UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

NASAL CARRIAGE OF METHICILLIN-RESISTANT COAGULASE-POSITIVE STAPHYLOCOCCI AMONG CATS AND DOGS HOSPITALIZED IN THE VETERINARY TEACHING HOSPITAL OF THE FACULTY OF VETERINARY MEDICINE – TECHNICAL UNIVERSITY OF LISBON, PORTUGAL

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DISSERTAÇÃO DE MESTRADO EM MEDICINA VETERINÁRIA

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À minha família, aos meus amigos e ao meu namorado.

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Third, I would like to thank Professor Luca Guardabassi for giving me the opportunity to learn more at the laboratory of Microbiology, Faculty of Life Sciences, Copenhagen and for taking me under his Co-Supervision.

Finally I would like to thank all the veterinarians, especially Dr. Gonçalo Vicente, and veterinary assistants that work at the Teaching Hospital of the Faculty of Veterinary Medicine - UTL, for their help with the collection of the swabs, and to the veterinarians working at the Laboratory of Antimicrobial Resistance, especially Drª. Felisbela Loução, for their help with the lab work.

Abstract - Nasal carriage of methicillin-resistant coagulase-positive staphylococci among cats and dogs hospitalized in the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine – Technical University of Lisbon, Portugal

Methicillin-resistant coagulase-positive staphylococci (MRCPS) colonization in companion animals is an emerging and significant problem in public and animal health. During one year, nasal swabs were obtained from 40 cats and 146 dogs admitted to the Teaching Hospital of the Faculty of Veterinary Medicine - Technical University of Lisbon. MRCPS colonization was screened by plating enrichment cultures on a selective medium, Chrom MRSA ID. Bacterial species and *mecA* were confirmed by PCR. Clonality of the isolates was assessed by pulsed-field gel electrophoresis (PFGE). All isolates were subjected to *spa* and SCC*mec* typing. They were also tested by PCR for the *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL) in *Staphylococcus aureus* and Luk-I and *Staphylococcus intermedius* exfoliative toxin (SIET) in *Staphylococcus pseudintermedius*. Methicillin-resistant *S. aureus* (MRSA) was found in two cats (5 %) and one dog (0.6 %). Isolates were *spa* type t032, SCC*mec* IV and shared identical PFGE profiles. These were similar to the EMRSA-15 human clone. Strains were PVL-negative. Nine dogs carried methicillin-resistant *S. pseudintermedius* (MRSP) (6 %), whereas none of the cats was positive. The PFGE type A strain (*n*=1) showed identical characteristics as the American MRSP clone strains (CC68-MRSP-V), while PFGE cluster B grouped European MRSP isolates (CC71-MRSP-III) (*n*=8). All isolates were SIET-negative. The 8 European MRSP isolates were positive for the *lukF/lukS* genes and the American MRSP isolate was negative for both genes. Strains were multidrug-resistant, which represents a major challenge for veterinarians in terms of antibiotic therapy.

Keywords: colonization, companion animals, methicillin resistance, *Staphylococcus aureus*, *Staphylococcus pseudintermedius*

Resumo - Frequência de colonização por staphylococci coagulase-positivo meticilina-resistente em cães e gatos internados no hospital escolar da Faculdade de Medicina Veterinária – Universidade Técnica de Lisboa, Portugal

A colonização por staphylococci coagulase-positivo meticilina-resistente (MRCPS) é um problema emergente e de grande importância em termos de saúde animal e pública. Durante um ano, zaragatoas nasais de 146 cães e 40 de gatos foram obtidas de animais internados no Hospital Escolar da Faculdade de Medicina Veterinária - Universidade Técnica de Lisboa. A colonização por MRCPSfoi pesquisada por inoculação de culturas de enriquecimento num meio selectivo, Chrom MRSA ID. As espécies de MRCPS e a amplificação do gene *mecA* por feita por PCR. A clonalidade dos isolados foi confirmada por PFGE. Todos os isolados foram sujeitos a tipagem *spa* e SCC*mec*. Os isolados de *S. aureus* meticilina-resistente (MRSA) e de *S. pseudintermedius* meticilina-resistente (MRSP) foram testados por PCR para a presença dos genes *lukF/lukS* que codificam, respectivamente, a leucocidina Panton-Valentine (PVL) e a leucocidina-I (Luk-I). Os isolados de MRSP foram ainda testados para a presença da toxina exfoliativa do *S. intermedius* (SIET). Nesta amostra, 0,6 % (*n*=1) dos cães testados e 5 % (*n*=2) dos gatos apresentaram MRSA. Os isolados de MRSA eram *spa* tipo t032, SCC*mec* IV e partilhavam padrões idênticos de PFGE. As estirpes eram idênticas ao clone humano EMRSA-15. Os 3 isolados eram PVL negativos. Nove cães apresentaram MRSP (6 %), enquanto nenhum dos gatos foi positivo. PFGE tipo A mostrou características idênticas ao clone americano de MRSP (CC68-MRSP-V) e PFGE tipo B agrupou os isolados europeus de MRSP (CC71-MRSP-III) (*n*=8). Os isolados de MRSP PFGE tipo B eram Luk-I positivos mas SIET negativos. Todas as estirpes de MRSP eram multirresistentes a várias classes de antibióticos, o que representa um desafio para os médicos veterinários em termos de estratégias de antibioterapia.

Palavras-chave: β-lactâmicos, colonização, resistência, *Staphylococcus aureus*, *Staphylococcus pseudintermedius*

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Abbreviations

AST	Antimicrobial Susceptibility Testing
bp	Base Pairs
CC	Clonal Complex
CHEF	Clamped Homogeneous Electric Field
CIISA	Interdisciplinary Centre of Research in Animal Health
CIP	Ciprofloxacin
CLSI	Clinical Laboratories Standards Institute
CN	Gentamicin
DA	Clindamycin
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide Triphosphates
E	Erythromycin
EARSS	European Antimicrobial Resistance Surveillance System
EMRSA-15	Epidemic Methicilin-Resistant *Staphylococcus aureus*-15
ENR	Enrofloxacin
EURL	European Union Reference Laboratory of Antimicrobial Resistance
FMV-UTL	Faculty of Veterinary Medicine – Technical University of Lisbon
IgG	Immunoglobulin G
IWG-SCC	International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements
K	Kanamycin
LEV	Levofloxacin
Luk-I	Leukocidin I
MAR	Marbofloxacin
MRS	Methicillin-Resistant staphylococci
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSP	Methicillin-Resistant *Staphylococcus pseudintermedius*
MSSA	Methicillin-Susceptible *Staphylococcus aureus*
MSSP	Methicillin-Susceptible *Staphylococcus pseudintermedius*
MLST	Multilocus Sequence Typing
MXF	Moxifloxacin
NA	Not Applicable
NOR	Norfloxacin

OFX	Ofloxacin
ORF	Open Reading Frame
PBP	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
PVL	Panton-Valentine Leukocidin
QRDR	Quinolone Resistance Determining Regions
R	Resistant
RFLP	Restriction Fragment Length Polymorphism
S	Susceptible
SIG	*Staphylococcus intermedius* Group
spa	Staphylococcal Protein A
SCC*mec*	Staphylococcal Chromosome Cassette *mec*
SIET	*Staphylococcus intermedius* Exfoliative Toxin
SSR	Short Sequence Repeat
ST	Sequence Type
TE	Tetracycline
TSST-1	Toxic Shock Syndrome Toxin-1
W	Trimethoprim

1 Preface

In February 2008, I initiated a project with Professor Constança Pomba and Dr. Paula Tilley, both teachers at the Faculty of Veterinary Medicine – Technical University of Lisbon (FMV-UTL). The objective of the study was to investigate the carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) strains among horses with Chronic Obstructive Pulmonary Disease. My work consisted on inoculating the nasal swabs in an enrichment broth, collected by a colleague, Joana Simões, and posterior characterization of the isolated strains. This work was performed at the Laboratory of Antimicrobial and Biocide Resistance of the FMV-UTL, run by Professor Constança. No MRSA strains were isolated, but we found a high frequency of isolation of methicillin-resistant coagulase-negative staphylococci strains from horses affected by the disease. Dr. Paula presented an oral communication at the 3rd AMVE Congress 2008 entitled "Estudo da Colonização no Cavalo por *Staphylococcus* Meticilina-Resistentes" [Study of the colonization by methicillin-resistant *Staphylococcus* in horses] (Annex I).

During the same year, I collaborated in the identification of *Staphylococcus* species in a CIISA (Interdisciplinary Centre of Research in Animal Health) project about Canine Atopy. An oral communication describing the first case of pyoderma caused by methicillin-resistant *Staphylococcus simulans* in a dog with atopic dermatitis was presented to the 17th APMVEAC National Congress 2008 entitled "Primeiro caso de infecção cutânea por *Staphylococcus simulans* meticilina resistente num cão com dermatite atópica em Portugal" [First case of a cutaneous infection by a methicillin-resistant *Staphylococcus simulans* in a dog with atopic dermatitis in Portugal] (Annex II). I also participated in the Portuguese European Union baseline study for the detection of MRSA in breeding pigs and in the identification of MRSA in pigs with exudative epidermiditis.

In April of 2008, we started a new project: Determination of the frequency of isolation of methicillin-resistant coagulase-positive staphylococci strains among cats and dogs hospitalized at the Teaching Hospital of the FMV-UTL, which became later my Master dissertation subject. The Teaching Hospital receives small animal patients for first consults in internal medicine, radiology and surgery. Also receives patients for first and second opinion consults on dermatology, neurology, cardiology, ophthalmology, endocrinology, orthopedics and animal behavior. The hospital has a vast casuistic and many animals are admitted to the internment for various medical reasons. Normally, I collected nasal swabs in the morning, which is usually the period of animal internment. During a one year period (from April 2008 to April 2009) I swabbed 146 dogs and 40 cats admitted to the Teaching Hospital and

processed them at the Laboratory of of Antimicrobial and Biocide Resistance of the FMV-UTL.

In 2009, we presented an oral communication at the 18th APMVEAC National Congress 2009 under the title "Frequência de colonização por staphylococci coagulase-positivo em gatos internados no hospital escolar da FMV-UTL" [Frequency of colonization by methicillin-resistant coagulase-positive staphylococci in cats hospitalized at the teaching hospital of FMV-UTL] (Annex III), three posters at the ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications, designated "Nasal carriage of methicillin-resistant coagulase-positive staphylococci among cats and dogs hospitalized in a Veterinary Teaching Hospital in Portugal" (Annex IV), "First description of an MRSA skin infection in a dog and attending veterinarian in Portugal" (Annex V) and "High-frequency of clindamycin associated resistance among MRSA CC398 from breeding swine herds in Portugal" (Annex VI) and one poster at the VI OMV Congress 2009, named "Frequência de colonização por staphylococci coagulase-positivo meticilina-resistente em cães internados no hospital escolar da FMV-UTL" [Frequency of colonization by methicillin-resistant coagulase-positive staphylococci in dogs hospitalized at the teaching hospital of FMV-UTL] (Annex VII).

During three months (from October 2009 to December 2009) I undertook the LLP/Erasmus Program and went to Copenhagen to work in the Microbiology Laboratory of the Faculty of Life Sciences, University of Copenhagen. I was working under the supervision of Professor Luca Guardabassi and Doctor Arshnee Moodley, on the molecular characterization of my MRSP isolates and collaborating in other small projects.

In late 2009, we submitted a case report with the name "Treatment of a lower urinary tract infection in a cat caused by a multidrug-methicillin-resistant *Staphylococcus pseudintermedius* and *Enterococcus faecalis*" (Annex VIII) in collaboration with Doctor Arshnee Moodley, to the Journal of Feline Medicine, which was accepted early this year.

In February of this year, Professor Constança challenged me to present a small clinical case and some results of the study in a Strategies of Antimicrobial Therapy in Veterinary Medicine class, which I accepted gladly.

In April we submitted an article to the Veterinary Microbiology Journal, entitled "Nasal colonization of methicillin-resistant *Staphylococcus aureus* (EMRSA-15) and methicillin-resistant *Staphylococcus pseudintermedius* (CC71 and CC68) clones among hospitalized cats and dogs" (Annex IX), in co-authorship with Doctor Arshnee Moodley and Professor Luca Guardabassi.

2 Introduction

Staphylococci are important pathogenic bacteria, long known to cause infection in mammals. There are different species, but the main classification of staphylococci is based on their ability to produce coagulase, an enzyme that causes blood clot formation (Blair, 1962). In early times, only coagulase-positive staphylococci were thought to have the ability to cause infection (Blair, 1962). Due to their ubiquitous nature and relatively low virulence, coagulase-negative staphylococci were considered to be clinically insignificant contaminants when isolated from clinical specimens (Pfaller & Herwaldt, 1988). However, in recent years coagulase-negative staphylococci have become increasingly recognized as important agents of nosocomial infection, especially those related to indwelling devices (Pfaller & Herwaldt, 1988).

Coagulase-positive staphylococci can cause a variety of diseases, which vary in morbidity and mortality. Wound and cut infections, minor skin infections (pimples, impetigo, boils, cellulitis, folliculites, carbuncules, scalded skin syndrome and abscesses) and transient food poisoning have low mortality rates (Blair, 1962). On the other hand, pneumonia, toxic shock syndrome, meningitis, endocarditis, osteomyelitis, bacteremia and sepsis are life-threatening diseases caused by more virulent strains (Lowy, 1998). Staphylococci can produce a wide variety of toxins that can be divided in two categories: those which enable the colonization of the host and infection (e.g. coagulase, hyaluronidase, leukocidin) and those which damage the tissues or affect their normal functions (e.g. enterotoxins) (Blair, 1962).

Inside the coagulase-positive staphylococci group, *Staphylococcus aureus* and *Staphylococcus pseudintermedius* are the two most important species in Veterinary Medicine, due to their ability to cause infection and their zoonotic potential (Weese & van Duijkeren, 2010). Methicillin resistance emerged in these two organisms, raising the concern for animal and public health (Weese & van Duijkeren, 2010).

2.1 Methicillin-resistant *Staphylococcus aureus*

In 1882, Ogston first described staphylococcal disease and its role in sepsis and abscess formation. More than 100 years later, *S. aureus* remains an adaptable and dangerous pathogen in humans (Lowy, 1998). Treatment of infections with *S. aureus* has become more difficult because of the emergence of multidrug-resistant strains (Lowy, 1998).

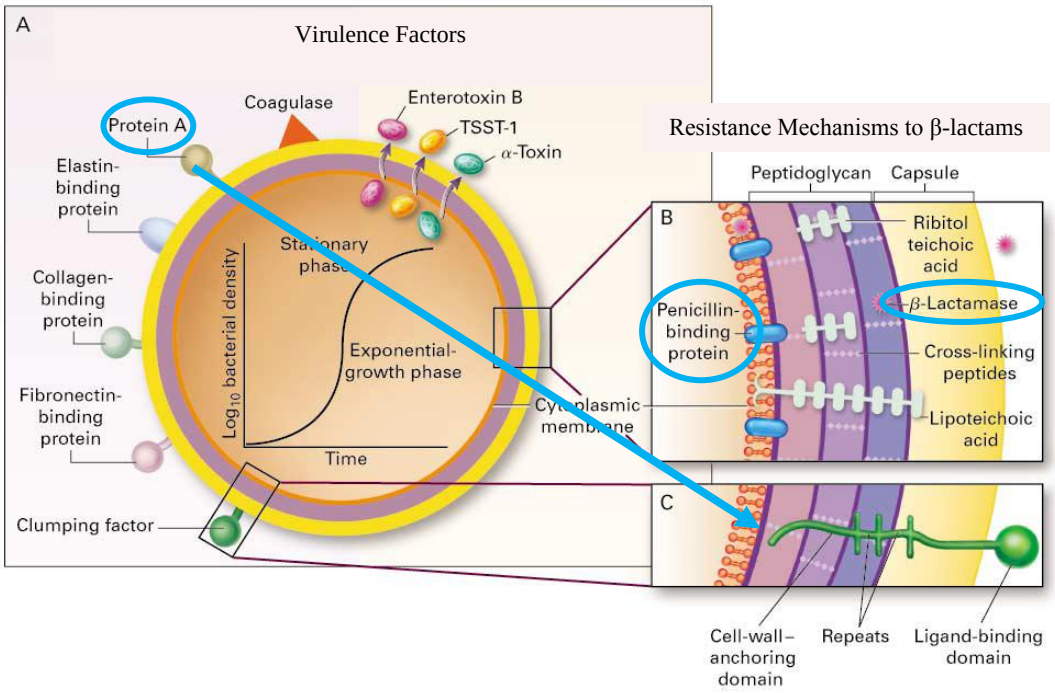
The genome of *S. aureus* consists of a circular chromosome, with prophages, plasmids and transposons (Lowy, 1998). Genes encoding virulence and resistance to antibiotics are found

on the chromosome and in extrachromosomal elements (Lowy, 1998), which enables the possibility of gene transference between staphylococci strains, species or other gram-positive bacterial species (Schaberg & Zervos, 1986).

S. aureus produces numerous toxins that are grouped on the basis of their mechanisms of action (Lowy, 1998). The synthesis of many of these proteins is dependent on the growth phase (Lowy, 1998). Nearly all strains secrete a group of enzymes and cytotoxins that cause pore formation and induce proinflammatory changes in mammalian cells (Lowy, 1998; Dinges, Orwin & Schlievert, 2000). Cytotoxins include four hemolysins (alpha, beta, gamma and delta), nucleases, proteases, lipases, hyaluronidase and coagulase (Dinges et al., 2000). These bacterial products may facilitate the spread of infection to other tissues, but their role in the pathogenesis of disease is not well defined (Lowy, 1998). Some strains produce one or more additional exoproteins, which include toxic shock syndrome toxin-1 (TSST-1), the staphylococcal enterotoxins, the exfoliative toxins and leukocidin (Dinges et al., 2000). Panton-Valentine leukocidin (PVL) is made as two non-associated secreted proteins, referred as to S and F components (S for slow- and F for fast-eluting proteins in an ion-exchange column) (Woodin, 1959; Woodin, 1960). PVL is leukotoxic (by pore induction) for rabbit and human polymorphonuclear cells and macrophages (Lina et al., 1999; Dinges et al., 2000). PVL appears to be a virulence factor associated with necrotic lesions of the skin (Prévost et al., 1995a) and subcutaneous tissues and necrotic pneumonia (Gillet et al., 2002), but its role in the progression of staphylococcal disease remains unclear (Lina et al., 1999; Dinges et al., 2000). Many staphylococcal surface proteins have certain structural features in common (Lowy, 1998). These proteins have a ligand-binding domain at the N-terminal that is exposed on the surface of the bacterial cell enabling some of these proteins to function as adhesins (Foster & McDevitt, 1994). Protein A is a prototype of these proteins, which include also elastin-binding protein, collagen-binding protein, fibronectin-binding protein and clumping factor (see Figure 1) (Lowy, 1998). Protein A has antiphagocytic properties that are based on its ability to bind to the Fc portion of immunoglobulin G (IgG) (Uhlén et al., 1984). Protein A also exhibits an ability to bind to von Willebrand factor, a protein present at sites of damage of endothelium, and as a result, it can play a role in adherence and induction of endovascular diseases by *S. aureus* (Hartleib et al., 2000).

Figure 1 – Structure of *Staphylococcus aureus.* Panel A shows the surface and secreted proteins, considered virulence factors. Altered penicillin-binding proteins and production of β-lactamases (shown inside the circles in panel B) are the two most important mechanisms of β-lactams resistance.

Many of the surface proteins have a structural organization similar to that of clumping factor and protein A, including repeated segments of amino acids (Panel C) (Adapted from Lowy, 1998).



β-lactam antibiotics reach bacterial killing by binding to penicillin-binding proteins (PBP), proteins located in the cytoplasmic membrane that are involved in cell-wall assembly (see Figure 1) (Lowy, 1998; Gardam, 2000). By binding to these PBP, β-lactams consequently inhibit the crosslink of bacterial cell wall (Gardam, 2000). The initial mechanism of staphylococci resistance involved the production of β-lactamases, which hydrolyze the cyclic amide bond of the β-lactam ring (see Figure 1) (Gardam, 2000). Subsequently, after introduction of penicillinase-stable penicillins (like methicillin and oxacillin), a new mechanism of resistance developed and the *S. aureus* strains began producing a unique penicillin-binding protein, PBP2a or PBP2', which has a much lower affinity for β-lactam antibiotics (including penicillins, cephalosporins, carbapenems) (Gardam, 2000; Weese & van Duijkeren, 2010). The gene encoding for PBP2a is *mecA*, which is part of a 21-to-60 kb mobile genetic element, termed staphylococcal chromosome cassette *mec* (SCC*mec*) (Lloyd, Boag and Loeffer, 2007). The origin of MRSA is matter of considerable controversy (Gardam, 2000). Kreiswirth et al. (1993), based on analysis of restriction fragment length polymorphisms (RFLP) generated by *Cla*I digestion of chromosomal Deoxyribonucleic acid (DNA) followed by hybridization with Tn*554* and *mecA* probes, proposed that all MRSA descended from a single ancestral *S. aureus* strain that acquired the *mecA* gene on only one occasion. The same authors concluded that the horizontal transfer of the *mecA* gene between staphylococcal species was an extremely rare event (Kreiswirth et al., 1993). However there is

a considerable genetic diversity between MRSA, meaning that *mecA* has been transferred between *S. aureus* lineages (Enright et al., 2002). Robinson and Enright (2003) summarized how the application of typing techniques, like multilocus sequence typing (MLST) and SCC*mec* typing, revealed evolutionary models of the emergence of MRSA: first it had been shown that MRSA arose on multiple occasions out of successful MSSA lineages; second methicillin resistance emerged in five phylogenetic distinct lineages (CC5, CC8, CC22, CC30 and CC45); third only a small number of pandemic clones caused most MRSA disease. It is now accepted that modern MRSA represent independent acquisitions of SCC*mec* by different genetic lineages of *S. aureus* (Enright, 2003). The source of *mecA* gene is also subject of investigation (Gardam, 2000). The *ccr* and *mec* genes, which are the basis of SCC*mec*, are thought to have initially been introduced into coagulase-negative staphylococci from an unknown source and then into *S. aureus* (Enright et al., 2002). It is not known which staphylococcal species donated the SCC*mec* types found among MRSA, but some speculate that it could have come from *Staphylococcus sciuri* (Couto et al., 1996; Wu, Piscitelli, de Lencastre & Tomasz, 1996). Robinson and Enright (2003) indicated that at least 20 acquisitions of SCC*mec* have occurred in *S. aureus*. Of these 20, 16 were acquisitions of SCC*mec* by a MSSA clone and 4 putative reacquisitions of SCC*mec* by an MRSA clone (Robinson & Enright, 2003).

