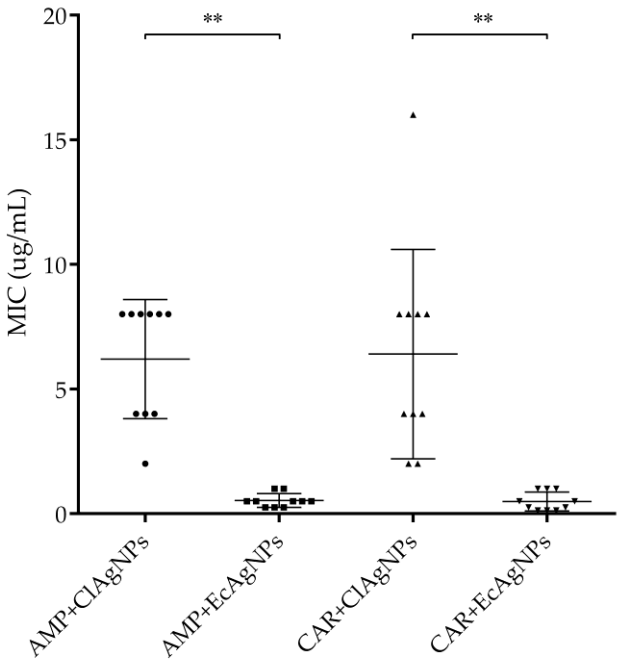

Fig. 6. MIC of NPs in combination with ampicillin and carbenicillin.

Statistical differences were found between the combination ampicillin+ClAgNPs and the same antibiotic adjuvated with EcAgNPs (*p-value*= 0.002). Similarly, carbenicillin showed lower MIC when in synergistic combination with EcAgNPs instead of ClAgNPs (*p-value*= 0.0023).

Antibiofilm properties

The formation of biofilm is a highly coordinated process during which bacteria adhere to each other and to biotic/abiotic surfaces, resulting in the formation of a complex community enclosed in a self-produced extracellular matrix.

Following preliminary assays for the screening of biofilm-forming ability (Supplementary figure 1), 8 and 7 strains among, respectively *P. aeruginosa* and *S. pseudintermedius*, were selected for their biofilm-forming ability.

These strains were grown for 24 h in microtiter plates and then exposed to ClAgNPs and EcAgNPs following a 1:20 dilution. The results (Fig. 7) confirm the anti-biofilm ability of silver nanoparticles but only for P. aeruginosa strains the inhibition was statistically significant ($p<0.05$) between the non-treated group and both nanoparticles. For staphylococcal strains, no differences were found between NT and both nanoparticles, but a negative trend could be observed between NT and ClAgNPs.

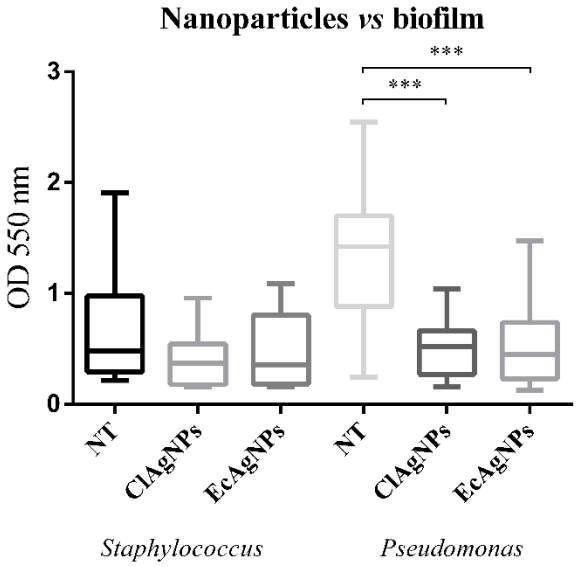

Fig. 7. Anti-biofilm properties of NPs

Statistical differences were found only among *Pseudomonas* strains (*p value: 0,05-0,01; **p value: <0.01; ***p value: <0,001).

Discussion

In this study, the antibacterial and anti-biofilm properties of silver nanoparticles, synthesized using two biological methods were determined using different *in vitro* assays against *P. aeruginosa* and MDR *S. pseudintermedius* strains isolated from clinical patients from the veterinary teaching hospital of the University of Milan. To achieve these objectives silver nanoparticles were synthesized using a) *Curcuma longa* powder, and b) the supernatant of *E. coli* ATCC® 25922; then their characterization was performed using UV-vis spectroscopy and TEM analysis. Finally, the disk diffusion test, the determination of the MIC values and the time-killing assay demonstrated the high bactericidal ability of NPs against both Pseudomonas and Staphylococcus. The ability to disrupt mature biofilm was determined using a microtiter assay.