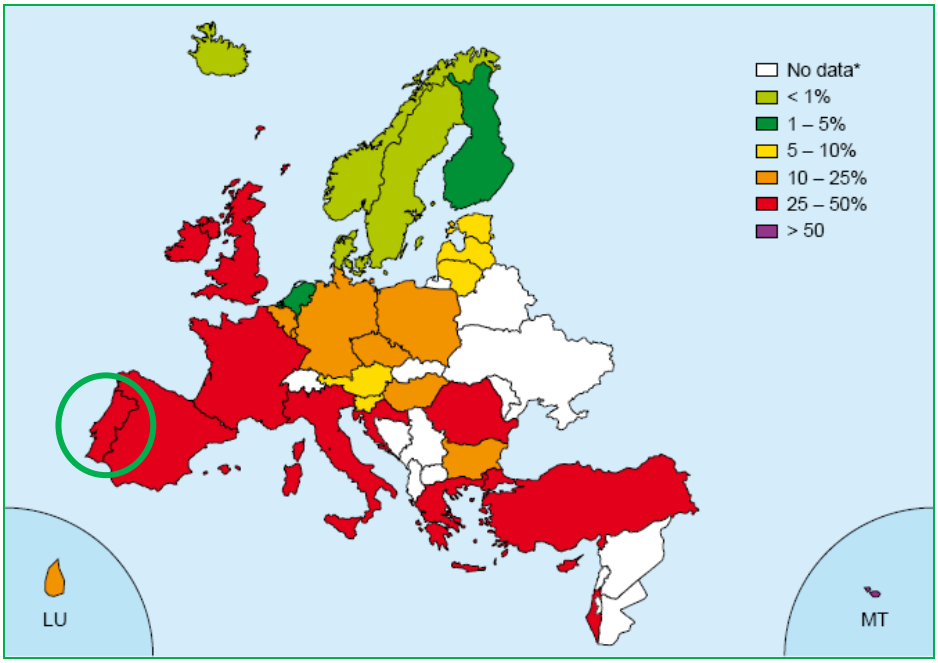
Isolation of methicillin-resistant *S. aureus* (MRSA) from animals was first described in 1972 after its detection in milk from mastitic cows (Devriese, Vandamme & Fameree, 1972). This is not unexpected, since *S. aureus* is an important cause of mastitis in cattle and there is a widespread usage of intramammary antibiotics in that species (Leonard & Markey, 2008). MRSA was first isolated in dogs in the same year in Nigeria (Ojo, 1972). In Europe, MRSA was first described only in 1988, in a cat (Scott, Thomson, Malone-Lee & Ridgway, 1988). Since then MRSA has been increasingly reported as an emerging pathogen in veterinary medicine (Leonard & Markey, 2008). MRSA is capable of colonizing healthy animals and can affect all ages, breed and genders (Weese & van Duijkeren, 2010). However the actual rates of carriage of MRSA by dogs and cats are not known (Lloyd et al., 2007). Colonization rates in dogs range from 0 to 9 % (Baptiste et al., 2005; Loeffler et al., 2005; Lefebvre et al., 2006; Rich & Roberts, 2006; Vengust, Anderson, Rousseau, & Weese, 2006; Bagcigil, Moodley, Baptiste, Jensen & Guardabassi, 2007; Hanselman, Kruth & Weese, 2008; Boost, O'Donoghue & James, 2008; Griffeth, Morris, Abraham, Shofer & Rankin, 2008; Kottler, Middleton, Weese & Cohn, 2008), while in cats ranges from 0 to 4 % (Baptiste et al., 2005; Loeffler et al., 2005; Abraham, Morris, Griffeth, Shofer, & Rankin, 2007; Hanselman et al., 2008; Kottler et al., 2008). However, there has been less investigation in cats then in dogs

(Weese and van Duijkeren, 2010). MRSA can develop opportunistic infections at various body sites, but the most often reported are wound infections, surgical site infections, pyoderma, otitis and urinary tract infections (Tomlin et al., 1999; Baptiste et al., 2005; Leonard et al., 2006; Morris, Mauldin, O'Shea, Shofer & Rankin, 2006a; Morris, Rook, Shofer & Rankin, 2006b; Vitale, Gross & Weese, 2006; Weese et al., 2006; Griffeth et al., 2008). Risk factors for MRSA infection and colonization have been poorly investigated (Weese & van Duijkeren, 2010). Faires & Weese (2008) concluded that antimicrobial therapy, particularly with fluoroquinolones, appeared to be a risk factor for MRSA versus MSSA infection in dogs and cats. MRSA infections have also been associated with exposure to extensive wounds, prolonged hospitalization and immunosuppression (Duquette & Nuttall, 2004). Other risk factors for acquisition of MRSA infections are: presence of a urinary catheter or a joint infection (Faires & Weese, 2008). Animals in contact with human hospitals and contact with children are in higher risk for MRSA colonization (Lefebvre, Reid-Smith, Waltner-Toews & Weese, 2009).

Epidemiological studies have revealed the occurrence of indistinguishable clones in animals and in humans: e.g. CMRSA-5, EMRSA-15 and EMRSA-16 are major human epidemic clones that have been isolated from dogs and cats (Baptiste et al., 2005; Loeffler et al., 2005; O'Mahony et al., 2005; Hanselman et al., 2008; Pomba, Hasman, Cavaco, Couto & Aarestrup, 2009). These MRSA strains found in small animals tend to be those that predominate in people in the region (Weese & van Duijkeren, 2010). Loeffler et al. (2005) isolated EMRSA-15 from dogs in United Kingdom (UK), which is one of the two EMRSA strains (EMRSA-15 and EMRSA-16) currently prevalent in UK human hospitals. Weese et al. (2006) recovered Canadian epidemic MRSA-2 from household pets in Canada, which is the predominant community-associated MRSA human clone in Canada. This strongly suggests that the principal origin for MRSA colonization and infection in companion animals is through contact with humans infected with or carrying MRSA (Lloyd et al., 2007). Companion animals can then serve as reservoirs and act as a source of re-infection or re-colonization (Leonard & Markey, 2008).

The prevalence of MRSA in European countries varies, with Portugal having one of the highest (45 %) just behind Malta and Romania (see Figure 2) (European Antimicrobial Resistance Surveillance System [EARSS], 2007).

Figure 2 – European map showing the *Staphylococcus aureus* proportion of invasive isolates resistant to oxacillin (MRSA) in 2007 (EARSS, 2007).



Portuguese human hospitals have been analyzed since the early 1990s (Aires-de-Sousa, Conceição, Simas and de Lencastre, 2005; Aires-de-Sousa, Correia, de Lencastre & Multilaboratory Project Collaborators, 2008). Aires-de-Sousa et al. (2005) reported that at least three epidemiological important events were recorded in Portugal: (i) in 1992 and 1993, the Portuguese clone (ST239-MRSA-III) was replaced by the Iberian clone (ST247-MRSA-IA); (ii) in 1994 and 1995, the emergence of the Brazilian clone (ST239-MRSA-III/IIIA) and its rapid dissemination; and (iii) recently (2001) the Brazilian clone (ST239-MRSA-III/IIIA) was replaced by the EMRSA-15 clone (ST22-MRSA-IV). EMRSA-15 clone is one of the two major clones (together with EMRSA-16) in the UK (Johnson et al., 2001). EMRSA-15 is replacing previous established clones in various regions of the world, including Europe (Aires-de-Sousa et al., 2008). The three dominant MRSA clones in Portuguese hospitals (Iberian-ST247, Brazilian-ST239 and EMRSA-15-ST22), or their ancestral genotypes, were not detected or scarcely found among an MSSA collection (Aires-de-Sousa et al., 2005). These facts suggest the three major clones have not originated from the introduction of SCC*mec* into main MSSA backgrounds present in the Portuguese nosocomial or community background but were probably imported from out of the country (Aires-de-Sousa et al., 2005). EMRSA-15 has been described in the community in healthy young adults in the absence of risk factors (Mollaghan, Lucey, Coffey & Cotter, 2010), in outpatients (Amorim et al., 2009; Marchese, Gualco, Maioli & Debbia, 2009), healthcare workers (Amorim et al., 2009) and veterinary surgeons (Pomba et al., 2009).

There have been some studies considering that veterinarians and veterinary personnel are at high risk of nasal MRSA colonization (Loeffler et al., 2005; O'Mahony et al., 2005; Hanselman et al., 2006; Moodley et al., 2006; McLean & Ness, 2008). However none of the studies proved that these colonization rates derived from acquisition of MRSA from animals (Weese and van Duijkeren, 2010). Still these higher rates provide support of possible occupational origin (Weese and van Duijkeren, 2010). MRSA infections in owners with involvement of their companion animals are recognized too (van Duijkeren et al., 2004; van Duijkeren, Wolfhagen, Heck & Wannet, 2005; Leonard & Markey, 2008; Morgan, 2008). A recently study has proved that veterinary staff and owners of infected pets are risk groups for MRSA carriage (Loeffler et al., 2010).

There are no treatment protocols for MRSA infections in animals (Lloyd et al., 2007). When deciding for the best treatment protocol, veterinarians should take in account the antibiotic sensitivity pattern of the MRSA isolated, the severity of the infection and other co-morbid conditions or the underlying disease (Lloyd et al., 2007). The antimicrobial therapy should be chosen considering the culture and antimicrobial susceptibility testing results and MRSA clones prevalent in the geographical area, which can help set an empirical therapy while waiting for the microbiological results (Lloyd et al., 2007). In Portugal, only one MRSA strain was isolated in a dog and it was co-resistant to fluoroquinolones (Pomba et al., 2009). Potentiated sulphonamides, tetracyclines and lincosamides are usually the antimicrobials of choice for systemic therapy (Loeffler, 2008). If the infection is in the superficial layers of the skin, the treatment should also include topical antibacterial therapy such as ointments, creams or shampoos (Loeffler, 2008).

There are no guidelines for when decolonization should be performed in animals, however treatment of carrier animals should be accomplished if there is a possibility of re-infection or if the carriers have not been infected but present a risk to other animals or to humans in contact with them (Lloyd et al., 2007). Decolonization can be achieved with topical therapy with fusidic acid, mupirocin, chlorhexidine and benzoyl peroxide (Lloyd et al., 2007; Loeffler, 2008). Decolonization should only be performed after carriage has been confirmed, through swabbing of the nose, mouth or perineum, and when infection is being resolved (Loeffler, 2008).

2.2 Methicillin-resistant *Staphylococcus pseudintermedius*

In 1976, a new species within the genus *Staphylococcus*, *Staphylococcus intermedius*, was characterized as coagulase positive staphylococci isolated from pigeons, dogs, minks and

horses (Hajék, 1976). However, strains isolated from different animal species showed sometimes diverse characteristics and confusion about the classification of *S. intermedius* was a problem during some decades (Weese & van Duijkeren, 2010). In 2005, Devriese and co-workers described a new species, *Staphylococcus pseudintermedius* which had high similarities with *Staphylococcus delphini*, *S. intermedius* and *Staphylococcus schleiferi* (Devriese et al., 2005). On this basis *S. pseudintermedius* was classified within the *S. intermedius* group (SIG). In order to determine the prevalence of each species within the SIG, Sasaki and colleagues (2007b) reclassified 117 *S. intermedius* originated from cats, dogs, humans, pigeons, horses, minks and dolphins. They concluded that phenotypically identified *S. intermedius* strains were reclassified into at least four clusters (*S. intermedius*, *S. pseudintermedius*, *S. delphini* groups A and B). Moreover, all dog strains were identified as being *S. pseudintermedius* strains (Sasaki et al., 2007b). Bannoehr et al. (2007) came to the same conclusion and for this reason all strains isolated from dogs are now being classified as *S. pseudintermedius*, unless it's proven by genomic assays that the strains belong to another species within the genus (Devriese, Hermans, Baele & Haesebrouck, 2009). It should be taken in consideration that previous studies reporting *S. intermedius* from dogs might be in fact reporting *S. pseudintermedius* (Weese & van Duijkeren, 2010).

Differentiation between the members of the SIG by phenotypical tests is very difficult. *S. intermedius* is easily differentiated from SIG strains by a combination of phenotypical characteristics such as positive arginine dihydrolase and acid production from ß-gentiobiose test and D-mannitol (Sasaki et al., 2007b). In contrast, there is no difference in the biochemical profile between *S. pseudintermedius* and *S. delphini* (Sasaki et al., 2007b). Commercial identification systems for the fast and correct identification of *S. pseudintermedius* are not available to date, because the phenotypic properties of *S. intermedius*, *S. delphini* and *S. pseudintermedius* resemble one another (Sasaki et al., 2007a). In many cases *S. pseudintermedius* isolates will be erroneously identified as *S. intermedius* or *S. aureus* (Devriese et al., 2005; Van Hoovels, Vankeerberghen, Boel, Van Vaerenbergh & De Beenhouwer, 2006; Schwarz, Kadlek & Strommenger, 2008).

S. pseudintermedius integrates the normal flora of the nares, mouth, anus, groin and forehead of healthy dogs and cats (Abraham et al., 2007; Griffeth et al., 2008). As well as *S. aureus* in humans, *S. pseudintermedius* is an opportunistic pathogen that can cause infections of the skin and ears (Moodley, Stegger, Zakour, Fitzgerald & Guardabassi, 2009), post-operative wound infections (Moodley et al., 2009), urinary tract infections (Wettstein, Descloux, Rossano & Perreten, 2008) and respiratory tract infections (Schwarz et al., 2008). Furthermore *S. pseudintermedius*, and not *S. intermedius*, is the primary pathogen isolated from canine

pyoderma (Bannoehr et al., 2007). However, little is known about the pathogenesis of *S. pseudintermedius* (Fitzgerald, 2009). Still, like other staphylococci, *S. pseudintermedius* has the ability to produce a number of virulence factors, some closely related to those produced by *S. aureus*. *S. pseudintermedius* produces enzymes such as coagulase, proteases and thermonuclease and toxins, including haemolysins, exfoliative toxins and enterotoxins (Fitzgerald, 2009). *Staphylococcus intermedius* exfoliative toxin (SIET) is a well known exfoliative toxin that seems to be the causative agent of canine pyoderma (Lautz et al., 2006). Terauchi and colleagues proved that dogs injected with purified SIET developed clinical signs like erythema, exfoliation and crusting which are equal to the signs of canine pyoderma and human staphylococcal scalded skin syndrome (Terauchi et al., 2003). *S. pseudintermedius* also produces a leukotoxin known as Luk-I, which is very similar to PVL from *S. aureus* (Futagawa-Saito et al., 2004). Luk-I is encoded as a *luk-I* operon with co-transcribed genes, *lukS* and *lukF*, encoding LukS and LukF, respectively (Prévost, Bouakham, Piemont & Monteil, 1995b). Luk-I shows a strong leukotoxicity on various polymorphonuclear cells, including activity on human leukocytes, but only a minor hemolytic activity on rabbit erythrocytes (Prévost et al., 1995b). In 2009, Geoghegan and colleagues determined that *S. pseudintermedius* expresses surface proteins that resemble those from *S. aureus* (Geoghegan, Smith, Speziale & Foster., 2009). They discovered that *S. pseudintermedius* has the capacity to bind to fibrinogen, fibronectin and cytokeratin 10, which could explain how *S. pseudintermedius* adheres to canine corneocytes in colonization and in infection (Geoghegan et al., 2009). Moodley and co-workers, in 2009 reported that *S. pseudintermedius* produces an immunoglobulin-binding protein similar to *S. aureus*, the so called protein A. Protein A is encoded by the staphylococcal protein A gene (*spa*). The *spa* gene of *S. pseudintermedius* has 68 % nucleotide and 55 % predicted amino acid identity with the homologous gene in *S. aureus* (Moodley et al., 2009) and is thought to be a potential virulence factor (Moodley et al., 2009). Like most staphylococci, *S. pseudintermedius* has the capacity to form biofilms (Haenni, Bréchard & Madec, 2009).

Like in MRSA, methicillin resistance in *S. pseudintermedius* is mediated by the *mecA* gene (Loeffler et al, 2007; Moodley et al., 2009). Methicillin-resistant *S. pseudintermedius* (MRSP) was already isolated from dogs, cats and humans, which turns it to a zoonotic pathogen (Van Hoovels et al., 2006; Bannoehr et al., 2007; Hanselman et al., 2008; Sasaki et al., 2007b; Schwarz et al., 2008; Wettstein et al 2008; Chuang, Yang, Hsueh & Lee, 2010). MRSP infections were mostly associated with veterinary clinic epidemic outbreaks (Weese & van Duijkeren, 2010), although colonization of healthy animals and people working with animals has been described (Sasaki et al., 2007a; Hanselman et al., 2008; Boost, So & Perreten, 2009).

In Europe, a single common genotype (ST71) was found to be prevalent in the northern and central part of the continent (Bannoehr et al., 2007; Boost et al., 2009; Moodley et al., 2009; Perreten et al., 2010; Ruscher et al., 2010). ST71 is also prevalent in Hong Kong (Moodley et al., 2009). These MRSP isolates were generally distinct from MRSP isolated in North America (Bannoehr et al., 2007; Moodley et al., 2009; Perreten et al., 2010). Black et al. (2009) and Moodley et al. (2009) analyzed MRSP strains isolated in the United States of America by MLST and determined that the strains belonged to ST68. However, this clone was isolated in Portugal in a cat in 2009 (Pomba, Couto & Moodley, 2010). A single *spa* type (t06) was detected among the American isolates belonging to ST68 while multiple *spa* types (t02, t03, t04, t05 and t09) were identified within the European clone ST71 (Moodley et al., 2009). It was hypothesized that broad geographic dissemination of sucessful MRSP genetic lineages had happened and that the spread had occurred in an amazing short period of time (Bannoehr et al., 2007; Ruscher et al., 2010). Alternatively, Ruscher et al. (2010) postulated that ST71 could represent a very successful *S. pseudintermedius*-lineage that could co-evolved with the canine host and disseminated widely in European countries in the past, even though there is a lack of ST 71 MSSP. The same authors identified new spa types associated with the ST71 (t05, t06, t15 and t23). These ST71-associated spa types differ only slightly by the number of a certain repeat (on to five r03-repeats) at a central position on the spa gene. Therefore, variations in ST 71 related spa types were possibly a result of repeated loss of r03-repeats by deletion events (Ruscher et al., 2010). This is the first report of t06 associated with European ST71 isolates but further investigation of the functional aspects of the presumptive *spa* gene is necessary (Ruscher et al., 2010). ST71 and ST68 contain distinct SCC*mec* elements, type III and type V, respectively (Sasaki et al., 2007a; Moodley et al., 2009; Ruscher et al., 2009; Ruscher et al., 2010). Descloux and colleagues (2008) reported a new SCC*mec* type, SCC*mec* type II-III, associated with Swiss ST71 isolates. However, according to Kondo criteria (Kondo et al., 2007) this SCC*mec* element would have been classified as type III (class A *mec* complex and *ccrAB3* recombinase) (Moodley et al., 2009). This SCC*mec* consists of a combination of SCC*mec* II from *Staphylococcus epidermidis* and of SCC*mec* III from *S. aureus* and lacks the cadmium resistance operon (Descloux, Rossano & Perreten, 2008). Recently a new SCC*mec* element was described, SCC*mec* VII-241 (class A *mec* complex and *ccrA3/B5*), which is not related to SCC*mec* VII from *S. aureus* (Black et al., 2009; Perreten et al., 2010).

In addition to resistance to β-lactams, MRSP isolates are displaying resistance to many classes of antimicrobial agents (Perreten et al., 2010), sometimes more than 4 classes, which turns them into multidrug-resistant strains. In late 1990s and early 2000s resistance to several

antimicrobial classes was rare and *S. pseudintermedius* had never presented as a therapeutic problem in pets in Europe (Lloyd, Lamport & Feeney, 1996; Guardabassi, Loeber & Jacobson, 2004). Loeffler et al. (2005) reported the first MRSP in Europe. These MRSP strains had been isolated from dogs with skin and ear infections and were co-resistant to enrofloxacin, clindamycin, erythromycin, tetracycline and trimethoprim-sulfamethoxazole, but susceptible to gentamicin, rifampicin, fusidic acid and mupirocin. After, resistance to gentamicin, chloramphenicol, tobramycin, levofloxacin and ciprofloxacin has been reported among MRSP strains (Schwarz et al., 2008; Wettstein et al., 2008; Ruscher et al., 2009; Perreten et al., 2010; Ruscher et al., 2010).

As for MRSA, veterinarians and owners of dogs infected with MRSP are at higher risk of carrying an MRSP strain (Weese & van Duijkeren, 2010). In one study, owners of dogs with deep pyoderma were shown to be more often cultured positive for *S. pseudintermedius* than individuals without daily contact with dogs and they often carried the same *S. pseudintermedius* strain as their dogs (Guardabassi et al., 2004). Sasaki and colleagues (2007a) identified an MRSP among the veterinary staff sampled. Van Duijkeren et al. (2008) identified MRSP in environment samples and veterinary personnel in a clinic which were epidemiologically related. People working at veterinary clinics and hospitals should be aware of the risk of nosocomial transmission of MRSP and take precautions to avoid it (Weese & van Duijkeren, 2010).

The treatment of infections with MRSP is a new challenge in veterinary medicine because of the very limited therapeutic options (Wettstein et al. 2008). The multidrug-resistance patterns results in a potential pressure for veterinarians to use unauthorised antimicrobials available in human medicine which requires careful evaluation of extra-label drug use in veterinary medicine (Weese and van Duijkeren, 2010).

2.3 Typing of methicillin-resistant staphylococci strains

The epidemiology of infectious diseases relies on typing methods as tools for the characterization and discrimination of isolates based on either their genotypic or phenotypic characteristics, which may be used to establish clonal relationships between strains and to trace the geographic dissemination of bacterial clones (Faria, Carrico, Oliveira, Ramirez & de Lencastre, 2008).

Phenotypic typing methods comprise colonial characteristics, biochemical reactions, antibiotic susceptibility pattern, susceptibility to various phages and toxin production (Leonard & Markey, 2008).

Even though antibiotic susceptibility pattern is still frequently used as a tool to characterize bacterial isolates, nowadays, the classification of isolates is mostly based on molecular methods, which usually provide better discriminatory power than phenotypic methods (Faria et al., 2008). For molecular typing there are various methods proposed, but the most important and globally used are pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), *spa* and SCC*mec* typing.

2.3.1 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE separates DNA under conditions of alternating polarity allowing for the resolution of DNA fragments nearly 20-times larger than those separated by traditional agarose gel electrophoresis (Leonard & Markey, 2008). PFGE is used in combination with restriction enzymes to give a DNA fingerprint of the bacterial genome (Tenover et al., 1995). The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness (Tenover et al., 1995). The main advantage of this technique is that it provides great discrimination among strains and is useful in the investigation of outbreaks, by allowing differentiation of unrelated strains and another advantage is its high resolution (Prévost, Jaulhac & Piemont, 1992; Struelens, Deplano, Godard, Maes & Serruys, 1992; Schlichting et al., 1993; Struelens, Bax, Deplano, Quint & van Belkum, 1993; Tenover et al., 1994; Nada et al., 1996; Na'was et al., 1998; Schmitz et al., 1998; Olive & Bean, 1999; Oliveira et al., 2001; Montesinos, Salido, Delgado, Cuervo & Sierra, 2002; Strommenger et al., 2006; Faria et al., 2008; Leonardo & Markey, 2008). Disadvantages relate principally with difficulties with inter-laboratory comparison of results and consequently reliable comparison of strains between regions and internationally are not always possible (Leonard & Markey, 2008). Other disadvantages are the consuming time that it takes to get the results and the expensive cost of the method (Montesinos et al., 2002).

In 2003, Murchan et al., in a multicenter study, described a consensus PFGE protocol for typing of strains of MRSA which resulted in higher intercenter reproductibility, local acceptability and the establishment of a web-based database of harmonized MRSA *Sma*I restriction patterns (Murchan et al., 2003). This protocol has been adapted, with minor modification, for use in MRSP strains using the same restriction enzyme, *Sma*I (Perreten et al., 2010).

2.3.2 Multilocus Sequence Typing (MLST)

MLST was first developed for the identification of the hypervirulent lineages of *Neisseria meningitidis* (Maiden et al., 1998) and for assigning *Streptococcus pneumoniae* strains to major hypervirulent clones (Enright & Spratt, 1998; Enright, Fenoll, Griffiths & Spratt, 1999). MLST is a highly discriminatory method of characterizing bacterial isolates on the basis of the sequences of ~ 450 bp of internal fragments of seven housekeeping genes (Maiden et al., 1998). For each gene fragment, the different sequences are assigned as distinct alleles and each isolate is defined by the alleles at each of the seven housekeeping loci (the sequence type [ST]) (Enright, Day, Davies, Peacock & Spratt, 2000). MLST sequences of the seven gene fragments of some bacteria can be readily compared between laboratories via the internet (http://www.mlst.net). MLST reveals slowly accumulating changes in conserved genes that reflect long-term evolutionary changes and can identify global spread of the relatively small number of successful clones (Enright et al., 2002). MLST has become popular due to development in large-scale sequencing methodology, ease of data transfer and excellent comparability of results (Aires-de-Sousa et al., 2006), however MLST is not appropriate for routine infection control due to high cost, labor intensity and lack of broad access to high-throughput DNA sequencing (Strommenger et al., 2006).

In order to overcome the difficulty of comparing PFGE results and the genetic relatedness of the clones of EMRSA described by different laboratories, Enright and colleagues (2000) developed a multilocus sequence typing for MRSA. Out of the fourteen housekeeping gene fragments sequenced by Enright et al. (2000), the seven housekeeping gene fragments that provided the greatest number of alleles were chosen for use in the MLST scheme and included: carbamate kinase gene (*arcC*), shikimate dehydrogenase gene (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). The MLST scheme for *S. aureus* was developed in 2000 and the details of several isolates are available at the *S. aureus* MLST website http://saureus.mlst.net (Enright et al., 2000).

With the aim of investigating the population genetic structure of the SIG, Bannoehr and colleagues (2007) developed a multilocus sequencing approach that included five gene *loci* with a range of predicted nucleotide diversities to facilitate inter- and intraspecies differentiation. Five *loci*, the 16S rRNA (16S ribosomal RNA), *cpn60* (chaperonin 60), *tuf* (elongation factor Tu), *pta* (phosphate acetyltransferase) and *agrD* (autoinducing peptide-AIP) genes were selected for DNA sequence analysis based on previous single-*locus* approaches to differentiating staphylococcal species (16S rRNA, *cpn60* and *tuf*) and on

inclusion of some genes (*pta*, *cpn60* and *tuf*) into multilocus sequence typing schemes for differentiating strains of several different bacteria species (Bannoehr et al., 2007). The allelic variation of *agrD* was examined to further investigate both inter- and intraspecies discrimination (Bannoehr et al., 2007). There is no available *S. pseudintermedius* MLST website, therefore MLST sequences must be compared with allele sequences present in the NCBI nucleotide database in order to determine the allele number (Perreten et al., 2010). Sequence type numbers are assigned using the key table for MLST typing of SIG isolates (Bannoehr et al., 2007). The current *S. pseudintermedius* MLST scheme needs optimization, as most of MLST schemes have seven or more *loci* and none includes 16S rRNA (Perreten et a., 2010).

2.3.3 *spa* Typing

In 1996, Frenay and colleagues developed a single-*locus* sequence typing method for *S. aureus* using the sequence of polymorphic X or short sequence repeat (SSR) region of the *spa* gene as an alternative technique for the typing of *S. aureus* (Frenay et al., 1996). The polymorphic X region consists of a variable number of 21 bp to 27 bp repeats and is located upstream of the region encoding the C-terminal cell wall attachment sequence (Guss et al., 1984; Schneewind, Model & Fischetti, 1992; Uhlen et al, 1984). The diversity of the SSR region seems to arise from deletion and duplication of the repetitive units and also by point mutation (Brigido et al., 1991). The existence of well-conserved regions flanking the X region coding sequence in *spa* gene allows the use of primers for PCR amplification and direct sequence typing (Shopsin et al., 1999). Moreover, the determination of *spa* types was simplified when appropriate software synchronized with an accompanying public website was developed (http://www.SpaServer.ridom.de) (Harmsen et al., 2003). A potential problem with *spa* typing is that it involves sequencing of only one small region of the chromosome, which is subject to recombinant between unrelated clones (Cookson et al., 2007). This could result in isolates exhibiting the same *spa* type when they are shown to be unrelated by other methods (Cookson et al., 2007).

In 2009, Moodley and colleagues described the use of *spa* sequencing as a technique for *S. pseudintermedius* typing. The sequencing of the *spa* gene of *S. pseudintermedius* revealed that it was shorter then the *spa* gene of *S. aureus*, partly due to the lack of IgG-binding domain region B (Moodley et al., 2009). In *S. pseudintermedius* the polymorphic X region consists of variable numbers (between 6 and 10) of 30 bp tandem repeats (Moodley et al., 2009). All MRSP isolates tested yelded *spa* PCR products (100 %), but the typeability rate among

methicillin-susceptible isolates was lower (58 %) (Moodley et al., 2009). The *spa* products of non-typeable isolates could not be sequenced due to the presence of multiple bands, which was found to be related to the presence of two adjacent *spa* genes (Perreten et al., 2010). This second *spa* gene has three IgG-binding domains and a partial deletion of the X region, but the biological significance of this gene duplication is unclear (Perreten et al., 2010). Like in *S. aureus*, the variable region X in *S. pseudintermedius* consisted of duplications or deletions of whole repeats or point mutations within repeats (Moodley et al., 2009).

2.3.4 SCC*mec* Typing

SCC*mec* is a mobile genetic element characterized by the presence of terminal inverted and direct repeats, a set of site-specific recombinase (*ccr*) genes and the *mecA* gene complex (Ito, Katayama & Hiramatsu, 1999; Katayama, Ito & Hiramatsu, 2000). Each SCC*mec* element integrates at the same site ($attB_{SCC}$) at the 3' end of an open reading frame (ORF) of unknown function, designated *orfX* (Ito et al., 1999). There are five classes of *mec* gene complex (A to E), which vary in their genetic structure (Katayama, Ito & Hiramatsu, 2001; Lim, Chong, O'Brien & Grubb, 2003). SCC*mec* consists of three regions: a *mec* complex carrying an intact copy of *mecA*, when present, complete or truncated *mec* regulatory genes *mecI* and *mecR*, a *ccr* complex carrying cassette chromosome recombinase (*ccr*) genes and a serious of variable "junkyard" or "J" regions (Ito, Okuma, Ma, Yuzawa, Hiramatsu, 2003; Lim et al., 2003). According to the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) there are eight currently established SCC*mec* types (International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements [IWG-SCC], 2009).

3 Objectives of the study

The primary objective of the study was to investigate the frequency of MRSA and MRSP colonization in cats and dogs hospitalized at the Teaching Hospital of the Faculty of Veterinary Medicine, Technical University of Lisbon. The second objective was to characterize the isolates by molecular methods and relate them with previous described epidemic clones as well as their possible pathogenic potential (frequency of the PVL toxin in *S. aureus* and Luk-I and SIET in *S. pseudintermedius*).

For this purposes we defined our protocol considering previous studies. Nasal swabbing was chosen as the method for screening the small animals for colonization, since the nostrils are

the predominant site of colonization for both MRSA and MRSP (Weese & van Duijkeren, 2010). The inoculation broths were those proposed elsewhere for detection of MRSA in animals (Vengust et al., 2006). Chrom MRSA ID, the selective agar for screening of methicillin-resistance, was chosen because is sensitive and specific for differentiation between MSSA and MRSA *in vitro*, as showed by the study of Diederen et al. (2006).
MRS strains were typed by PFGE, *spa* typing and SCC*mec* typing. MLST was not used due to economical reasons. Since *spa* types can unambiguously be assigned to, a limited number of clonal lineages known to be prevalent (Strommenger et al., 2006), the MRS were assigned a Clonal Complex (CC) based on the *spa* type.

4 Materials and Methods

4.1 Sampling and collection of data on antimicrobial treatment

During a one year study (from April 2008 to April 2009), nasal swabs were obtained from cats and dogs immediately or at the most two hours after admission to the Teaching Hospital of the FMV-UTL. Forty cotton swabs from cats and 146 from dogs were rolled on the nasal mucosae of both nostrils and were immediately processed at the laboratory. A brief questionnaire about each animal was completed, that included gender, age, breed, presenting complaint, antimicrobial use in the last month and in the last year, hospitalization and previous surgery (Annex X).

4.2 Bacterial isolation and characterization

4.2.1 Strains isolation

The nasal swabs were inoculated in 3 ml of an enrichment broth consisting of 10 g/l mannitol, 65 g/l NaCl, 2,5 g/l yeast extract and 10 g/l tryptone for 24 h at 37º C (Vengust et al., 2006). After overnight incubation, 10 µl of bacterial suspension were inoculated in Columbia 5% blood sheep agar plates, again for 24 h at 37º C (bioMérieux, Marcy l'Etoile, France). Colonies were identified as coagulase-positive staphylococci based on colony morphology, ability to cause hemolysis, gram straining, positive catalase test, positive tube coagulase test (Fermentas, Ontario, Canada) and BBL™ Crystal™ typing system (BD BBL, New Jersey, USA). Coagulase test and BBL™ Crystal™ typing system were performed according to the manufacturs.

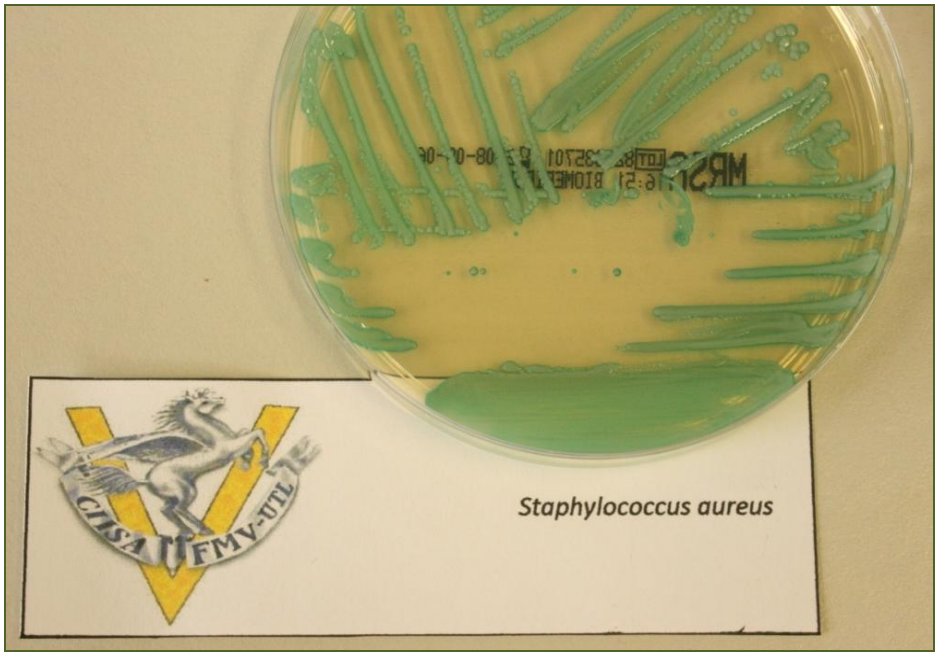
4.2.2 DNA Extraction

DNA extraction protocol was adapted from the European Union Reference Laboratory of Antimicrobial Resistance (EURL) protocol available on the Internet (http://www.crl-ar.eu). In brief, 1 ml of PBS was transferred to a 1.5-ml eppendorf tube and, using a disposable inoculation loop (white; 1µl), a loop full of bacteria was picked from a plate and transferred to the eppendorf tube. After, the eppendorf was centrifuged at 14,000 x *g* for 5 min. The supernatant was discarded and the pellet re-suspended in 100 µl TE 10:1. The suspension was boiled for 5-10 minutes and transferred directly to ice. Finally the lysed DNA was diluted 10 times in TE 10:1 and was stored at -20º C.

4.2.3 Methicillin resistance confirmation

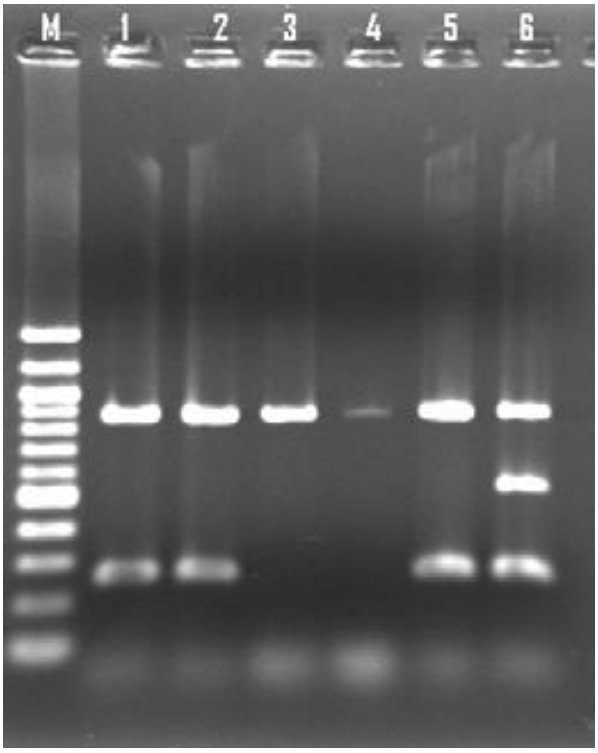
MRSA and MRSP carriage was screened by plating enrichment cultures on a selective medium, Chrom MRSA ID (bioMérieux) and suspected colonies (see Figure 3) were confirmed by PCR to have the *mecA* gene.

Figure 3 – Aspect of MRSA colonies on Chrom MRSA ID medium (bioMérieux).



Amplification of the *mecA*, 16S rRNA and *nuc* of *S. aureus* genes was carried out in a multiplex-PCR (M-PCR) adapted from the EURL protocol (http://www.crl-ar.eu) (see Figure 4).

Figure 4 – M-PCR profile of MRSA and *S.aureus*. M, DNA ladder; 1, 2 and 5, Test strains *S. aureus*; 3, 16S control coagulase-negative *Staphylococcus*; 4, Mastermix negative control; 6, MRSA.



M-PCR was performed in a final volume of 50 µl containing 5 µl of the DNA template, 2 µl of each of the following primers Mec-1 (10 µM), Mec-2 (10 µM), Nuc-1 (10 µM), Nuc-2 (10 µM), 16S-1 (10 µM), 16S-2 (10 µM) (see Table 1), 0.5 µl of NZYTech *Taq* (NZYTech, Lisbon, Portugal), 5 µl of 10x PCR buffer without $MgCl_2$, 6 µl of $MgCl_2$ (25 mM), 0.5 deoxyribonucleotide triphosphates (dNTPs) (25 mM) and 21 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (5 minutes at 94˚ C) followed by 30 cycles of denaturation (30 s at 94˚ C), annealing (30 s at 55˚ C) and elongation (60 s at 72˚ C), with a single final elongation (10 min at 72˚ C).

4.2.4 Species confirmation

Presumptive *S. aureus* and *S. pseudintermedius* isolates were confirmed by PCR amplification of the specific *nuc* gene. The *nuc* gene of *S. aureus* was amplified through the M-PCR described above (http://www.crl-ar.eu). The *nuc* gene of *S. pseudintermedius* was adapted from the protocol described by Sasaki et al. (2010). PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, 2 µl of each of the following primers Pse F(10 µM) and Pse R (10 µM) (see Table 1), 0.5 µl of Dream *Taq* (Fermentas), 5 µl of 10x PCR buffer, 0.5 µl dNTPs (25 mM) and 40 µl of MiliQ water. Thermal cycling reactions consisted

of an initial denaturation (2 min at 95˚ C) followed by 30 cycles of denaturation (30 s at 95˚ C), annealing (35 s at 56˚ C) and elongation (60 s at 72˚ C), with a single final elongation (2 min at 72˚ C).

Discrimination between *S. pseudintermedius* and *S. intermedius* was further investigated by restriction fragment length polymorphism (RFLP) analysis of the *pta* gene (Bannoehr, Franco, Iurescia, Battisti & Fitzgerald, 2009). PCR amplification of a 320 bp fragment of the *pta* gene was carried out in a 50 µl volume with 2 µl of each oligonucleotide primers (pta_f1 and pta_r1) (see Table 1), 0.8 µl dNTP's, 0.5 µl *Taq* DNA Polymerase (Fermentas) and 5 µl DNA template in a 10x reaction buffer. Thermocycling conditions included a 94º C incubation for 1 minutes, followed by 30 cycles of 94º C for 1 min, 53º C for 1 min and 72º C for 1 min, with a final incubation of 72º C for 7 min. Twenty five microliter samples of the PCR mixtures were incubated with 5 U of MboI (Fermentas) and 5 µl of 5x digestion buffer for 2 hours. The digestion products were resolved in 2 % (w/v) agarose by electrophoresis.

4.2.5 PVL, Luk-I and SIET detection

MRSA isolates were tested by PCR for the presence of *lukF/lukS* genes encoding PVL using the primers described by Lina et al. (1999). Briefly, PCR was performed in a final volume of 50 µl containing 2 µl of the DNA template, 0.5 µl of each of the following primers luk-PV-1 (10 µM) and luk-PV-2 (10 µM) (see Table 1), 0.5 µl of *Taq* DNA Polymerase (Fermentas), 5 µl of 10x PCR buffer, 0.5 dNTPs (25 mM) and 41 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (5 minutes at 94˚ C) followed by 30 cycles of denaturation (30 s at 94˚ C), annealing (30 s at 55˚ C) and elongation (60 s at 72˚ C), with a single final elongation (10 min at 72˚ C).