It is well known that classical chemical methods produce more homogeneous and long-term stable particles but contrary to biological methods, a lot of potentially hazardous chemicals are used to synthesize and stabilize silver nanoparticles (e.g. sodium borohydride and sodium citrate) (Zhang *et al*., 2011, 2016; Wei *et al*., 2016). Molecules from living organisms (e.g. proteins and amino acids) or extract from plants (e.g. phenols and aldehydes) act like reducing and capping agents, making the biological methods more eco-friendly and less expensive, but producing more heterogeneous particles (Sharma *et al*., 2009). The oxidation of aldehyde (naturally present in *Curcuma* root) to carboxylic groups allows to reduce Ag^{+} and control the shape of spherical nanoparticles (Shameli *et al*., 2010).

The proteins expressed during the bacterial life (e.g. nitrate reductase) actively reduce silver ions to silver nanoparticles, changes in pH and temperature affect this reaction with the final effect of producing nanoparticles of different dimensions (Gurunathan *et al*., 2009).

In this study the nanoparticles produced by *Curcuma* powder and *E. coli* shown heterogeneous dimensions as observed on previous studies (Gurunathan *et al*., 2009; Shameli *et al*., 2010; Kushwaha *et al*., 2015; Das *et al*., 2017). However, particles dimensions were

different (11.107±2.705 nm for *C. longa* and 27.282±2.68 nm for *E. coli*) from those observed in the literature in which NPs ranged from 6.3-2.64 nm (Shameli *et al*., 2010) to 50 nm (Gurunathan *et al*., 2009). One possible explanation could be the differences in terms of raw material use (e.g. quality of *Curcuma* powder) and the bacterial strain used.

The disk diffusion assay demonstrated the ability of NPs (alone or in combination with antibiotics) to kill more easily antibiotic-resistant bacteria. In particular, statistical differences were found in *S. pseudintermedius* between nanoparticles from *E. coli* and *Curcuma* underlying the major effect of bacterial-derived nanoparticles. These results allow us hypothesize that nanoparticles are broad-spectrum agents as described in literature (Lara *et al*., 2010). It has to be noted that, in the specific case of *S. pseudintermedius*, the measurement of the diameter in the Kirby-Bauer assay after NPs addition gives a resistant profile of these bacteria. The MIC values are in agreement with the literature in which NPs are reported as being more active against Gram-negative bacteria (Das *et al*., 2017). From the comparison of ClAgNPs and EcAgNPs between *S. pseudintermedius* and *P. aeruginosa*, only EcAgNPs were found statistical different in *S. pseudintermedius*, these results seem to be in contrast with MIC values, but could be explained by the presence of *E.coli* proteins residues (used for the synthesis of EcAgNPs) present on the surface of NPs that could interfere with the staphylococcal growth in a similar way of chemical synthesis of NPs (Shahverdi *et al*., 2007). It is well documented that the *quorum-sensing* pathways, devoted to bacterial growth, communication and expression of virulence factors, are linked to the expression of species-specific peptides, experimentally peptides derived from Gram-negative bacteria are able to inhibit the growth or expression of specific proteins of Gram-positive (Kong *et al*., 2006).

One of the most studied bacterial virulence factors is the ability to produce biofilm through the *quorum-sensing* communication system. Biofilm development is related to environmental signals and communication systems, that reflect on specific gene expression

(Hall and Mah, 2017). Nanoparticles from both *Curcuma* and bacterial synthesis are able to destroy the mature biofilm of *Pseudomonas*. The reason for this higher activity could be the adsorption of biomolecules on the surface of bacteria, due to electrostatic attraction (Kalishwaralal *et al*., 2010).

The synthesized AgNPs showed antibacterial activity alone and in combination with common antibiotics, suggesting a potential use as alternative therapies in case of Multi Drug Resistant-derived infections. Their ability to destroy mature biofilm has to be properly studied in terms of localization and elaboration of other nanoparticles-based approaches

Conclusions

Two different biological methods are used in this study to synthesize silver nanoparticles: one via *C. longa* powder, the second using the supernatant of *E. coli* ATCC® 25922 strain. UV-vis spectroscopy and transmission electron microscopy were used to analyze the neo-synthesized nanoparticles, the results confirmed the ability of natural products (present in Curcuma root) and bacterial proteins to reduce and cap silver ions generating nanoparticles very small in size.

The antibacterial abilities of these nanoparticles were tested against *P. aeruginosa* and MDR staphylococcal strains through commonly used assays. It was shown that AgNPs are particularly effective against Gram-negative bacteria even in small concentrations. The mature biofilm (produced by both Gram-positive and Gram-negative bacteria) can be though as a potential target for silver nanoparticles-based therapies.

Acknowledgments

This work was supported by the Unitech NOLIMITS Platform of the Università degli Studi di Milano responsible for TEM analysis. The authors express gratefulness to Dr. Gabriele Graziani for providing *Curcuma* powder and for his freely shared scientific background.