MRSP isolates were tested by two PCR for the presence of *lukF* and *lukS* genes encoding Luk-I using the primers described by Futagawa-Saito et al. (2004). Briefly, both PCR were performed in a final volume of 25 µl containing 2 µl of the DNA template, 2 µl of each of the following primers lukF pseud F (10 µM) and lukF pseud R (10 µM) or lukS pseud F (10 µM) and lukS pseudo R (10 µM) (see Table 1), 0.5 µl of Dream *Taq* Polymerase (Fermentas), 5 µl of 10x PCR buffer, 0.5 dNTPs and 12 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (2 minutes at 94˚ C) followed by 35 cycles of denaturation (60 s at 94˚ C), annealing (60 s at 55˚ C) and elongation (60 s at 72˚ C), with a single final elongation (10 min at 72˚ C).

SIET gene was amplified by PCR based on the protocol described by Lautz et al. (2006). This protocol consists of a first preparation of a PCR master mix consisting of 2 µl of the DNA

template, 2 µl of each of the following primers siet 1 toxin (10 µM) and siet 2 toxin (10 µM) (see Table 1), 0.5 µl of Dream *Taq* Polymerase (Fermentas), 2 µl of 10x PCR buffer, 0.8 dNTPs and 16.7 µl of MiliQ water in a final volume of 25 µl. The thermocycling conditions were the following: an initial denaturation (3 minutes at 94˚ C) followed by 35 cycles of denaturation (30 s at 94˚ C), annealing (30 s at 56˚ C) and elongation (60 s at 72˚ C), with a single final elongation (10 min at 72˚ C).

4.3 *spa* Typing

For MRSA, *spa* typing was performed as recommended by the EURL (http://www.crl-ar.eu). PCR was accomplished in a final volume of 50 µl containing 2 µl of the DNA template, 0.5 µl of each of the following primers SPA 1095F new (10 µM) and SPA extended_f (10 µM) (see Table 1), 0.5 µl of Dream *Taq* Polymerase (Fermentas), 5 µl of 10x PCR buffer, 0.5 dNTPs (25 mM) and 41 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (5 minutes at 94˚ C) followed by 35 cycles of denaturation (45 s at 94˚ C), annealing (45 s at 62˚ C) and elongation (90 s at 72˚ C), with a single final elongation (10 min at 72˚ C). For DNA sequencing, 100 µl of the PCR products were purified using the JETQUICK PCR Product Purification Spin Kit (GENOMED, GmbH Löhne, Germany). DNA sequencing was performed by STAB VIDA (Lisbon, Portugal). The *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/).

For MRSP, *spa* typing was performed according to Moodley et al. (2009). PCR was performed in a final volume of 25 µl containing 1.5 µl of the DNA template, 2.5 µl of each of the following primers SIspaF2 (5 µM) and SIspaR3 (5 µM) (see Table 1), 12.5 µl of Dream *Taq* Green Master Mix (Fermentas) and 6 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (10 minutes at 95˚ C) followed by 30 cycles of denaturation (30 s at 95˚ C), annealing (30 s at 58˚ C) and elongation (60 s at 72˚ C), with a single final elongation (10 min at 72˚ C). For DNA sequencing, 10 µl of the PCR products were purified adding 1 µl of Exonuclease enzyme (20 U/ µl) and 4 µl of Alkaline Phosphatase (1 U/ µl) and incubating at 37˚ C for 15 min and then at 80˚ C for 15 min. DNA sequencing was performed by Macrogen Inc. (Seoul, South Korea) using BigDye (Applied Biosystems). The sequences were interpreted manually according to the criteria proposed by Moodley et al. (2009).

Table 1 – List of primers used in the study.

Primers	Nucleotide Sequence	PCR Product Size
Mec-1	5'-GGGATCATAGCGTCATTATTC-3'	527 bp
Mec-2	5'-AACGATTGTGACACGATAGCC-3'	
Nuc-1	5'-TCAGCAAATGCATCACAAACAG-3'	255 bp
Nuc-2	5'-CGTAAATGCACTTGCTTCAGG-3'	
16S-1	5'-GTGCCAGCAGCCGCGGTAA-3'	886 bp
16S-2	5'-AGACCCGGGAACGTATTCAC-3'	
Pse F	5’-TRGGCAGTAGGATTCGTTAA-3’	926 bp
Pse R	5’-CTTTTGTGCTYCMTTTTGG-3’	
pta_f1	5’-AAAGACAAACTTTCAGGTAA-3’	320 bp
pta_r1	5’-GCATAA ACAAGCATTGTACCG-3’	
luk-PV-1	5’-ATCATTAGGTAAAATGTCTGGACATGATCCA-3’	433 bp
luk-PV-2	5’-GCATCAASTGTATTGGATAGCAAAAGC-3’	
lukF pseud F	5’-CCTGTCTATGCCGCTAATCAA-3’	572 bp
lukF pseud R	5’-AGGTCATGGAAGCTATCTCGA-3’	
lukS pseud F	5’-TGTAAGCAGCAGAAAATGGGG-3’	503 bp
lukS pseud R	5’- GCCCGATAGGACTTCTTACAA-3’	
siet1 tox	5’-ATGGAAAATTTAGCGGCATCTGG-3’	359 bp
siet2 tox	5’-CCATTACTTTTCGCTTGTTGTGC-3’	
SPA 1095F new	5'-AGACGATCCWTCAGTGAGC-3'	Variable
SPA extended_f	5'-TAATCCACCAAATACAGTTGTACC-3'	
SIspaF2	5’-AACCTGCGCCAAGTTTCGATGAAG-3’	Variable
SIspaR3	5’-CGTGGTTTGCTTTAGCTTCTTGGC-3’	

4.4 SCC*mec* Typing

Staphylococcal cassette chromosome *mec* types were determined using the multiplex PCR 1 and multiplex PCR 2 accordingly to Kondo and collaborators (2007) and accordingly to the IWG-SCC (2009). PCRs were performed in a final volume of 25 µl containing 0.5 µl of the DNA template, 5 µl of a 10x primer mix, 12.5 µl of Dream Taq Green Master Mix (Fermentas) and 7 µl of MiliQ water. Thermal cycling reactions consisted of an initial denaturation (5 minutes at 94˚ C) followed by 35 cycles of denaturation (2 min at 94˚ C), annealing (60 s at 57˚ C) and elongation (3 min at 72˚ C), with a single final elongation (8

min at 72˚ C). The molecular weight of each PCR amplicon was determined and assigned to a particular allele type in each strain. The combination of the type *ccr* and *mec* complex was used to assign SCC*mec* types.

4.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disk diffusion method. The results were registered after incubation at 37˚ C for 18h, measuring the diameter of the zone of inhibition, if present, for each antibiotic used. Inhibition zone diameters were interpreted according to the Clinical Laboratories Standards Institute clinical breakpoints for animals (CLSI, 2008) and, when breakpoints were unavailable for bacteria of animal origin, according to the human CLSI breakpoints (CLSI, 2009). For fusidic acid and mupirocin, inhibition zone diameters were interpreted using breakpoints proposed by Toma and Barriault (1995) and Fuchs et al. (1999), respectively. Susceptibility test breakpoints for oxacillin of *S. pseudintermedius* were those described by Bemis et al. (2009). For MRSA and MRSP, the following antimicrobial discs (Oxoid, Hampshire, United Kingdom) were used: amikacin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), enrofloxacin (5 μg), erythromycin (15 μg), fusidic acid (10 μg) gentamicin (10 μg), kanamycin (30 μg), levofloxacin (5 μg), linezolid (30 μg), marbofloxacin (5 μg), moxifloxacin (5 μg), mupirocin (5 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), ofloxacin (5 μg), oxacillin (1 μg), penicillin G (10 units), quinupristin/dalfopristin (15 μg), rifampicin (5 μg), tetracycline (30 μg), trimethoprim (5 μg), and vancomycin (30 μg). MRSA was also tested for cefoxitin (30 μg) susceptibility, since cefoxitin has been recently used to replace oxacillin in the phenotypic identification of MRSA isolated from humans (CLSI, 2009).

4.6 PFGE

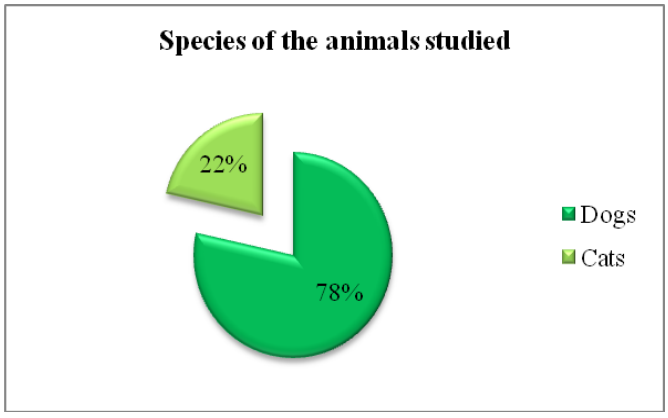
PFGE was performed as described by Murchan et al. (2003) with minor modifications. Briefly, bacterial cultures were grown overnight in brain heart infusion broth (BD Difco, New Jersey, USA) and were incorporated into 2 % (w/v) agarose discs, instead of plugs. After four hours lysis with lysostaphin (5 mg/ml), lysozyme (100 mg/ml) and RNase (32,5 mg/ml) at 37º C, the discs were incubated with proteinase K (20 mg/ml) overnight at 56º C and one disc was digested with *Sma*I (20 U/ml) overnight at room temperature. PFGE was performed by clamped homogeneous electric field (CHEF) electrophoresis with a CHEF-DR III System

(Bio-Rad Laboratories, San Diego, USA) in a 1,1 % (w/v) agarose gel with a pulsing switch time of 2-5 s at 5,6 V/cm for 24 h for MRSP and pulsing switch time of 5-15 s at 6 V/cm for 10 h followed by pulsing switch time of 15-60 s at 6 V/cm for 13 h for MRSA. EMRSA-15 and American MRSP clones, previously isolated in Portugal (Pomba et al., 2009; Pomba et al., 2010), were used as reference strains and included in the gel. The gels were inspected visually and band patterns were interpreted according to the criteria proposed by Tenover et al. (1995).

5 Results

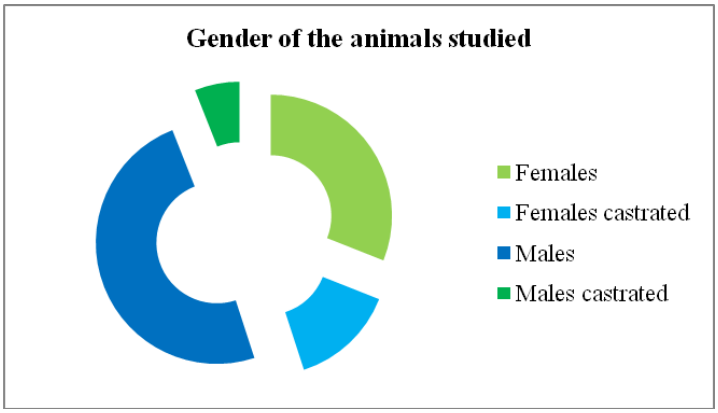
Twelve methicillin-resistant staphylococci were isolated from the nose of ten dogs and two cats out of a total of 186 animals screened. One hundred forty six animals were dogs (78 %) and forty were cats (22 %) (see Figure 5).

Figure 5 – Percentage of the species of the animals sampled.



Males were the predominant gender sampled (49 %), followed by females (31 %), females castrated (14 %) and males castrated (6 %) (see Figure 6).

Figure 6 – Percentage of each gender among the animals sampled.



One hundred seventeen animals (63 %) had received antimicrobial treatment within the month prior to sampling and forty nine animals (26 %) had received antimicrobial treatment within the year before sampling. Many classes of antimicrobials were prescribed, but the classes more often selected were β-lactams, metronidazole and fluoroquinolones (see Figure 7 and Figure 8).

Figure 7 – Number of antimicrobials prescribed to the animals in the study within the month prior to swab collection. Take in account that some animals received more than one antimicrobial.

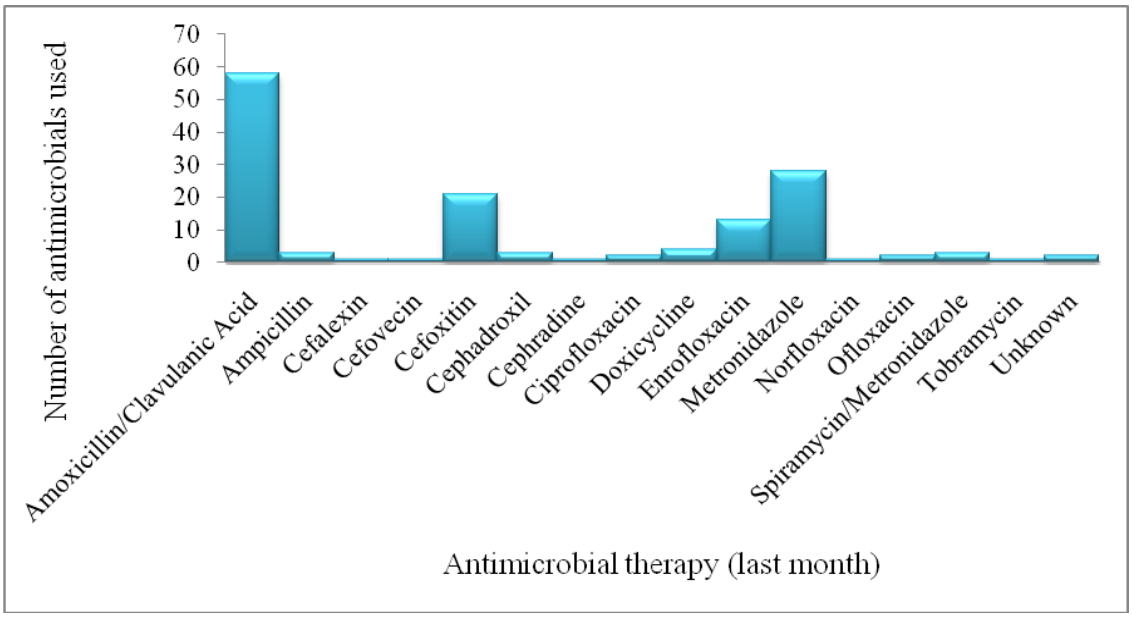
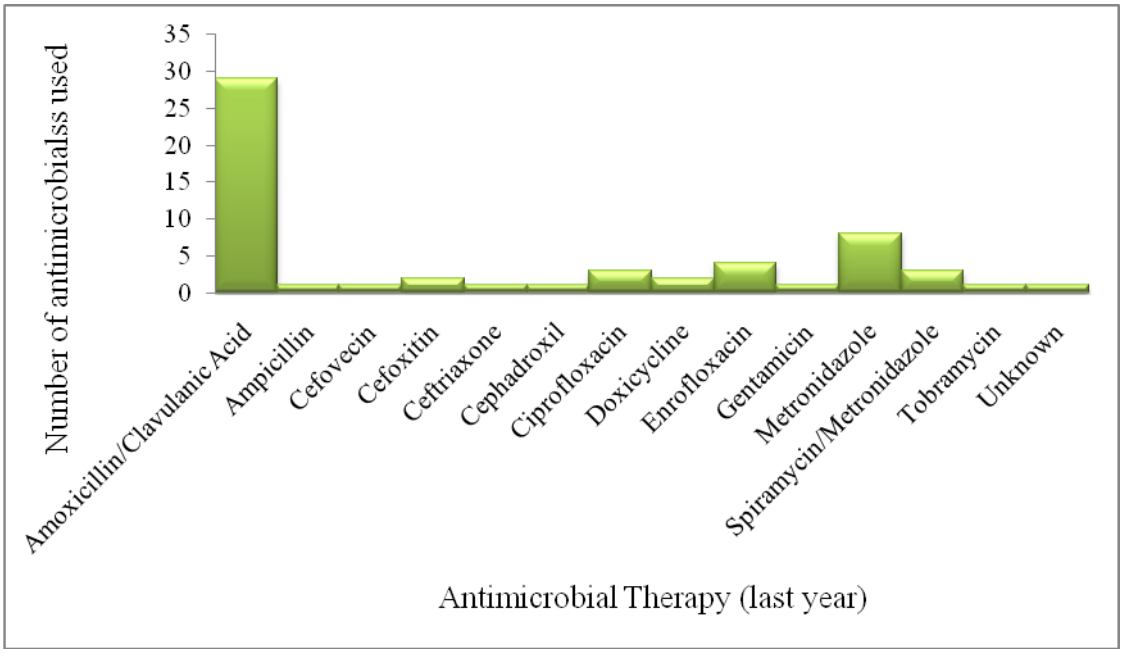


Figure 8 – Number of antimicrobials prescribed to the animals in the study within the year before the swab collection. Take in account that some animals received more than one antimicrobial.



The three animals carrying a MRSA isolate had received antimicrobial treatment the month before the nasal swab collection and one animal in the year before too (see Table 2). Six animals colonized with MRSP had received antimicrobial treatment one month previous to sampling and four one year before (see Table 2).

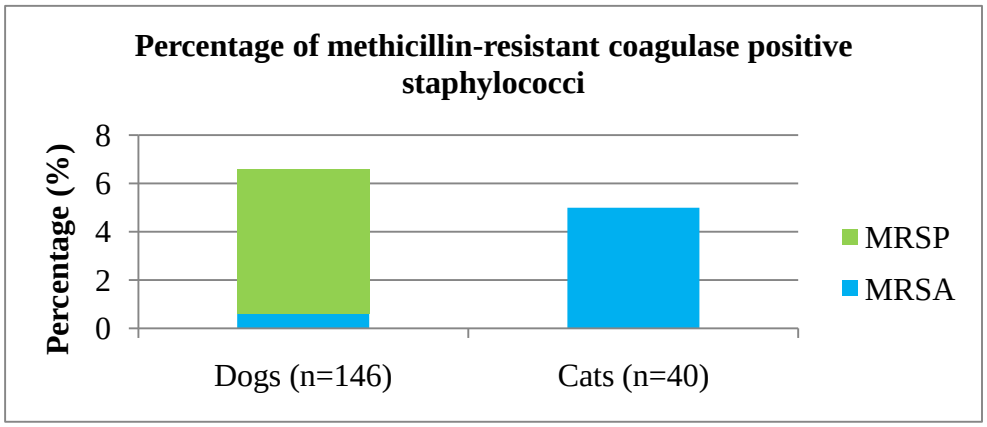
Table 2 – Species, gender, age and antimicrobial consumption of the companion animals colonized with MRSA and MRSP.

Case	Species	Gender	Age	Motive for admission	Antimicrobial Treatment (last month)	Antimicrobial Treatment (last year)
FMV 1	Canine	Female	Unknown	Surgery	Spiramycin/Metronidazole	Spiramycin/Metronidazole
FMV 2	Canine	Female	Unknown	Cushing's disease, thromboembolism	No	No
FMV 3	Canine	Male	Unknown	Urinary obstruction	Unknown	Unknown
FMV 4	Canine	Male	Unknown	Urinary tract infection	Unknown	Unknown
FMV 5	Canine	Female	3 years	Urinary tract infection, gastritis	Amoxicillin/Clavulanic Acid	Doxycycline
FMV 6	Canine	Female	3 months	Canine parvovirus suspicion	Amoxicillin/Clavulanic Acid and Metronidazole	Unknown
FMV 7	Canine	Female castrated	11 years	Intestinal obstruction	No	Amoxicillin/Clavulanic Acid
FMV 8	Canine	Female castrated	2 years	Surgery (castration)	Amoxicillin/Clavulanic Acid	Amoxicillin/Clavulanic Acid
FMV 9	Canine	Male	8 years	Surgery	Enrofloxacin, Cefoxitin and Metronidazole	Unknown
FMV 10	Feline	Male	Unknown	Multiple fractures	Amoxicillin/Clavulanic Acid	No
FMV 11	Feline	Female	11 years	Hypothermia, anorexia	Amoxicillin/Clavulanic Acid	Unknown
FMV 12	Canine	Male	2 years	Surgery (castration)	Amoxicillin/Clavulanic Acid	Unknown

MRSP strains were identified in 9 dogs, with a frequency of 6 % (see Figure 9). Two pattern of resistance were seen: FMV 1 strain demonstrated resistance to oxacillin, gentamicin, kanamycin, ciprofloxacin, enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin, ofloxacin, erythromycin and trimethoprim (see Table 3); the other 8 strains, FMV 2 to 9, demonstrated resistance to oxacillin, gentamicin, kanamycin, ciprofloxacin,

enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin, ofloxacin, clindamycin, erythromycin, tetracycline and trimethoprim (see Table 3). One of the isolates, FMV 1, was *spa* type t06 belonging to the CC68 and the other eight, FMV 2 to 9, were t02 and consequently were CC71. According to Kondo and colleagues criteria (2007), isolate FMV 1 harbored a SCC*mec* type V (class C *mec* complex and *ccrC* recombinase) and the other 8 isolates, FMV 2 to 9, harbored a variant of the SCC*mec* type III (class A *mec* complex and *ccrAB3* recombinase) (see Table 4). All isolates were typeable with *Sma*I-PFGE, resulting in the detection of two PFGE clusters (A and B) (see Figure10). The type B was subdivided into 3 subtypes (B1 to B3) (see Table 4). The PFGE type A strain showed identical characteristics as the American MRSP clone (ST68-MRSP-V), while PFGE cluster B grouped European MRSP isolates (CC71-MRSP-III) (see Table 4). PFGE typing results were in agreement with those obtained by *spa* and SCC*mec* typing. All isolates were SIET-negative. The 8 European MRSP isolates were positive for the *luF/lukS* genes and the American MRSP isolate was negative for both genes.

Figure 9 – Percentage of methicillin-resistant coagulase-positive staphylococci found in the study.



MRSA was found in two cats (5 %) and one dog (0.6%) (see Figure 9). The same pattern of resistance was seen for the three isolates (FMV 10, 11 and 12): oxacillin, ciprofloxacin, enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin and ofloxacin (see Table 3). Tandem repeat sequence analysis of the *spa* gene concluded that all isolates were t032 and thus belonged to the CC22. All isolates harbored SCC*mec* type IV (class B *mec* complex and *ccrAB2* recombinase) (see Table 4). A close relation to the European disseminated clone EMRSA-15 (ST22-MRSA-IV) was present: PFGE analysis revealed the same PFGE type (C) (see Figure 11) among the isolates, including with the previously described EMRSA-15 strain (ST22-MRSA-IV) causing skin infection in a dog and the attending veterinarian in Portugal (Pomba et al., 2009). All isolates were PVL negative.

Figure 10 – PFGE profiles (A, B) and subtypes (B1–B3) observed among MRSP isolated from dogs.

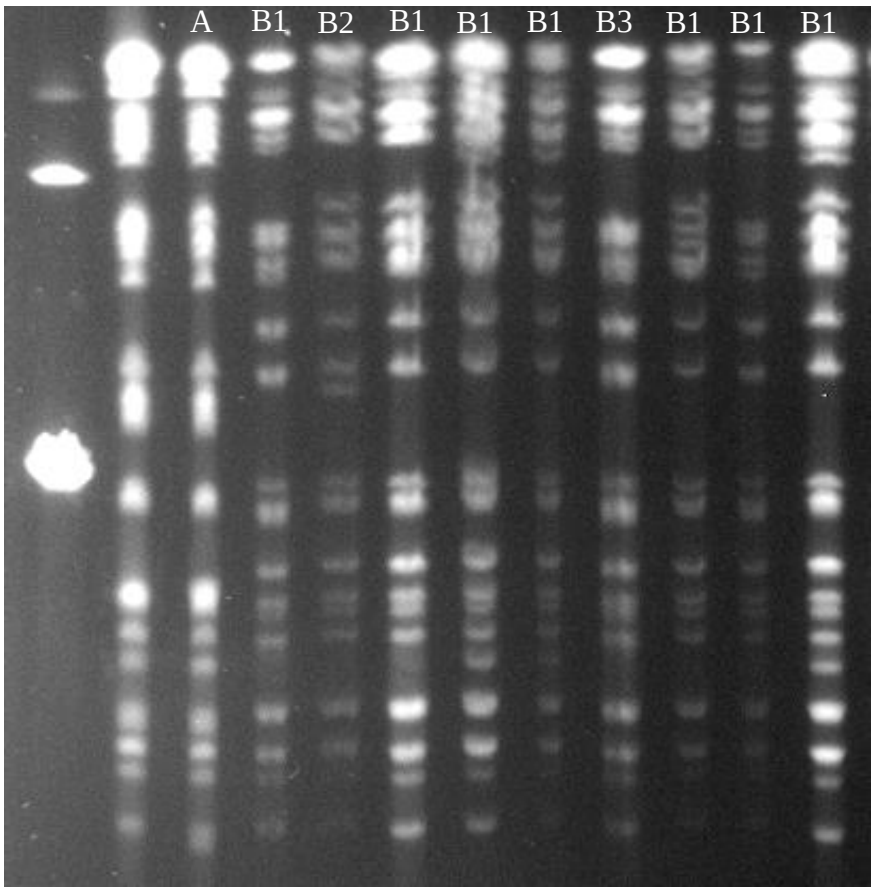


Figure 11 – PFGE profile (C) observed among MRSA isolates.

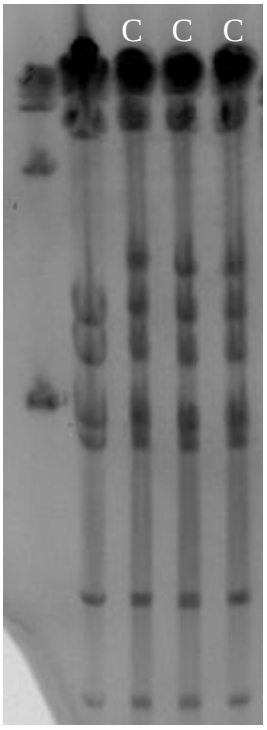


Table 3 – Susceptibility testing by disk diffusion of 27 antimicrobial agents of methicillin-resistant staphylococci strains.

Antibiotics	Disk Content	Clinical Breakpoints Criteria (Zone diameter in mm)				MRSP Strains									MRSA Strains		
		S	I	F	R	FMV 1	FMV 2	FMV 3	FMV 4	FMV 5	FMV 6	FMV 7	FMV 8	FMV 9	FMV 10	FMV 11	FMV 12
Amikacin[a]	30 µg	≥ 17	15-16	-	≤ 14	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin[a]	10 µg	≥ 15	13-14	-	≤ 12	R	R	R	R	R	R	R	R	R	S	S	S
Kanamycin[a]	30 µg	≥ 18	14-17	-	≤ 13	R	R	R	R	R	R	R	R	R	S	S	S
Rifampicin[a]	5 µg	≥ 20	17-19	-	≤ 16	S	S	S	S	S	S	S	S	S	S	S	S
Cephalothin[a]	30 µg	≥ 18	15-17	-	≤ 14	S	R	R	R	R	R	R	R	R	S	S	S
Cefoxitin[b]	30 µg	≥ 20	-	-	≤ 19	S	R	R	R	S	R	R	S	R	S	S	S
Oxacillin[a,c]	1 µg	≥ 13	11-12	-	≤ 10	NA	NA	NA	NA	NA	NA	NA	NA	NA	R	R	R
Oxacillin[d,e]	1 µg	≥ 20	-	-	< 20	R	R	R	R	R	R	R	R	R	NA	NA	NA
Penicillin G[a]	10 U	≥ 29	-	-	≤ 28	R	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin[b]	5 µg	≥ 21	16-20	-	≤ 15	R	R	R	R	R	R	R	R	R	R	R	R
Enrofloxacin[a]	5 µg	≥ 23	-	17-22	≤ 16	R	R	R	R	R	R	R	R	R	R	R	R
Levofloxacin[b]	5 µg	≥ 19	16-18	-	≤ 15	R	R	R	R	R	R	R	R	R	R	R	R
Marbofloxacin[a]	5 µg	≥ 20	15-19	-	≤ 14	R	R	R	R	R	R	R	R	R	R	R	R
Moxifloxacin[b]	5 µg	≥ 24	21-23	-	≤ 20	R	R	R	R	R	R	R	R	R	R	R	R
Norfloxacin[b]	10 µg	≥ 17	13-16	-	≤ 12	R	R	R	R	R	R	R	R	R	R	R	R
Ofloxacin[b]	5 µg	≥ 18	15-17	-	≤ 14	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin[a]	30 µg	≥ 15	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin[a]	2 µg	≥ 21	15-20	-	≤ 14	I	R	R	R	R	R	R	R	R	S	S	S
Erythromycin[a]	15 µg	≥ 23	14-22	-	≤ 13	R	R	R	R	R	R	R	R	R	S	S	S
Linezolid[b]	30 µg	≥ 21	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol[a]	30 µg	≥ 18	13-17	-	≤ 12	S	S	S	S	S	S	S	S	S	S	S	S
Quinupristin/Dalfopristin[b]	15 µg	≥ 19	16-18	-	≤ 15	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline[a]	30 µg	≥ 19	15-18	-	≤ 14	S	R	R	R	R	R	R	R	R	S	S	S
Trimethoprim[b]	5 µg	≥ 16	11-15	-	≤ 10	R	R	R	R	R	R	R	R	R	S	S	S
Nitrofurantoin[b]	300 µg	≥ 17	15-16	-	≤ 14	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic Acid[f]	10 µg	≥ 21	20	-	≤ 19	S	S	S	S	S	S	S	S	S	S	S	S
Mupirocin[g]	5 µg	≥ 14	-	-	≤ 13	S	S	S	S	S	S	S	S	S	S	S	S

Abbreviations: NA, not applicable; R, resistant; S, susceptible.

Inhibition zone diameters were interpreted according to: [(a)] CLSI M31-A3; [(b)] CLSI M100-S19; [(c)] Bemis et al., 2009; [(d)] Criteria only for MRSA; [(e)] Criteria only for MRSP; [(f)] Toma and Barriault 1995; [(g)] Fuchs et al., 1990

Table 4 – Origin, genotypic characteristics and antimicrobial resistant patterns of *mecA*-positive *S. aureus* and *S. pseudintermedius* strains isolated.

Strains	Origin	Antimicrobial associated co-resistance patterns	SCC*mec* element	*spa* type	Tandem Repeat Sequence	CC	PFGE Subtype
FMV 1	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, E^R, W^R	V	t06	r01r02 r03 r03 r06 r05	68	A
FMV 2	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 3	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B2
FMV 4	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 5	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 6	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B3
FMV 7	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 8	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 9	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 10	Feline	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C
FMV 11	Feline	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C
FMV 12	Canine	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C

Abbreviations: Cn, gentamicin; K, kanamycin; Cip, ciprofloxacin; Enr, enrofloxacin; Lev, levofloxacin; Mar, marbofloxacin; Mxf, moxifloxacin; Nor, norfloxacin; Ofx, ofloxacin; Da, clindamycin; E, erythromycin; Te, tetracycline; W, trimethoprim; CC, clonal complex.

6 Discussion

This is the first study to report the prevalence of MRSA and MRSP colonization in small animals in Portugal. MRSP was the predominant coagulase positive methicillin-resistant *Staphylococcus* in dogs, while MRSA was the main in cats. This is in accordance with previous reports (Hanselman et al., 2008). The origin of MRSA and MRSA in these small animals is unclear, but the genetic variety of isolates suggests that the two organisms may be spread in the dog and cat population, as has been proposed before (Sasaki et al., 2007; Hanselman et al., 2008). There were several limitations to this study. All data was collected at a single geographic location with no follow-up on colonized animals and therefore we cannot conclude the source or duration of colonization. Although there was a high prevalence of methicillin-resistant coagulase-positive staphylococci, the small sample size was prohibitive for assessment of potential risk factors for colonization.

MRSI colonization was not identified which is in agreement with previous reports (Sasaki et al., 2007a; Hanselman et al., 2008).

The incidence of MRSA in Human Portuguese Hospitals remains one of the highest in Europe, with dominance of the EMRSA-15 clone (ST22-MRSA-IV) (Aires-de-Sousa et al., 2008). Thus, it is not surprising that the MRSA colonization strains isolated from our companion animals belong to this epidemic clone. Furthermore, MRSA ST22-SCC*mec* IV had been previously isolated from a skin infection in a dog and attending veterinarian in Portugal (Pomba et al., 2009). All isolates showed the same resistance profile as the epidemic EMRSA-15 (resistance to methicillin and ciprofloxacin). The origin of EMRSA-15 isolates in small animals remains unknown but several authors suggest that MRSA isolated from companion animals have a human source (Loeffler et al., 2005). As in our study, Loeffler and colleagues (2005) demonstrated the occurrence of EMRSA-15 in dogs in the United Kingdom, and pointed the epidemiological impact since it proves that this nosocomial pathogen is not restricted to hospitals and can be carried and disseminated by healthy humans and animals in the community. Little information is available to date on the risk of antimicrobial usage with regard to MRSA colonization in small animals (Catry et al., 2010). According to previous case reports, many animals infected or colonized with MRSA have been treated with antimicrobials prior to the diagnosis (Catry et al., 2010). Interestingly, our 3 animals colonized with MRSA received antimicrobial treatment with amoxicillin-clavulanic acid one month prior to the nasal swab collection. Nevertheless, our sample is too small to prove antimicrobial administration was a risk factor and 63 % of non-colonized animals were

also exposed to antimicrobial administration, including amoxicillin-clavulanic acid, without development of MRSA colonization.

In 2009, the first MRSP infection was identified in Portugal. The isolate was the co-source (together with *Enterococcus faecalis*) of a lower urinary tract infection in a cat and after molecular characterization was found to be associated with the American MRSP clone (ST68-MRSP-V) (Pomba et al., 2010). To that date, this clone had been exclusively reported in the United States of America and Canada. The isolation of a second strain belonging to this clonal complex raises the question if it is already spreading through the European continent, too. More studies should be conducted to identify the real prevalence of this clone in Portugal and other European countries.

The European MRSP clone (ST71-MRSP-III) has been implicated as responsible for infections in companion animals in Germany, Sweden and Switzerland (Wettstein et al., 2008; Moodley et al., 2009) and more recently was isolated also from clinical samples throughout Europe (Perreten et al., 2010; Ruscher et al., 2010). Interestingly, European MRSP isolates show a multidrug-resistant pattern (resistance to more than 4 antimicrobial classes), with resistance to some aminoglycosides, quinolones, lincosamides, macrolides, tetracyclines, and folate pathway inhibitors (trimethoprim) (Loeffler et al., 2007; Wettstein et al., 2008; Ruscher et al., 2009; Perreten et al., 2010; Ruscher et al., 2010) and therefore they should be referred as multidrug-resistant *S. pseudintermedius* (MDRSP). The isolates in our study show exactly the same multidrug-resistant pattern. Surprisingly, our isolates show resistance not only to second and third generation quinolones, but also to a fourth generation fluoroquinolone, moxifloxacin, which has enhanced activity against gram-positive cocci (Ince, Zhang, & Hooper, 2003). This is an interesting fact since moxifloxacin is not licensed for veterinary use and prescription in Human Medicine is licensed only for the treatment of respiratory and skin infections. Intorre et al. (2007) found moxifloxacin-bordeline-intermediate-resistant *S. intermedius* strains according to CLSI breakpoints and in 2009 resistance to moxifloxacin was described in 45 clinical strains out of 46 MRSP strains (Ruscher et al., 2009). Intorre and co-workers (2007) found that resistance to the older fluoroquinolones was correlated with a double mutation in the quinolone-resistance-determining regions (QRDR) within the genes *gyrA* (subunit of DNA gyrase) and *grlA* (subunit of topoisomerase IV) and speculated that an additional alteration in *grlA* was necessary for fourth generation fluoroquinolone's resistance (Intorre et al., 2007). However the true mechanism of moxifloxacin-resistance in *S. pseudintermedius* remains unknown and further studies are required. This development of resistance to moxifloxacin and the observation of a multidrug-resistant pattern along European and American MRSP strains can

indicate that these strains have a high capacity of mutation or can easily acquire mobile elements, like transposons or plasmids, encoding resistance genes.

Wettstein and colleagues (2008) also found resistance to chloramphenicol in their European MRSP strains, but our isolates do not demonstrate chloramphenicol resistance. This multidrug-resistance pattern represents a major challenge for veterinarians in terms of antibiotic therapy. Most of the drugs to which these bacteria remain susceptible are used off-label in small animal practice (like amikacin, chloramphenicol and nitrofurantoin), others are used for human decolonization (fusidic acid and mupirocin) and others are last-resort drugs in human medicine (linezolid, quinupristin/dalfopristin, rifampicin and vancomycin). Moreover, linezolid, quinupristin/dalfopristin and vancomycin are considered antimicrobial limited for therapy of multidrug-resistant *S. aureus* infections by the joint Report of the FAO/WHO/OIE Expert Meeting (FAO/OIE/WHO, 2008). Thus, this represents a serious ethical dilemma, even when applying the "cascade principle". Since there are no guidelines for treatment of infections with these MDRSP, it is up to every veterinarian to decide which antimicrobial to use. Most infections with MDRSP are skin and wound infections, otitis externa, respiratory infections and urinary tract infections (UTIs). Biocides, like chlorhexidine, triclosan, Tris-EDTA and benzoyl peroxide, have been recommended for topical use in superficial skin infections and otitis externa. This approach is very successful and does not seem to select for antimicrobial resistance (Guardabassi, Ghibaudo & Damborg, 2009). However, care should be taken in order to also avoid development of resistance to biocides. Treatment of respiratory infections and UTIs with these MDRSP will be far more complicated to solve and precautions should be taken when choosing the antimicrobial therapy.

The presence of Luk-I and SIET in MRSP CC71 has been previously described by Ruscher and colleagues in 2010. Our strains were SIET-negative but Luk-I-positive. Luk-I shows a strong toxicity on various polymorphonuclear cells, including human leukocytes, but only a slight hemolytic activity on rabbit erythrocytes (Futagawa-Saito et al., 2004). Because phagocytosis by polymorphonuclear cells is an important defense mechanism of the host, Luk-I may play a primary role in bacterial evasion to the host defense mechanisms, enhancing the opportunity of the cocci to colonize and set up an infection. Nevertheless the exact role by which Luk-I enables staphylococci colonization and infection is not completely understood and so its true meaning may be underestimated.

The different colonization rates between dogs and cats of the two staphylococci species can indicate that the relative risk of MRSA and MRSP colonization might vary between the two host species, with MRSP being more frequent among dogs and MRSA among cats. The high rate of colonization of MRSP by dogs is likely to be due to host specificity, given that *S.*

pseudintermedius is the pathogen more frequently isolated from dogs (Sasaki et al., 2010). However, the same is not true for cats and the higher rate of MRSA colonization is not fully understood. Weese and van Duijkeren in 2010 referred that MRSA colonization might be transient in dogs and cats perhaps because *S. aureus* is not naturally a predominant commensal in these species. With *S. pseudintermedius* being the most prevailing commensal at least in dogs the duration of colonization is probably longer. Yet further studies on the duration of colonization are needed to evaluate this.

Routine decolonization therapy is not recommended in human or animals that have mucosae colonized with MRSA (Catry et al., 2010). Nevertheless decolonization with antimicrobial therapy may be measured in individual MRSA colonized animals as an option to control transmission of MRSA between animals or from animals to humans (Catry et al., 2010). Animal confinement may also be considered. Still no antimicrobial drugs for veterinary use have been effectively studied and approved for local or systemic application intended to resolve MRSA carrier status. For MRSP decolonization there are no recommendations for therapy of colonized animals and not even if decolonization should be performed.

7 Conclusion

In conclusion, even though the frequency of MRSA colonization was low, carriage of MRSA by companion animals in the community is a serious public health problem and adequate measures are necessary to prevent MRSA from becoming endemic in the animal population.

The frequency of isolation of MRSP in our study was high (6 %), considering that MRSP was first described in Europe in 2006 and in Portugal in 2009. This carriage rate suggests that these bacteria are quickly spreading through the canine population through all Europe, including Portugal. How this dissemination has occurred is not completely understood (Perreten et al., 2010; Ruscher et al., 2010), however colonization of healthy animals can be an important way of transferring the pathogen from animal to animal.

Although isolation of MRSP is infrequent in humans, transmission of strains between animals and humans has occurred in the past (Weese & van Duijkeren, 2010; Chuang et al., 2010). More important, the MDRSP strains can serve as reservoirs of antimicrobial resistance genes that can, possibly, be transferred to other bacterial species like *S. aureus*, since the location of many of these resistance genes is on mobile elements. Subsequent clonal spread of such a new MRSA clone might be a threat for human health in the future. The same may be applied for virulent toxins encoding genes among MRSP CC71 strains, which can play an important role in the dissemination of this epidemic clone. Recommendations for treatment of MDRSP

infections and decolonization are urgently needed and new treatments without the use of antimicrobials are required.

More importantly, monitoring of MRSA and MRSP in companion animals should be promoted in veterinary surveillance programmes on antimicrobial resistance.

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Annex I – Abstract of the oral communication presented at the 3rd AMVE Congress 2008

Estudo da colonização no cavalo por *Staphylococcus* meticilina-resistente

[Study of the colonization by methicillin-resistant *Staphylococcus* in horses]

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O *Staphylococcus aureus* é reconhecido como agente patogénico tanto em medicina humana como em medicina veterinária, podendo provocar desde infecções cutâneas ligeiras a situações de bacteriémia com risco de vida para o indivíduo. Surgiu pela primeira vez como agente patogénico com relevância para o ser humano na década de 70, nomeadamente em infecções hospitalares. Nos anos 90 já constituía uma infecção nosocomial séria a nível mundial, tendo nos últimos 10 anos vindo a ser relatado com importância crescente nos animais. As estirpes de MRSA ("methicillin-resistant *Staphylococcus aureus*") são resistentes a todos os β-lactâmicos actualmente em uso em medicina veterinária, sendo a resistência mediada pelo gene *mecA*. Este gene codifica uma proteína que se liga à penicilina, PBP2a, a qual é expressa na parede celular bacteriana e tem baixa afinidade para antibióticos β-lactâmicos. A prevalência de MRSA no homem varia muito com a área geográfica, sendo da ordem dos 60 % em algumas regiões dos Estados Unidos da América, 40 % no Sul da Europa e abaixo de 1 % no Norte da Europa (Leonard & Markey, 2008). Há evidência de que pode ocorrer transferência de estirpes de MRSA dos animais para o homem e vice-versa. Dados epidemiológicos obtidos por classificação fenotípica e molecular mostram que os isolados de cães e gatos não diferem dos do ser humano mas os isolados de cavalos e de pessoal que lida com eles são diferentes, estando ainda mal estudados. No cavalo as infecções por MRSA ocorrem preferencialmente em feridas cutâneas e em situações pós-operatórias, à semelhança do que ocorre no cão (Leonard & Markey, 2008). Encontram-se descritas infecções como artrite séptica, infecção no local de introdução do cateter endovenoso, pneumonia, infecção da ferida cirúrgica, mastite, rinite ou abcessos cutâneos. A principal via de transmissão parecem ser as mãos dos médicos veterinários. Também têm sido descritas infecções por estafilococos coagulase-negativo resistentes à meticilina ("methicillin-resistant coagulase negative staphylococci"- MRCoNS) (Trostle et al., 2001).