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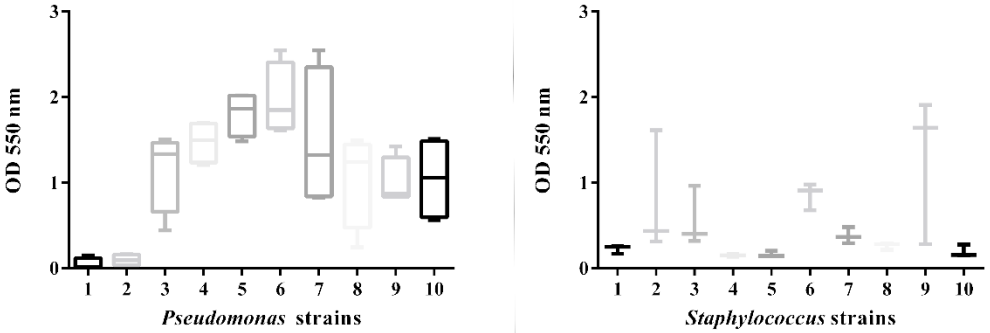
Appendix A: Supplementary materials

Supplementary figure 1

Biofilm forming ability of *Pseudomonas* and *Staphylococcus* strains.



Optical densities of all the strains as measured following the method previously described (Stepanović *et al*., 2007). After MtP assay, among the 10 *Pseudomonas* strains, 8 were considered strongly biofilm producers. Among the 10 *S. pseudintermedius* strains 4 resulted in non-biofilm-producers, 2 weakly producers and 4 strongly producers.

General discussion and conclusions

Canine pyoderma is the result of different associated factors: the host, the pathogen and the surrounding environment. After the introduction of more discriminative diagnostic techniques, the reclassification of *S. intermedius* finally identified in *S. pseudintermedius* the most frequently pyoderma-associated pathogen.

This thesis focused on four areas associated with *S. pseudintermedius*, in order to: i) better describe the molecular characterization of this pathogen, ii) evaluate the emerging problem of antibiotic-resistance through a comparison between two different veterinary teaching hospitals, iii) analyze the zoonotic potential of some strains related with their ability to produce biofilm and to resist to antibiotic treatment, and iv) find a valid alternative to antibiotic-based therapies using silver ions as precursor for nanoparticles.

Our survey put the basis for further investigations, one of the future plans consisting of the study of resistome and all resistance-associated genes. In fact, in literature, the OMICs-based approaches are currently fast-growing research areas able to deeper investigate the origin and evolution of antibiotic-resistance.

It is already known that the evolution of traditional antibiotics will be based on the attenuation of bacterial virulence using peptides or small interfering RNAs. *S. pseudintermedius* offers a large variety of possible molecular targets first of all the 18 Cell Wall Anchored proteins without which the adhesion will not take place, avoiding the downstream penetration and multiplication bacterial phases.

Scientific publications

Meroni G., Zamarian V., Prussiani C., Bronzo V., Lecchi C., Martino P.A., Ceciliani F. The bovine acute phase protein α1-acid glycoprotein (AGP) can disrupt *Staphylococcus aureus* biofilm (2019) *Veterinary Microbiology*, 235: 93-100.

Meroni G., Filipe S. JF., Drago L., Martino P.A. Investigation on Antibiotic-Resistance, Biofilm Formation and Virulence Factors in Multi Drug Resistant and Non Multi Drug Resistant *Staphylococcus pseudintermedius* (2019) *Microorganisms*, 7 (12) 702-713.

Submitted papers

Meroni G., Filipe S. JF., Martino P.A. In vitro antibacterial activity of biological-derived silver nanoparticles: preliminary data. Paper submitted to Veterinary Sciences, Special Issue *Italian Society of the Veterinary Sciences SISVet 2019.*

Nocera FP., **Meroni G**., Fiorito F., De Martino L., Martino PA. Prevalence and antimicrobial susceptibility patterns of canine *Staphylococcus pseudintermedius* strains isolated from two different Italian university veterinary hospitals. Paper submitted to Veterinary Research Communications.

Abstract

G. Meroni and P.A. Martino (2019) "Green synthesis" of silver nanoparticles: characterization and in vitro antibacterial activity against *P. aeruginosa* and *S. pseudintermedius* isolates. Poster presented at 47th National Congress of the Italian Society of Microbiology " SIM. Rome 18-21 September 2019

C. Rendina, **G. Meroni**, P.A. Martino (2019) A preliminary study of the antibacterial effect of manuka honey and propolis against *S. pseudintermedius* strains. Poster presented at LXIII Convegno Federazione SISVet. Olbia 19-22 June 2019

G. Meroni, P.A. Martino (2019) "Green synthesis" of silver nanoparticles: characterization and in vitro antibacterial activity against *P. aeruginosa* and *S. pseudintermedius* isolates. Oral presentation at LXIII Convegno Federazione SISVet. Olbia 19-22 June 2019

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