Nesta perspectiva procedemos ao estudo da possível colonização nasal de 10 equídeos por MRSA. Recolheu-se uma zaragatoa nasal de cada cavalo. As zaragatoas foram inoculadas em meio de enriquecimento de Manitol selectivo para estafilococos e incubadas 37º C 18 h. Em seguida inocularam-se 10 µl em meio de Manitol com cefoxitina (3,5 mg/l) e aztreonam (75 mg/l). Após incubação, 10 µl de suspensão bacteriana foram semeados em meio Chrom ID

MRSA (bioMérieux). Não foi detectado nenhum portador de MRSA. No entanto, 5 cavalos apresentaram outras espécies de estafilococos resistentes à meticilina: *Staphylococcus sciuri* (*n=2*), *Staphylococcus lentus* (*n=1*), *Staphylococcus vitulus* e *S. sciuri* (*n=1*), *Staphylococcus* spp. (n=1). Assim, no caso dos equinos este meio selectivo parece ser pouco específico não seleccionando exclusivamente MRSA, como acontece nos seres humanos para o qual foi desenvolvido (Diederen et al., 2006). Estas espécies de MRCoNS fazem parte da flora normal dos equinos (Vengust et al., 2006) e podem potencialmente causar infecção.

A infecção por MRSA e MRCoNS é extremamente relevante em termos de saúde pública. Os animais podem ser fonte de infecção estafilocócica do ser humano, podendo funcionar como reservatório, nomeadamente no que respeita a seres humanos imunodeprimidos. Embora seja de extrema importância fazer o despiste de MRSA, alargá-lo a toda a população equina e ao pessoal que com ela trabalha pode ser economicamente inviável. Este facto reforça a importância acrescida de um estudo mais aprofundado da população do nosso país, de forma a poder formular algumas directrizes com vista à elaboração de *guidelines*, as quais, de futuro, nos permitam fazer uma vigilância direccionada a uma população equina em risco mais restrita.

Annex II – Abstract of the oral communication presented at the 17th APMVEAC National Congress 2008

Primeiro caso de infecção cutânea por *Staphylococcus simulans* meticilina resistente num cão com dermatite atópica em Portugal

[First case of a cutaneous infection by a methicillin-resistant *Staphylococcus simulans* in a dog with atopic dermatitis in Portugal]

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Apesar do *Staphylococcus intermedius* ser o agente patogénico mais frequente na piodermite canina, os estafilococos coagulase negativos (ECNs), também têm sido associados a infecção cutânea quer no homem quer no cão (Medleau *et al.*, 1986). Neste trabalho descrevemos um caso de piodermite superficial causada por uma estirpe multirresistente de *Staphylococcus simulans* num Teckel atópico.

O cão apresentou-se à consulta no início de 2005 com problemas dermatológicos crónicos. Foi diagnosticada uma dermatite atópica complicada por uma piodermite secundária grave. O perfil de sensibilização incluía os grupos de fungos (*Malassezia pachydermatis*), penas (psitacídeos) e ácaros do pó e de armazenamento. A gravidade da atopia foi avaliada através do CADESI 02, tendo-se obtido um valor de 187, compatível com um quadro severo. Um ano após o diagnóstico de atopia em presença de um quadro de piodermite superficial, foi feita citologia cutânea e colheita para exame microbiológico. A citologia confirmou o quadro de piodermite. Foi isolado *S. simulans* em cultura predominante em associação com *Escherichia coli*. A estirpe de *S. simulans* era resistente à oxacilina com uma CIM > 4 μg/ml e por PCR possuía o gene *mecA*. Este isolado era também resistente às fluoroquinolonas de 2ª, 3ª e 4ª geração, gentamicina, cotrimoxazol, tetraciclina, cloranfenicol, clindamicina, eritromicina e azitromicina. Foi instituído tratamento *per os* com cefadroxil e tópico com peróxido de benzoílo 2%.

Apesar de não haver indicação para o tratamento das infecções por ECNs meticilina resistentes por qualquer antibiótico do grupo dos β-lactâmicos, a resposta à terapêutica combinada foi positiva provavelmente pelo componente tópico. O caso descrito difere de dados anteriores quanto à existência deste mecanismo de resistência em *S. simulans* isolados de cães (Lilenbaum *et al.*, 2000). A epidemiovigilância da resistência à meticilina em medicina veterinária é uma preocupação mundial pelo seu potencial zoonótico.

Annex III – Abstract of the oral communication presented at the 18th APMVEAC National Congress 2009

Frequência de colonização por staphylococci coagulase-positivo em gatos internados no hospital escolar da FMV – UTL

[Frequency of colonization by methicillin-resistant coagulase-positive staphylococci in cats hospitalized at the teaching hospital of FMV-UTL]

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A colonização por staphylococci coagulase-positivo (SCP), em particular SCP meticilina resistente (SCPMR) é extremamente relevante em termos de saúde pública e de controlo da infecção hospitalar.

Foram realizadas zaragatoas nasais a 40 gatos de diversas idades e de ambos os sexos no internamento do hospital escolar da Faculdade de Medicina Veterinária de Abril de 2008 a Abril de 2009. Cada zaragatoa foi inoculada primeiro em meio de enriquecimento de Manitol selectivo para estafilococos e incubada a 37º C 24 horas e depois em agar Columbia. As colónias suspeitas (β-hemólise, cor amarela) foram semeadas Chrom ID MRSA (bioMérieux) durante 48 horas. Realizou-se um Multiplex-PCR para pesquisa do gene *mecA*, do gene *nuc*, e do gene *16S*. As estirpes foram ainda caracterizadas pela coloração de Gram, prova da catalase e coagulase e identificação pelo sistema BBL Gram positive.

Nesta amostra, 12,5 % (*n*=5) dos gatos testados apresentaram colónias hemolíticas possíveis SCP, 80 % (*n*=32) apresentaram outras colónias não hemolíticas e 7,5 % (*n*=3) não apresentaram qualquer tipo de colónias. Das 5 estirpes hemolíticas, 2 (5 %) foram caracterizadas como *S. aureus* meticilina resistente (MRSA) (*nuc* +, *mecA* +, *16S* +), uma como *S. aureus* meticilina sensível (MSSA) (*nuc* +, *16S*+), uma (2,5 %) como *Staphylococcus intermedius* meticilina resistente (MRSI) (*mecA* +, *16S* +), uma como *S. intermedius* meticilina sensível (MSSI) (*16S* +).

A frequência de colonização encontrada por SCPMR foi baixa e enquadra-se nos valores encontrados em outros Países. A detecção da colonização por staphylococci coagulase-positivo é muito importante por estes poderem ser vectores de genes de resistência a antibióticos e pela gravidade das infecções que podem provocar.

Annex IV – Poster presented at the ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications 2009

Nasal carriage of methicillin-resistant coagulase-positive staphylococci among cats and dogs hospitalized in a Veterinary Teaching Hospital in Portugal

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Many authors have emphasized the importance of methicillin-resistant staphylococci carriage in companion animals, as these bacteria are emerging as a significant problem in veterinary medicine, including both animal and public health standpoints. The objective of this study was to investigate the frequency of methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) carriage in a random sample of 40 cats and 146 dogs hospitalized in the Faculty of Veterinary Medicine Teaching Hospital, Lisbon. Nasal swabs were collected at the time of patient admission to the hospital and inoculated in 3 ml of an enrichment broth (Weese *et al.*, 2006). After overnight incubation, 10 µl of bacterial suspension were inoculated in Columbia 5% blood sheep agar plates. Colonies were identified as coagulase-positive staphylococci based on colony morphology, ability to cause hemolysis, gram straining, positive catalase test, positive tube coagulase test and BBL™ Crystal™ typing system. MRSA and MRSP carriage was screened by plating enrichment cultures on a selective medium, Chrom MRSA ID (bioMérieux) and suspected colonies were confirmed by PCR identification of *mecA* (http://www.crl-ar.eu). Presumptive *S. aureus* isolates were confirmed by PCR amplification of the *nuc* gene and discrimination between *S. pseudintermedius* and *S. intermedius* was done by restriction fragment length polymorphism (RFLP) analysis of *pta*. All isolates were tested by PCR for the presence of lukF/lukS genes encoding Panton-Valentine leukocidin (PVL). MRSA was only found in two cats (5%) and one dog (0.6%). Nine dogs carried MRSP (6%), whereas none of the cats was found to be positive. All isolates were PVL-negative. The results indicate that the relative risk of MRSA and MRSP carriage might vary between the two host species, with MRSP being more frequent among dogs and MRSA among cats. The observed MRSA carriage rates were not surprising considering the high prevalence of MRSA in humans in Portugal. As sampling was performed immediately after admission to the hospital, the observed MRSP carriage rate is likely to reflect the prevalence among dogs in the community. MRSP were first described in

Europe in 2006 and the relatively high MRSP carriage rate observed in dogs suggest that these bacteria are quickly spreading in the canine population of this country.

Annex V − Poster presented at the ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications 2009

First description of an MRSA skin infection in a dog and attending veterinarian in Portugal

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Methicillin-resistant *Staphylococcus aureus* (MRSA) have been increasingly reported in veterinary medicine. We hereby report a case of an MRSA skin infection associated with hepatocutaneous syndrome (HS) in a geriatric female dog, as well as the colonization of one of the attending veterinarians. An eleven year old female Labrador Retriever presented five years ago at a veterinary hospital in the Lisbon area with a chronic right forelimb pododermatitis and footpad hyperkeratosis. Leishmaniosis was diagnosed. Two episodes of acute onset hepatitis developed during leishmaniosis treatment with allopurinol and levamisol. Cutaneous lesions became exudative, ulcerative and purulent. Several courses of antimicrobial therapy allowed temporary remission. The infection extended to the other paws and ano-genital skin. A fistulae swab was cultured. *S. aureus* was isolated in predominant culture along with *Enterobacter cloacae*. Antimicrobial susceptibilities were determined by the disk diffusion method and by minimal inhibitory concentration with DADE MicroScan panels and interpreted according to CLSI guidelines M31-A3 and M100-S17. MRSA nasal carriage by the attending vet was evaluated three weeks after exposure. The two MRSA isolates were identified by PCR for the *mecA* gene, subjected to staphylococcal protein A typing (*spa*), staphylococcal cassette chromosome *mec* typing, and underwent multilocus sequence typing (MLST). Isolates were also tested for the *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL). The animal and human MRSA isolates were identified as ST22, *spa* type t032 and SCC*mec* IV. None of the MRSA isolates carried the PVL genes. The isolates were also resistant to fluoroquinolones (enrofloxacin, marbofloxacin, ciprofloxacin, levofloxacin) and susceptible to aminoglycosides (gentamicin, tobramycin, netilmicine, amikacin), chloramphenicol, trimethoprim-sulfamethoxazole, and vancomycin. The *E. cloacae* was resistant to amoxicillin, amoxicillin-clavulanate, cephalexin, cefuroxime and tetracycline. Treatment with gentamicin 5 mg/kg IM *sid* was carried out for five days. Although therapy was performed the cutaneous infection aggravated and clinical condition worsened. The owner asked for euthanasia. MRSA are an important cause of human

nosocomial infections in Portugal. It seems that this dog's MRSA infection may be community acquired, possibly through the owner or transmitted through the environment. The severity of the footpads skin lesions, associated with the HS and the previous antimicrobial usage may have predisposed to the MRSA infection. To our knowledge, this is the first report of the epidemic clone EMRSA-15 in a dog in Portugal.

Annex VI – Poster presented at the ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications 2009

High-frequency of clindamycin associated resistance among MRSA CC398 from breeding swine herds in Portugal

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Recent evidence of a community-acquired methicillin-resistant *Staphylococcus aureus* case of invasive infection caused by MRSA ST398 multi-resistant strain of swine origin in a pig-farm worker in Cremona, Italy, reinforces the emerging problem of animal MRSA as a human occupational health hazard (Pan *et al.*, 2009). The aim of this study was to characterize the antimicrobial susceptibility of MRSA strains isolated during the Portuguese European Union baseline study for the detection of MRSA in breeding pigs. Dust swabs were taken from 171 randomly selected breeding and production pig farms from distinct regions of Portugal. MRSA isolates were identified by polymerase chain reaction (PCR) for the *mecA* gene (http://www.crl-ar.eu), subjected to staphylococcal protein A (*spa*) (http://www.seqnet.org/) typing, and a new variant underwent multilocus sequence typing (MLST) (http://www.mlst.net). Isolates were also tested for the *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL). Antimicrobial susceptibilities were determined by minimal inhibitory concentration with DADE MicroScan panels, and interpreted according to CLSI guidelines M31-A2 and M100-S17. Dust swabs from 21 herds tested positive for MRSA. The MRSA isolates from Portuguese herds were all identified as CC398 with *spa* types t108 (*n*=12), t011 (*n*=7), and t1255 (*n*=1), except for a newly identified ST398 *spa* variant named t4854. None of the MRSA isolates carried the PVL genes. Susceptibility testing revealed resistance to tetracycline in all MRSA isolates. Seven isolates of the 21 were resistant to clindamycin only and 6 both to clindamycin and erythromycin. Eight isolates were resistant to trimethoprim/sulfamethoxazole. Two strains were gentamicin resistant. One strain was resistant to all fluoroquinolones tested (ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin), and also chloramphenicol. All isolates were susceptible to mupirocin, fosfomycin, fusidic acid, rifampin, nitrofurantoin, linezolid, quinupristin/dalfopristin, teicoplanin and vancomycin. The occupational hazard for livestock associated MRSA colonization through the intensity of pig contact has been confirmed. Recent evidence-based

guidelines for the prophylaxis and treatment of human MRSA skin and soft tissue infections (Gould et al., 2009) include the use in monotherapy or eradication therapy of tetracyclines, clindamycin and trimethoprim/sulfamethoxazole. The high frequency of resistance found in this study towards these antimicrobials in CC398 MRSA of animal origin is important. Further studies are required to elucidate the mechanism of resistance towards clindamycin with macrolide susceptibility in these isolates. The emergence of associated resistance in MRSA animal isolates is a concerning fact for the pig industry, compromising antimicrobial therapy of possible human or animal infections.

Annex VII – Poster presented at the VI OMV Conference 2009

Frequência de colonização por staphylococci coagulase-positivo meticilina-resistente em cães internados no hospital escolar da FMV – UTL

[Frequency of colonization by methicillin-resistant coagulase-positive staphylococci in dogs hospitalized at the teaching hospital of FMV-UTL]

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Os staphylococci coagulase-positivos meticilina resistentes, particularmente *Staphylococcus aureus* (MRSA) e *Staphylococcus pseudintermedius* (MRSP), são agentes oportunistas de grande importância. Estes organismos têm vindo a ser relatados com cada vez mais frequência em cães, o que os torna relevantes em termos de saúde animal e pública. O objectivo deste estudo baseou-se na investigação da frequência de colonização por MRSA e MRSP em cães internados no Hospital Escolar da Faculdade de Medicina Veterinária.

Foram realizadas zaragatoas nasais a 146 cães de diversas idades e de ambos os sexos no internamento do hospital escolar de Abril de 2008 a Abril de 2009. Cada zaragatoa foi inoculada primeiro em meio de enriquecimento de manitol selectivo para estafilococos e incubada a 37ºC 24 horas e depois em agar Columbia. As colónias suspeitas (β-hemólise, cor amarela) foram semeadas Chrom ID MRSA (bioMérieux) durante 48 horas. A resistência à meticilina foi confirmada por amplificação do gene *mecA* por PCR. Os possíveis isolados de *S. aureus* foram confirmados por amplificação do gene *nuc* e a diferenciação entre *S. intermedius* e *S. pseudintermedius* foi feita por análise do gene *pta* por RFLP. As estirpes foram ainda caracterizadas pela coloração de Gram, prova da catalase e coagulase e identificação pelo sistema BBL™ Crystal™. Todos os isolados foram testados por PCR para a presença dos genes *lukF/lukS* que codificam a leucocidina Panton-Valentine (PVL).

Nesta amostra, 0,6 % (*n*=1) dos cães testados apresentaram MRSA e 6% (*n*=9) MRSP. Todos os isolados eram PVL negativos.

Os resultados revelam que o risco de colonização por MRSP é superior ao risco de colonização por MRSA. Como a amostra foi colhida durante a admissão ao internamento, a frequência de colonização por MRSP reflecte a sua prevalência nos cães na comunidade. Tendo em conta que o primeiro MRSP descrito na Europa foi em 2006, a elevada frequência encontrada neste estudo indica que esta bactéria se está rapidamente a disseminar pela população canina do país.

Annex VIII –Paper article submitted and accepted to the *Journal of Feline Medicine and Surgery*

Treatment of a lower urinary tract infection in a cat caused by a multidrug-methicillin-resistant *Staphylococcus pseudintermedius* and *Enterococcus faecalis*

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Keywords: MRSP, *E. faecalis*, urinary tract infection, nitrofurantoin, cat

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Abstract

Staphylococci and enterococci are common causes of urinary tract infections in cats. However, both species are rarely implicated together as causes of lower urinary tract infections associated with urethral obstruction. This report describes the first case of a multidrug-methicillin-resistant *Staphylococcus pseudintermedius* belonging to *spa* type t06 and *Enterococcus faecalis* urinary infection in a cat with pre-existing and recurrent urethral obstruction. Both species were isolated at $>10^5$CFU/ml from cystocentesis urine specimen. Clinical and ultrasound features, results from urinalysis, urine culture, molecular typing and susceptibility testing by minimal inhibitory concentrations determination are described. Oral treatment with nitrofurantoin was the only antimicrobial agent that constituted a viable therapeutical option and had a positive outcome.

A 5-year-old, neutered male Persian cat, living in an apartment was presented with urethral obstruction. The cat was fed a commercial dry cat food. The owner reported a 4-day history of inappetence, reduced water intake, dysuria, stranguria and constipation and generalized weakness. On physical examination, its bladder was enlarged and painful and the cat appeared depressed. Blood samples were taken for routine laboratory evaluation. The CBC revealed mild leucocytosis (20.5x $10^3/\mu$L, reference range 3.8-19x $10^3/\mu$L) with neutrophilia (18,655, reference range 1290-15 950/μL) and all red blood cell parameters were within reference range. Serum biochemical analysis revealed severe azotemia (BUN, > 300 mg/dL, reference range 10-30 mg/dL; Creatinine > 10 mg/dL, reference range 0.8-2.0 mg/dL) and hyperkalaemia (Potassium 9.6 mmol/L, reference range 3.5-5.1 mmol/L). Tests for feline

leukaemia virus and feline immuno-deficiency virus were negative (Snap Combo FeLV Ag/FIV Ab Test Kit, Idexx Labs.). The cat was treated with diazepam (Diazepam; Intervet, 0.2 mg/kg, IV) and butorphanol (Torbugesic, Fort Dodge) at the dosage of 0.4mg/kg and a sterile catheter was passed through the urethra to the bladder, and then sutured to the perineal skin. Haemorrhagic urine was removed and analysed (see Table 1). Urinalysis revealed pH 7.0 and the absence of crystalluria. The cat was treated with intravenous physiological saline solution 0.9% to resolve the post-renal uraemia and hyperkalaemia, ranitidine (Zantac; GlaxoSmithKline, 2 mg/kg, bid, IV) to reduce gastric acidity, and butorphanol (0.4 mg/kg, tid, IM) ensured analgesia. On day 3, the urethral catheter was removed and the cat was seen to urinate on its own. Haematology and serum biochemistry parameters were within reference ranges. After catheterization, an antimicrobial treatment course of amoxicillin combined with clavulanic acid (Synulox, Pfizer) at the dose of 12.5 mg/Kg body weight, twice daily, for one week was instituted. The cat was discharged from the hospital with feline URINARY SO® diet. On day 15 the cat was re-evaluated for persistent haematuria, pollakiuria and stranguria due to a partial urethral obstruction. The urinalysis was compatible with inflammation in the absence of bacterial infection (Table 1). Abdominal ultrasound examination detected acoustic shadowing in the bladder consistent with cellular debris, but no calculi, mass, thickening or mineralisation of the wall was observed. The left kidney had normal form, size, and echogenicity. The right kidney had a cystic cortical lesion.The prescription diet was maintained and flavoxate hydrochloride was prescribed (50mg, PO, bid) for urethral spasm control. On day 23 a second complete urethral obstruction occurred, this time with a urinalysis compatible with lower urinary tract infection (LUTI). Urine culture was performed with a sample obtained by cystocentesis. A mixed LUTI was found caused by *Staphylococcus pseudintermedius* (>10^5CFU/ml) and *Enterococcus faecalis* (>10^5CFU/ml) isolates. Antimicrobial susceptibility testing was performed using the microbroth dilution method (DADE Behring Microscan PM21, USA), and interpreted according to CLSI guidelines M31-A3 and M100-S17 (CLSI 2008, 2007) (Table 2). Discrimination between *S. pseudintermedius* and *S. intermedius* was done by *pta* PCR- restriction fragment length polymorphism analysis (Bannoehr et al 2009). Methicillin resistance in *S. pseudintermedius* was confirmed by PCR detection of *mecA* (http://www.crl-ar.eu). The methicillin-resistant *S. pseudintermedius* (MRSP) harbored a SCC*mec* V cassette using MPCR1 and MPCR2 described by Kondo et al (2007), was PVL negative (Lina et al 1999), and had *spa* type t06 (repeat sequence r01r02r03r03r06r05) (Moodley et al. 2009).

Possible therapeutic options were based on the following principles: i) both strains had to be susceptible to the antimicrobial agent; ii) the agent had to have good pharmacodynamic and

pharmokinetic characteristics for use in LUTI; iii) the antimicrobial agent could not be one of the critically important antimicrobials for human medicine (FAO/OIE/WHO, 2008). Nitrofurantoin was the only antimicrobial to fit all three criteria (Table 2), and was prescribed at 4 mg/kg, tid, for 60 days (one fourth of the content of a 100mg capsule was administrated PO after dilution in 5 mL of water with a syringe). The motivated owners of the cat were a veterinary student and her mother. This ensured compliance. There were clear signs of clinical improvement after 5 days and further urine samples collected on days 5, 20, 25 and 60 after commencing nitrofurantoin treatment were culture negative (Table 1). However, on day 51 after the first episode of urethral obstruction, a third episode of total obstruction occurred (Table 1), and was not related to the LUTI but probably due to the underlying presence of a feline idiopathic cystitis obstructive form. This required a perineal urethrostomy to alleviate the obstruction.

Obstructive idiopathic cystitis (OIC) constitutes a common medical problem of the lower urinary tract in male cats and Persian cats appear to be predisposed (Gunn-Moore 2003, Eggertsdóttir et al 2007). Urethral obstruction in cats may be present with a concurrent urinary tract infection at the time of obstruction relief (Eggertsdóttir et al 2007). Risk factors for the occurrence of a LUTI secondary to OIC remain to be elucidated. In a retrospective case-control study at three veterinary referral hospitals significant risk factors for the acquisition of a methicillin-resistant *Staphylococcus aureus* infection compared to a methicillin-susceptible *Staphylococcus aureus* infection was the presence of a urinary catheter or co-infection (Faires and Weese 2008). The third consecutive urethral obstruction in our cat was associated with the presence of a mixed LUTI with a multi-drug resistant MRSP. In our case, repeated bladder over-distension and urinary catheterisation may have predisposed to LUTI. The lack of a closed collection system at the time of the urinary catheterisation and the use of antimicrobial therapy during and after the first catheterisation procedure could have been contributing risk factors for the LUTI. Antimicrobials and their selective pressure have already been implicated in companion animal MRSP infection or colonization (Sasaki et al 2007). The systematic utilization of sterile closed collection systems attached to urethral catheters may overcome the occurrence of a LUTI secondary to OIC and result in antimicrobial prudent usage.

The recent clonal spread of MRSP in Europe is largely due to the emergence of a single clone (t02, ST71, SCC*mec* II-III) (Moodley et al 2009, Kadlec et al 2009). *Spa* type t06 and SCC*mec* V identical to those found in our MRSP, are associated with an MRSP clone currently circulating in North America (t06, ST68, SCC*mec* V) (Black et al 2009, Moodley et al 2009). In addition to β-lactam resistance, resistance in this isolate was observed to five

antimicrobial classes; fluoroquinolones, lincosamides, macrolides, aminoglycosides and trimethoprim/sulfamethoxazole. Susceptibility was observed to tetracycline and chloramphenicol. This susceptibility pattern is also observed in the ST68 MRSP U.S.A. clone but not in the European one (Andersson et al 2009). The MRSP causing LUTI in our patient is related to the ST68 clone which is a common cause of LUTI and pyoderma in the U.S.A.

The *E. faecalis* also involved in the LUTI was ampicillin susceptible and exhibited low-level gentamicin resistance which is common in Portugal and in other European countries (Delgado et al 2007, Damborg et al 2008). Therapeutic problems associated with enterococcal infections are often the result of intrinsic resistance of this genus towards cephalosporins, penicillinase-resistant penicillins, polymyxines, low concentrations of aminoglycosides, clindamycin, fluoroquinolones, streptogramines (*E. faecalis*) and trimethoprim/sulfamethoxazole.

This case report describes for the first time a co-infection with a multidrug-MRSP and *E. faecalis* in a feline lower urinary tract infection with pre-existing and recurrent urethral obstruction. In Switzerland, multidrug-resistant MRSP were previously found as a single causative pathogen in three cats with urinary tract infection (Wettstein et al 2008). The multi-resistance profile of MRSP strains spreading in Europe and North America typically includes resistance to all oral antimicrobials routinely used for treatment of infections in small animal medicine. In the present case report, therapeutic choices were even further narrowed by the co-infection. Nitrofurantoin usage is not authorized in animals. However, the rational for its use was the avoidance of using one of the critically important antimicrobials in human medicine, e.g. vancomycin. Infections with MRSP represent a real therapeutic challenge. The increased pressure to use last resort antimicrobials that are saved for the treatment of serious human infections raises important ethical questions that demand revision of regulatory and preventive measures to control this important animal health problem.

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Declaration of interest

None

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TABLE 1. Urinalysis and urine culture results during treatment and follow-up

Parameter and normal range	Day 1 (1st urethral obstruction)	Day 15	Day 23 (2nd urethral obstruction and LUTI)	Day 31 (5 days after the beginning of nitrofurantoin treatment, 8 days from LUTI diagnosis)	Day 46 (20 days after the beginning of nitrofurantoin treatment)	Day 51 (3rd urethral obstruction and urethrostomie/ 25 days after the beginning of nitrofurantoin treatment)	Day 86 (60 days after the beginning of nitrofurantoin treatment)
Specific gravity (1035-1060)	1037	1035	1015	1010	1030	1035	1020
pH (6-7)	7	7	7,5	8	6	7	6,5
Protein (0 to trace) mg/dl	300	> 2000	300	low	negative	300	negative
Glucose (negative)	negative	negative	negative	negative	negative	negative	negative
Bilirubin (negative)	negative	negative	negative	negative	negative	negative	negative
RBC/HPF (<5)	> 300	40 to 50	> 300	rare	0 to 1	> 300	1 to 2
WBC/HPF (<5)	0 to 1	0 to 1	8 to 10	0	1 to 2	2	0 to 1
Crystals (none)	none	none	none	none	none	none	none
Epithelial cells (occasional)	rare	rare	occasional	occasional	rare	rare	rare
Bacteria (none if cystocentesis used)	none	none	present	present	none	none	none
Culture	not available	not available	*Staphylococcus pseudintermedius* (>10^5CFU/ml) and *Enterococcus faecalis* (>10^5CFU/ml)	negative	negative	negative	negative

RBC/HPF, Red blood cell average count under high power field (400X); WBC/HPF, white blood cell average count under high power field (400X)

TABLE 2. *In vitro* activity of twenty-eight selected antimicrobial agents tested against uropathogenic bacterial strains and possible therapeutical options

Antimicrobial Agent	Uropathogenic bacterial strains						Combined MRSP /*E. faecalis* MIC interpretation	Possible therapeutical options for UTI treatment
	Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP)			*Enterococcus faecalis*				
	MIC (μg/ml)			MIC (μg/ml)				
		Clinical breakpoints			Clinical breakpoints			
		Susceptible	Resistant		Susceptible	Resistant		
Amoxicillin-Clavulanic Acid	4/2	≤ 4/2	≥ 8/4	-	NA	NA	R	No
Ampicillin	> 8	≤ 0.25	≥ 0.5	< 0.25	≤ 8	≥ 16	R	No
Azithromycin	> 4	≤ 2	≥ 8	-	NA	NA	R	NoA
Cefotaxime	< 0.5	≤ 8	≥ 64	-	NA	NA	R	No
Chloramphenicol	8	≤ 8	≥ 32	8	≤ 8	≥ 32	S	NoA
Ciprofloxacin	> 2	≤ 1	≥ 4	> 2	≤ 1	≥ 4	R	No
Clindamycin	> 2	≤ 0.5	≥ 4	-	NA	NA	R	NoA
Erythromycin	> 4	≤ 0.5	≥ 8	> 4	≤ 0.5	≥ 8	R	No
Fosfomycin	< 32	≤ 32	> 32	-	NA	NA	S	NoB
Fusidic Acid	< 2	≤ 2	≥ 32	-	NA	NA	S	NoB
Gatifloxacin	4	≤ 2	≥ 8	-	NA	NA	I	NoB
Gentamicin	> 8	≤ 4	≥ 16	-	NA	NA	R	No
Gentamicin High Level	-	NA	NA	< 500	< 500	≥ 500	S	NA
Levofloxacin	4	≤ 2	≥ 8	> 4	≤ 2	≥ 8	R	NoB
Linezolid	2	≤ 4	> 4	< 1	≤ 2	≥ 8	S	NoB
Moxifloxacin	0.5	≤ 0.5	≥ 2	> 2	≤ 1	≥ 2	R	NoB
Mupirocin	< 4	≤ 4	≥ 256	-	NA	NA	S	NA
Netilmicin	8	≤ 8	≥ 32	-	NA	NA	S	NoB
Nitrofurantoin	< 32	≤ 32	≥ 128	< 32	≤ 32	≥ 128	S	**Yes**

Oxacillin [a]	> 4	≤0,25	≥ 0,25	-	NA	NA	R	No
Penicillin	> 8	≤ 0.12	≥ 0.25	2	≤ 8	≥ 16	R	No
Quinupristin- Dalfopristin	< 0.5	≤ 1	≥ 4	> 2	≤ 1	≥ 4	R	NoB
Rifampin	< 0.5	≤ 1	≥ 4	2	≤ 1	≥ 4	I	NoB
Streptomycin High Level	-	NA	NA	< 1000	< 1000	≥ 1000	S	NoA
Teicoplanin	< 1	≤ 8	≥ 32	< 1	≤ 8	≥ 32	S	NoB
Tetracycline	< 2	≤ 4	≥ 16	> 8	≤ 4	≥ 16	R	No
Trimethoprim/Sulfamethoxazole	> 2/38	≤ 2/38	≥ 4/76	-	NA	NA	R	No
Vancomycin	2	≤ 2	≥ 16	2	≤ 4	≥ 32	S	NoB

[a] according to the new clinical breakpoints for *S. pseudintermedius* (Bemis et al 2009)

NA - not applicable; No – therapy not possible due to antimicrobial resistance; NoA – therapy not applicable due to pharmacodynamic and pharmacokinetic antimicrobial agent characteristics; NoB - critically important antimicrobial agents for humans as categorized by the Joint FAO/WHO/OIE Expert Meeting on Critically Important Antimicrobials Report (FAO/OIE/WHO, 2008).

Clinical breakpoint categorization: S, susceptible bacterial isolate; I, intermediate bacterial isolate; R, resistant bacterial isolate.

Annex IX – Paper article submitted to the *Veterinary Microbiology* Journal

Nasal Colonization of Methicillin-resistant *Staphylococcus aureus* (EMRSA-15) and Methicillin-resistant *Staphylococcus pseudintermedius* (CC71 and CC68) clones among hospitalized cats and dogs

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Abstract

Colonization and infection with methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) in companion animals is an emerging and significant problem in veterinary medicine. During one year, nasal swabs were obtained from cats and dogs admitted to the Teaching Hospital of the Faculty of Veterinary Medicine, Lisbon. Forty cats and 146 dogs were swabbed immediately. MRSA and MRSP colonization was screened by plating enrichment cultures on a selective medium, Chrom MRSA ID. Bacterial species and *mecA* were confirmed by PCR. Clonality of the isolates was assessed by pulsed-field gel electrophoresis (PFGE). All isolates were subjected to *spa* and SCC*mec* typing. They were also tested by PCR for the *lukF/lukS* genes encoding Panton-Valentine leukocidin (PVL) in *S. aureus* and Luk-I and *Staphylococcus intermedius* exfoliative toxin (SIET) in *S. pseudintermedius*. MRSA was found in two cats (5 %) and one dog (0,6 %). Isolates were *spa* type t032, SCC*mec* IV and shared identical PFGE profiles. These were similar to the EMRSA-15 human clone. Strains were co-resistant to all fluoroquinolone and were PVL-negative. Nine dogs carried MRSP (6 %), whereas none of the cats was positive. PFGE cluster B grouped European MRSP isolates (CC71-MRSP-III) (*n=8*), while the PFGE type A strain (*n=1*) showed identical characteristics as the American MRSP clone strains (CC68-MRSP-V). All isolates were SIET negative. The 8 European MRSP isolates were positive for the *luF/lukS* genes and the American MRSP isolate was negative for both genes. Strains were multidrug-resistant, which represents a major challenge for veterinarians in terms of antibiotic therapy.

Keywords: colonization, companion animals, methicillin resistance, *Staphylococcus aureus*,

Staphylococcus pseudintermedius

1. Introduction

Coagulase positive staphylococci, particularly *Staphylococcus aureus* and *Staphylococcus pseudintermedius* are major important pathogens in Veterinary Medicine, which can be isolated from various body sites of healthy animals (Hanselman et al., 2008; Griffeth et al., 2008; Weese and van Duijkeren, 2009). However, many opportunistic infections can develop, including pyoderma, otitis, wound infections, surgical site infections and urinary tract infections (Weese and van Duijkeren, 2009). The rising isolation of antibiotic resistant strains in household pets raises the concern for public health (van Duijkeren et al., 2004; Baptiste et al., 2005; Loeffler et al., 2005; O'Mahony et al., 2005; Hanselman et al., 2008; Loeffler et al., 2007; Wettstein et al., 2008; Weese and van Duijkeren, 2009; Perreten et al., 2010; Ruscher et al., 2010). Epidemiological studies have revealed the occurrence of indistinguishable clones in animals and in humans: e.g. CMRSA-5, EMRSA-15 and EMRSA-16 are major human epidemic clones that have been isolated from dogs and cats (Baptiste et al., 2005; Loeffler et al., 2005; O'Mahony et al., 2005; Hanselman et al., 2008).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major world nosocomial pathogen in humans and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has already been implicated in human infections (Van Hoovels et al., 2006; Chuang et al., 2010). Unfortunatelly, these MRSP isolates from humans were not characterized genetically and we do not know their relation to the animal clones. Furthermore MRSA and MRSP often express a multidrug-resistant pattern (van Duijkeren et al., 2004; Baptiste et al., 2005; Moodley et al., 2009; Wettstein et al., 2008; Ruscher et al., 2010) which narrows therapeutic options in already complicated infections and serve as reservoirs of antimicrobial resistance genes that can be transferred from pet animals to humans. This transmission of antimicrobial resistance might be enhanced by the fact that pet animals are in close physical contact with humans, with touching, petting and licking occurring at high frequency and animals being considered as part of the family (Guardabassi et al., 2004). Another important fact is the prescription of the same classes of antimicrobial agents in human medicine and in small animal practice, like penicillins, cephalosporins, quinolones, tetracyclines, aminoglycosides and macrolides (Guardabassi et al., 2004). All these classes of antimicrobial agents are considered critically important for both human and veterinary medicine (FAO/OIE/WHO, 2008), which raises the problem of prudent use of antimicrobials.

Data on MRSA and MRSP colonization in cats is scarce, particularly in Europe. Furthermore, data on clonality and genotypic characteristics of MRSP colonization isolates recovered from

pets is also limited. The primary objective of the study was to investigate the frequency of MRSA and MRSP colonization in cats and dogs hospitalized at the Teaching Hospital of the Faculty of Veterinary Medicine, Lisbon. The second objective was to characterize the possible isolates by molecular methods and relate them with previous described epidemic clones as well as their possible pathogenic potential (prevalence of the PVL toxin in *S. aureus* and Luk-I and SIET in *S. pseudintermedius*).

2. Material and Methods

2.1 Sampling and collection of data on antimicrobial treatment

During a one year study (from April 2008 to April 2009), nasal swabs were obtained immediately or at the most two hours after admission from cats and dogs entering the Teaching Hospital of the Faculty of Veterinary Medicine. Forty cotton swabs from cats and 146 from dogs were rolled on the nasal mucosae of both nostrils and were immediately processed at the laboratory.

2.2 Bacterial isolation and characterization

2.2.1 Strains isolation

The nasal swabs were inoculated in 3 ml of an enrichment broth consisting of 10 g/l mannitol, 65 g/l NaCl, 2,5 g/l yeast extract and 10 g/l tryptone (Vengust et al., 2006). After overnight incubation, 10 µl of bacterial suspension were inoculated in Columbia 5% blood sheep agar plates (bioMérieux, Marcy l'Etoile, France). Colonies were identified as coagulase-positive staphylococci based on colony morphology, ability to cause hemolysis, gram straining, positive catalase test, positive tube coagulase test and BBL™ Crystal™ typing system.

2.2.2 Methicillin resistance confirmation

MRSA and MRSP colonization was screened by plating 112 enrichment cultures on a selective medium, Chrom MRSA ID (bioMérieux) and suspected colonies were confirmed by PCR to have the *mecA* gene (http://www.crl-ar.eu).

2.2.3 Species confirmation

Presumptive *S. aureus* and *S. pseudintermedius* isolates were confirmed by PCR amplification of the *nuc* gene (http://www.crl-ar.eu; Sasaki et al., 2010) and discrimination between *S. pseudintermedius* and *S. intermedius* was further investigated by restriction fragment length polymorphism (RFLP) analysis of the *pta* gene (Bannoehr et al., 2009).

2.2.4 PVL, Luk-I and SIET detection

All MRSA isolates were tested by PCR for the presence of *lukF/lukS* genes encoding PVL using the primers described by Lina et al., 1999. All MRSP isolates were tested by PCR for the presence of *lukF/lukS* genes encoding Luk-I using the primers described by Futagawa-Saito et al. (2004) and the detection of SIET was carried out using primers described by Lautz and colleagues in 2006.

2.3 spa Typing

For MRSA, the primers used for *spa* typing were those recommend by the European UnionReference Laboratory of Antimicrobial Resistance (http://www.crl-ar.eu), and *spa* types were assigned through the Ridom web server (http://www.ridom .de/spaserver/).

For MRSP, *spa* typing was performed according to Moodley et al. (2009) and the sequences were interpreted manually according to the criteria proposed by the same author.

2.4 Staphylococcal Cassette Chromosome mec (SCCmec) Typing

SCC*mec* types were determined using the multiplex PCR 1 and the multiplex PCR 2 accordingly to Kondo and collaborators (2007). The molecular weight of each PCR amplicon was determined and assigned to a particular allele type in each strain. The combination of the type *ccr* and *mec* complex was used to consign SCC*mec* types.

2.5 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by the disk diffusion method. The results were registered after incubation at 37˚ C for 18h, measuring the diameter of the zone of inhibition, if present, for each antibiotic used. Inhibition zone diameters were interpreted according to the Clinical Laboratories Standards Institute clinical breakpoints for animals (CLSI, 2008) and, when breakpoints were unavailable for bacteria of animal origin, according to the human CLSI breakpoints (CLSI, 2009). For fusidic acid and mupirocin, inhibition zone diameters were interpreted using breakpoints proposed by Toma and Barriault (1995) and Fuchs et al. (1999), respectively. Susceptibility test breakpoints for oxacillin of *S. pseudintermedius* were those described by Bemis et al. (2009). For MRSA and MRSP, the following antimicrobial discs (Oxoid, Hampshire, United Kingdom) were used: amikacin (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), enrofloxacin (5 μg), erythromycin (15 μg), fusidic acid (10 μg) gentamicin (10 μg), kanamycin (30 μg), levofloxacin (5 μg), linezolid (30 μg), marbofloxacin (5 μg), moxifloxacin (5 μg), mupirocin (5 μg), nitrofurantoin (300 μg), norfloxacin (10 μg), ofloxacin

(5 μg), oxacillin (1 μg), penicillin G (10 units), quinupristin/dalfopristin (15 μg), rifampicin (5 μg), tetracycline (30 μg), trimethoprim (5 μg), and vancomycin (30 μg). MRSA was also tested for cefoxitin (30 μg) susceptibility, since cefoxitin has been recently used to replace oxacillin in the phenotypic identification of MRSA isolated from humans (CLSI, 2009).

2.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed as described by Murchan et al. (2003) with minor modifications. Briefly, bacterial cultures were grown overnight in brain heart infusion broth (BD Difco, New Jersey, USA) and were incorporated into 2 % (w/v) agarose discs, instead of plugs. After four hours lysis with lysostaphin (5 mg/ml), lysozyme (100 mg/ml) and RNase (32,5 mg/ml) at 37º C, the discs were incubated with proteinase K (20 mg/ml) overnight at 56º C and one disc was digested with *Sma*I (20 U/ml) overnight at room temperature. PFGE was performed by clamped homogeneous electric field (CHEF) electrophoresis with a CHEF-DR III System (Bio-Rad Laboratories, San Diego, USA) in a 1,1 % (w/v) agarose gel with a pulsing switch time of 2-5 s at 5,6 V/cm for 24 h for MRSP and pulsing switch time of 5-15 s at 6 V/cm for 10 h followed by pulsing switch time of 15-60 s at 6 V/cm for 13 h for MRSA. EMRSA-15 and American MRSP clones, previously isolated in Portugal (Pomba et al., 2009; Pomba et al., 2010), were used as reference 176 strains and included in the gel. The gels were inspected visually and band patterns were interpreted according to the criteria proposed by Tenover et al. (1995).

3. Results

Twelve methicillin-resistant staphylococci were isolated from the nose of ten dogs and two cats out of a total of 186 animals screened. One hundred forty six animals were dogs (78 %) and only forty were cats (22 %). Males were the predominant gender sampled (49 %), followed by females (31 %), females castrated (14 %) and males castrated (6 %). One hundred seventeen animals (63 %) had received antimicrobial treatment within the month prior to sampling and forty nine animals (26 %) had received antimicrobial treatment within the year before sampling. Many classes of antimicrobials were prescribed, but the two classes more often selected were β-lactams and fluoroquinolones. The three animals carrying a MRSA isolate had received antimicrobial treatment the month before the nasal swab collection and one animal in the year before too (see Table 1). Six animals colonized with MRSP had received antimicrobial treatment one month previous to sampling and four one year before (see Table 1).

MRSP strains were identified in 9 dogs, with a frequency of 6 %. No MRSP were isolated from cats. Two pattern of resistance were seen: FMV 1 strain demonstrated resistance to oxacillin, gentamicin, kanamycin, ciprofloxacin, enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin, ofloxacin, erythromycin and trimethoprim (see Table 2); the other 8 strains, FMV 2 to 9, demonstrated resistance to oxacillin, gentamicin, kanamycin, ciprofloxacin, enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin, ofloxacin, clindamycin, erythromycin, tetracycline and trimethoprim (see Table 2).One of the isolates, FMV 1, was *spa* type t06 belonging to the clonal complex (CC) 68 and the other eight, FMV 2 to 9, were t02 and consequently were CC71. According to Kondo and colleagues criteria (2007), isolate FMV 1 harbored a SCC*mec* type V (class C *mec* complex and *ccrC* recombinase) and the other 8 isolates, FMV 2 to 9, harbored a variant of the SCC*mec* type III (class A *mec* complex and *ccrAB3* recombinase). All isolates were typeable with *Sma*I-PFGE, resulting in the detection of two PFGE clusters (A and B). The type B was subdivided into 3 subtypes (B1 to B3) (Table 3). The PFGE typing results were in agreement with those obtained by *spa* and SCC*mec* typing. PFGE cluster B grouped European MRSP isolates (CC71-MRSP-III), while PFGE type A strain showed identical characteristics as the American MRSP clone (ST68-MRSP-V) (see Table 3). All isolates were SIET-negative. The 8 European MRSP isolates were positive for the *luF/lukS* genes and the 208 American MRSP isolate was negative for both genes.

MRSA was found in two cats (5 %) and one dog (0.6%). Therefore, MRSA were the only staphylococci isolated from cats. The same pattern of resistance was seen for the three isolates (FMV 10, 11 and 12): oxacillin, ciprofloxacin, enrofloxacin, levofloxacin, marbofloxacin, moxifloxacin, norfloxacin and ofloxacin (see Table 2). Tandem repeat sequence analysis of the *spa* gene concluded that all isolates were t032 and thus belonged to the CC22. All isolates harbored SCC*mec* type IV (class B *mec* complex and *ccrAB2* recombinase). A close relation to the European disseminated clone EMRSA-15 (CC22/ST22-MRSA-IV) was present: PFGE analysis revealed the same PFGE type (C) among the isolates, including with the previously described EMRSA-15 strain (ST22-MRSA-IV) causing skin infection in a dog and the attending veterinarian in Portugal (Pomba et al., 2009) (see Table 3). All isolates were PVL negative.

4. Discussion

The incidence of MRSA in Human Portuguese Hospitals remains one of the highest in Europe, with dominance of the EMRSA-15 clone (ST22-MRSA-IV) (Aires-de-Sousa et al., 2008). Thus, it is not surprising that the MRSA colonization strains isolated from our

companion animals belong to this epidemic clone. Furthermore, MRSA ST22-SCC*mec* IV had been previously isolated from a skin infection in a dog and attending veterinarian in Portugal (Pomba et al., 2009). All isolates showed the same resistance profile as the epidemic EMRSA-15 (resistance to methicillin and ciprofloxacin). The origin of EMRSA-15 isolates in small animals remains unknown but several authors suggest that MRSA isolated from companion animals have a human source (Loeffler et al., 2005). As in our study, Loeffler and colleagues (2005) demonstrated the occurrence of EMRSA-15 in dogs in the United Kingdom, and pointed the epidemiological impact since it proves that this nosocomial pathogen is not restricted to hospitals and can be carried and disseminated by healthy humans and animals in the community. Little information is available to date on the risk of antimicrobial usage with regard to MRSA colonization in small animals (Catry et al., 2010). According to previous case reports, many animals infected or colonized with MRSA have been treated with antimicrobials prior to the diagnosis (Catry et al., 2010). Interestingly, our 3 animals colonized with MRSA received antimicrobial treatment with amoxicillin-clavulanic acid one month prior to the nasal swab collection. Nevertheless, our sample is too small to prove antimicrobial administration was a risk factor and 63 % of non-colonized animals were also exposed to antimicrobial administration, including amoxicillin-240 clavulanic acid, without development of MRSA colonization.

In 2009, the first MRSP infection was identified in Portugal. The isolate was the co-source (together with *Enterococcus faecalis*) of a lower urinary tract infection in a cat and after molecular characterization was found to be associated with the American MRSP clone (ST68-MRSP-V) (Pomba et al., 2010). To that date, this clone had been exclusively reported in the United States of America and Canada. Furthermore, to the best of our knowledge we detected for the first time a case of colonization with the American MRSP clone (ST68-MRSP-V) in Europe. The isolation of a second strain belonging to this clonal complex raises the question if it is already spreading through the European continent, too. More studies should be conducted to identify the real prevalence of this clone in Portugal and other European countries.

The European MRSP clone (ST71-MRSP-III) has been implicated as responsible for infections in companion animals in Germany, Sweden and Switzerland (Wettstein et al., 2008; Moodley et al., 2009) and more recently was isolated also from clinical samples throughout Europe (Perreten et al., 2010; Ruscher et al., 2010). Interestingly, European MRSP isolates show a multidrug-resistant pattern (resistance to more than 4 antimicrobial classes), with resistance to some aminoglycosides, quinolones, lincosamides, macrolides, tetracyclines, and folate pathway inhibitors (trimethoprim) (Loeffler et al., 2007; Wettstein et al., 2008; Ruscher et al., 2009; Perreten et al., 2010; Ruscher et al., 2010) and therefore they should be

referred as multidrug-resistant *S. pseudintermedius* (MDRSP). The isolates in our study show exactly the same multidrug-resistant pattern. Surprisingly, our isolates show resistance not only to second and third generation quinolones, but also to a fourth generation fluoroquinolone, moxifloxacin, which has enhanced activity against gram-positive cocci (Ince et al., 2003). This is an interesting fact since moxifloxacin is not licensed for veterinary use and prescription in Human Medicine is licensed only for the treatment of respiratory and skin infections. Intorre et al. (2007) found moxifloxacin-bordeline-intermediate-resistant *S. intermedius* strains according to CLSI breakpoints and in 2009 resistance to moxifloxacin was described in 45 clinical strains out of 46 MRSP strains (Ruscher et al., 2009). Intorre and co-workers (2007) found that resistance to the older fluoroquinolones was correlated with a double mutation in the quinolone-resistance-determining regions (QRDR) within the genes *gyrA* (subunit of DNA gyrase) and *grlA* (subunit of topoisomerase IV) and speculated that an additional alteration in *grlA* was necessary for fourth generation fluoroquinolone's resistance. However the true mechanism of moxifloxacin-resistance in *S. pseudintermedius* remains unknown and further studies are required. This development of resistance to moxifloxacin and the observation of a multidrug-resistant pattern along European and American MRSP strains can indicate that these strains have a high capacity of mutation or can easily acquire mobile elements, like transposons or plasmids, encoding resistance genes.

Wettstein and colleagues (2008) also found resistance to chloramphenicol in their European MRSP strains, but our isolates do not demonstrate chloramphenicol resistance. This multidrug resistance pattern represents a major challenge for veterinarians in terms of antibiotic therapy. Most of the drugs to which these bacteria remain susceptible are used off-label in small animal practice (like amikacin, chloramphenicol and nitrofurantoin), others are used for human decolonization (fusidic acid and mupirocin) and others are last-resort drugs in human medicine (linezolid, quinupristin/dalfopristin, rifampicin and vancomycin). Moreover, linezolid, quinupristin/dalfopristin and vancomycin are considered antimicrobial limited for therapy of multidrug-resistant *S. aureus* infections by the joint Report of the FAO/WHO/OIE Expert Meeting (FAO/OIE/WHO, 2008). Thus, this represents a serious ethical dilemma, even when applying the "cascade principle". Since there are no guidelines for treatment of infections with these MDRSP, it is up to every veterinarian to decide which antimicrobial to use. Most infections with MDRSP are skin and wound infections, otitis externa, respiratory infections and urinary tract infections (UTIs). Biocides, like chlorhexidine, triclosan, Tris-EDTA and benzoyl peroxide, have been recommended for topical use in superficial skin infections and otitis externa. This approach is very successful and does not seem to select for antimicrobial resistance (Guardabassi et al., 2009). However, care should be taken in order to

also avoid development of resistance to biocides. Treatment of respiratory infections and UTIs with these MDRSP will be far more complicated to solve and precautions should be taken when choosing the antimicrobial therapy.

The presence of Luk-I and SIET in MRSP CC71 has been previously described by Ruscher and colleagues in 2010. Our strains were SIET-negative but Luk-I-positive. Luk-I shows a strong toxicity on various polymorphonuclear cells, including human leukocytes, but only a slight hemolytic activity on rabbit erythrocytes (Futagawa-Saito et al., 2004). Because phagocytosis by polymorphonuclear cells is an important defense mechanism of the host, Luk-I may play a primary role in bacterial evasion to the host defense mechanisms, enhancing the opportunity of the cocci to colonize and set up an infection. Nevertheless the exact role by which Luk-I enables staphylococci colonization and infection is not completely understood and so its true meaning may be underestimated.

The different colonization rates between dogs and cats of the two staphylococci species can indicate that the relative risk of MRSA and MRSP colonization might vary between the two host species, with MRSP being more frequent among dogs and MRSA among cats. The high rate of colonization of MRSP by dogs is likely to be due to host specificity, given that *S. pseudintermedius* is the pathogen more frequently isolated from dogs (Sasaki et al., 2010). However, the same is not true for cats and the higher rate of MRSA colonization is not fully understood. Weese and van Duijkeren (2009) referred that MRSA colonization might be transient in dogs and cats perhaps because *S. aureus* is not naturally a predominant commensal in these species. With *S. pseudintermedius* being the most prevailing commensal at least in dogs the duration of colonization is probably longer. Yet further studies on the duration of colonization are needed to evaluate this.

Routine decolonization therapy is not recommended in human or animals that have mucosae colonized with MRSA (Catry et al., 2010). Nevertheless decolonization with antimicrobial therapy may be measured in individual MRSA colonized animals as an option to control transmission of MRSA between animals or from animals to humans (Catry et al., 2010). Animal confinement may also be considered. Still no antimicrobial drugs for veterinary use have been effectively studied and approved for local or systemic application intended to resolve MRSA carrier status. For MRSP decolonization there are no recommendations for therapy of colonized animals and not even if decolonization should be performed.

5. Conclusion

In conclusion, even though the frequency of MRSA colonization was low, carriage of MRSA by companion animals in the community is a serious public health problem and adequate measures are necessary to prevent MRSA from becoming endemic in the animal population.

The frequency of isolation of MRSP in our study was high (6 %), considering that MRSP was first described in Europe in 2006 and in Portugal in 2007. This carriage rate suggests that these bacteria are quickly spreading through the canine population through all Europe, including Portugal. How this dissemination has occurred is not completely understood (Perreten et al., 2010; Ruscher et al., 2010), however colonization of healthy animals can be an important way of transferring the pathogen from animal to animal.

Although isolation of MRSP is infrequent in humans, transmission of strains between animals and humans has occurred in the past (Weese and van Duijkeren, 2009; Chuang et al., 2010). More important, the MDRSP strains can serve as reservoirs of antimicrobial resistance genes that can, possibly, be transferred to other bacterial species like *S. aureus*, since the location of many of these resistance genes is on mobile elements. Subsequent clonal spread of such a new MRSA clone might be a threat for human health in the future. The same may be applied for virulent toxins encoding genes among MRSP CC71 strains, which can play an important role in the dissemination of this epidemic clone. Recommendations for treatment of MDRSP infections and decolonization are urgently needed and new treatments without the use of antimicrobials are required. More importantly, monitoring of MRSA and MRSP in companion animals should be promoted in veterinary surveillance programmes on antimicrobial resistance.

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Table 1 – Species, gender, age and antimicrobial consumption of the companion animals colonized with MRSA and MRSP.

Case	Species	Gender	Age	Motive for admission	Antimicrobial Treatment (last month)	Antimicrobial Treatment (last year)
FMV 1	Canine	Female	Unknown	Surgery	Spiramycin/Metronidazole	Spiramycin/Metronidazole
FMV 2	Canine	Female	Unknown	Cushing's disease, thromboembolism	No	No
FMV 3	Canine	Male	Unknown	Urinary obstruction	Unknown	Unknown
FMV 4	Canine	Male	Unknown	Urinary tract infection	Unknown	Unknown
FMV 5	Canine	Female	3 years	Urinary tract infection, gastritis	Amoxicillin/Clavulanic Acid	Doxycycline
FMV 6	Canine	Female	3 months	Canine parvovirus suspicion	Amoxicillin/Clavulanic Acid and Metronidazole	Unknown
FMV 7	Canine	Female castrated	11 years	Intestinal obstruction	No	Amoxicillin/Clavulanic Acid
FMV 8	Canine	Female castrated	2 years	Surgery (castration)	Amoxicillin/Clavulanic Acid	Amoxicillin/Clavulanic Acid
FMV 9	Canine	Male	8 years	Surgery	Enrofloxacin, Cefoxitin and Metronidazole	Unknown
FMV 10	Feline	Male	Unknown	Multiple fractures	Amoxicillin/Clavulanic Acid	No
FMV 11	Feline	Female	11 years	Hypothermia, anorexia	Amoxicillin/Clavulanic Acid	Unknown
FMV 12	Canine	Male	2 years	Surgery (castration)	Amoxicillin/Clavulanic Acid	Unknown

Table 2 – Susceptibility testing by disk diffusion of 27 antimicrobial agents of methicillin-resistant staphylococci strains.

Antibiotics	Disk Content	Clinical Breakpoints Criteria (Zone diameter in mm)				MRSP Strains									MRSA Strains		
		S	I	F	R	FMV 1	FMV 2	FMV 3	FMV 4	FMV 5	FMV 6	FMV 7	FMV 8	FMV 9	FMV 10	FMV 11	FMV 12
Amikacin[a]	30 µg	≥ 17	15-16	-	≤ 14	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin[a]	10 µg	≥ 15	13-14	-	≤ 12	R	R	R	R	R	R	R	R	R	S	S	S
Kanamycin[a]	30 µg	≥ 18	14-17	-	≤ 13	R	R	R	R	R	R	R	R	R	S	S	S
Rifampicin[a]	5 µg	≥ 20	17-19	-	≤ 16	S	S	S	S	S	S	S	S	S	S	S	S
Cephalothin[a]	30 µg	≥ 18	15-17	-	≤ 14	S	R	R	R	R	R	R	R	R	S	S	S
Cefoxitin[b]	30 µg	≥ 20	-	-	≤ 19	S	R	R	R	S	R	R	S	R	S	S	S
Oxacillin[a,c]	1 µg	≥ 13	11-12	-	≤ 10	NA	NA	NA	NA	NA	NA	NA	NA	NA	R	R	R
Oxacillin[d,e]	1 µg	≥ 20	-	-	< 20	R	R	R	R	R	R	R	R	R	NA	NA	NA
Penicillin G[a]	10 U	≥ 29	-	-	≤ 28	R	R	R	R	R	R	R	R	R	R	R	R
Ciprofloxacin[b]	5 µg	≥ 21	16-20	-	≤ 15	R	R	R	R	R	R	R	R	R	R	R	R
Enrofloxacin[a]	5 µg	≥ 23	-	17-22	≤ 16	R	R	R	R	R	R	R	R	R	R	R	R
Levofloxacin[b]	5 µg	≥ 19	16-18	-	≤ 15	R	R	R	R	R	R	R	R	R	R	R	R
Marbofloxacin[a]	5 µg	≥ 20	15-19	-	≤ 14	R	R	R	R	R	R	R	R	R	R	R	R
Moxifloxacin[b]	5 µg	≥ 24	21-23	-	≤ 20	R	R	R	R	R	R	R	R	R	R	R	R
Norfloxacin[b]	10 µg	≥ 17	13-16	-	≤ 12	R	R	R	R	R	R	R	R	R	R	R	R
Ofloxacin[b]	5 µg	≥ 18	15-17	-	≤ 14	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin[a]	30 µg	≥ 15	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin[a]	2 µg	≥ 21	15-20	-	≤ 14	I	R	R	R	R	R	R	R	R	S	S	S
Erythromycin[a]	15 µg	≥ 23	14-22	-	≤ 13	R	R	R	R	R	R	R	R	R	S	S	S
Linezolid[b]	30 µg	≥ 21	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol[a]	30 µg	≥ 18	13-17	-	≤ 12	S	S	S	S	S	S	S	S	S	S	S	S
Quinupristin/Dalfopristin[b]	15 µg	≥ 19	16-18	-	≤ 15	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline[a]	30 µg	≥ 19	15-18	-	≤ 14	S	R	R	R	R	R	R	R	R	S	S	S
Trimethoprim[b]	5 µg	≥ 16	11-15	-	≤ 10	R	R	R	R	R	R	R	R	R	S	S	S
Nitrofurantoin[b]	300 µg	≥ 17	15-16	-	≤ 14	S	S	S	S	S	S	S	S	S	S	S	S
Fusidic Acid[f]	10 µg	≥ 21	20	-	≤ 19	S	S	S	S	S	S	S	S	S	S	S	S
Mupirocin[g]	5 µg	≥ 14	-	-	≤ 13	S	S	S	S	S	S	S	S	S	S	S	S

Abbreviations: NA, not applicable; R, resistant; S, susceptible.

Inhibition zone diameters were interpreted according to:

(a) CLSI M31-A3

[b] CLSI M100-S17 (M2)

[c] Bemis et al., 2009

[d] Criteria only for MRSA

[e] Criteria only for MRSP

[f] Toma and Barriault 1995

[g] Fuchs et al., 1990

Table 3 - Origin, genotypic characteristics and antimicrobial resistant patterns of *mecA*-positive *S. aureus* and *S. pseudintermedius* strains isolated.

Strains	Origin	Antimicrobial associated co-resistance patterns	SCC*mec* element	*spa* type	Tandem Repeat Sequence	CC	PFGE Subtype
FMV 1	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, E^R, W^R	V	t06	r01r02 r03 r03 r06 r05	68	A
FMV 2	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 3	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B2
FMV 4	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 5	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 6	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B3
FMV 7	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 8	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 9	Canine	Cn^R, K^R, Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R, Da^R, E^R, Te^R, W^R	III	t02	r01r02 r03 r03 r03 r06 r05	71	B1
FMV 10	Feline	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C
FMV 11	Feline	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C
FMV 12	Canine	Cip^R, Enr^R, Lev^R, Mar^R, Mxf^R, Nor^R, Ofx^R	IV	t032	r26 r23 r23 r13 r23 r31 r29 r17 r31 r29 r17 r25 r17 r25 r16 r28	22	C

Abbreviations: Cn, gentamicin; K, kanamycin; Cip, ciprofloxacin; Enr, enrofloxacin; Lev, levofloxacin; Mar, marbofloxacin; Mxf, moxifloxacin; Nor, norfloxacin; Ofx, ofloxacin; Da, clindamycin; E, erythromycin; Te, tetracycline; W, trimethoprim; CC, clonal complex.

Annex X – Inquiry considering characteristics of the sampled animals

Nome do Animal [Animal Name]					Idade [Age]	
Nome do Dono [Owner Name]					Gato [Cat]	Cão [Dog]
Sexo do Animal [Gender]	M	F	MC	FC		
Vacinado? [Vaccinated?]	Sim [Yes]	Não [No]				
Desparasitado? [Dewormed?]	Sim [Yes]	Não [No]				
Vive com outros animais? [Living with other animals?]	Sim [Yes]	Não [No]		Cão [Dog]	Gato [Cat]	
Tem acesso à rua? [Indoor/outdoor animal?]	Sim [Yes]	Não [No]				
Afecções anteriores [Previous diseases]	Sim [Yes]	Não [No]	Qual? [Which?]			
Fez no último ano algum antibiótico? [Antimicrobial therapy in the last year?]	Sim [Yes]	Não [No]	Qual? [Which?]			
Fez no último mês algum antibiótico? [Antimicrobial therapy in the last month?]	Sim [Yes]	Não [No]	Qual? [Which?]			
Já realizou alguma cirurgia? [Any previous surgery?]	Sim [Yes]	Não [No]	Qual? [Which?]			
Última vez que esteve no Médico Veterinário? [Last visit to the Veterinary]						
Internamentos Anteriores? [Previous admissions?]	Sim [Yes]	Não [No]	Quando? [When?]			
Já esteve em algum canil? [Previously in an animal shelter?]	Sim [Yes]	Não [No]	Quando? [When?]		Onde? [Where?]	
Motivo do Internamento [Motive for admission]						