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Prevalence of methicillin resistance in *Staphylococcus pseudintermedius* isolates from dogs with skin and ear infections in South Africa

By

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Dedication

This dissertation is dedicated to those who have supported me during this thesis. My parents, Lisa and Martin Prior, who have been the pillars of support throughout my long years of study. I would not be standing where I am today without your direction and encouragement to fulfil my dreams, "you lose everything when you don't love yourself".

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This dissertation is in honour of all of you.

Declaration of Originality/Plagiarism

By submitting this dissertation for the degree MSc (Veterinary Science) at the University of Pretoria, I declare that the entirety of the work contained therein is my own original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Pretoria University will not infringe any third party rights, and that I have not previously in its entirety or in part submitted it for obtaining any qualification at this or any other tertiary institution. Declaration of originality attached as appendix (**chapter 8**).

Ethical and Research Clearance

The author, whose name appears on the title page of this dissertation, has obtained, for research described in this work, the applicable research ethics approval. The author declares that he has observed the ethical standards required in terms of the University of Pretoria's *Code of Ethics for Researchers* and the *Policy guidelines for responsible research*. Ethical clearance certificate attached as appendix (**chapter 8**).

The author declares that the relevant Department of Agriculture, Forestry and Fisheries approval was obtained for permission to do research in terms of section 20 of the Animal Diseases Act, 1984 (Act no.35 of 1984)

Details of Presentations

Portions of the work included in this dissertation have been presented at the following events:

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List of Abbreviations

ADD	**A**gar **D**isc **D**issemination Process
AST	**A**ntimicrobial **S**usceptibility **T**esting
ATCC	**A**merican **T**ype **C**ulture **C**ollection
BSAVA	**B**ritish **S**mall **A**nimal **V**eterinary **A**ssociation
BTA	**B**lood **T**ryptose **A**gar
CDC	**C**entre for **D**isease **C**ontrol
CI	**C**onfidence **I**nterval
CLSI	**C**linical and **L**aboratory **S**tandards **I**nstitute
CoPS	**Co**agulase-**P**ositive**s**
CoNS	**Co**agulase-**n**egative **S**taphylococci
EOE	**E**rythematous **O**titis **E**xterna
EUCAST	**E**uropean **C**ommittee on **A**ntimicrobial **S**usceptibility **T**esting
HA-MRSA	**H**ospital-**a**cquired **m**ethicillin-**r**esistant ***S****taphylococcus* ***a****ureus*
Icli	**I**nducible **Cl**indamycin **R**esistance
INT	**I**ntegrons
IS	**I**nsertion **S**equence
IWG-SCC	**I**nternational **W**orking **G**roup on **C**lassification Of **S**taphylococcal **C**assette **C**hromosome **E**lements
Mcc	**MacC**onkey Medium
MDR	**M**ulti**d**rug **R**esistance
MDRSP	**M**ulti-**D**rug-**Resistant** ***S****. **p**seudintermedius*
MIC	**M**inimum **I**nhibitory **C**oncentration
MLEE	**M**ulti**l**ocus **e**nzyme **e**lectrophoresis
mPCR	**M**ultiplex **PCR**
MrCoNS	**M**ethicillin-**R**esistant **C**oagulase-**N**egative **S**taphylococcus
MRI	**M**agnetic **R**esonance **I**maging

MRS	**M**ethicillin-**R**esistant **S**taphylococci
MRSA	**M**ethicillin **R**esistant ***S****. **a**ureus*
MRSP	**M**ethicillin-**r**esistant ***S****taphylococcus **p**seudintermedius*
MSSA	**M**ethicillin-**s**usceptible ***S****taphylococcus **a**ureus*
MSSP	**M**ethicillin-**s**usceptible ***S****taphylococcus **p**seudintermedius*
OR	**O**dds **R**atio
OE	**O**titis **E**xterna
OI	**O**titis **I**nterna
OM	**O**titis **M**edia
PBP2a	**P**enicillin-**b**inding **p**rotein 2a
PFGE	**P**ulsed-**f**ield **g**el **e**lectrophoresis
SCC*mec*	**S**taphylococcal **C**hromosome **C**assette ***Mec***
SIG	**S**taphylococcus **i**ntermedius **g**roup
TCP	**T**issue **C**ulture **P**late

Glossary

Antibiotic	A drug used to treat bacterial infections.
Antimicrobial	A group of drugs that are used in the treatment of bacterial infection.
Dermatitis	A general term that describes a skin irritation.
Minimum Inhibitory Concentration (MIC)	The lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.
Otitis	Inflammation of the ear, usually distinguished as *otitis externa* (outer ear), *otitis media* (middle ear), and *otitis interna* (of the inner ear).
Resistance	Ineffectiveness of a particular antimicrobial to a pathogen.
Staphylococcus pseudintermedius	A bacterium of the genus Staphylococcus that is primarily found on domestic animals.
Susceptibility	A description of a particular group or subset of antimicrobials that are effective against bacteria.
Virulence	A pathogen's ability to infect a resistant host.

Abstract

Staphylococcus pseudintermedius is an important opportunistic commensal bacterium, often correlated with dermatitis and otitis in small animals. The emergence and rapid expansion of methicillin resistance giving rise to methicillin-resistant *S. pseudintermedius* (MRSP) is of concern as it is often correlated with multi-drug resistance, thereby reducing therapeutic options for these common veterinary conditions.

The study aims to 1) evaluate standard laboratory methods used at five regional veterinary laboratories in South Africa to identify *S. pseudintermedius*; 2) determine if an association exists between resistance to first and second tier antibiotics and the presence of the *mecA* gene; and 3) determine if there is an association between MRSPcarriage and previous antibiotic usage.

Sixty-eight presumptive MRSP clinical samples from five geographically dispersed laboratories in Republic of South Africa (RSA) were collected over a 24-month period. *S. pseudintermedius* was detected by a standard laboratory method and antimicrobial susceptibility testing done by means of disc diffusion. Presumptive MRSP isolates were identified when disc diffusion showed resistance to oxacillin. PCR confirmed MRSA clinical isolates by the presence of *mecA*.

Fifty-seven samples were confirmed to harbour *S. pseudintermedius* (83.8%) and 49 (85%) of those were further identified to carry *mecA*. Of the 49 *mecA* positive PCR isolates, 28 were isolated from pruritic patients (28/49, 57%) and 7/49 from otitis (14%).This study provides evidence that there is a high prevalence of *mecA* positive carriage (85% of samples) in methicillin resistant SP pyoderma and otitis in dogs in South Africa. Important risk factors for *mecA* positive carriage are previous hospital admission, pruritis and previous antibacterial failure.

Thus, the data suggests that there is an urgent need for better surveillance of dogs presenting with pyoderma and otitis in South Africa. Moreover, diligent antibiotic stewardship will be crucial to prevent a deterioration of this situation in the country.

Chapter 1: General Introduction

S. pseudintermedius is an opportunistic pathogen found in domestic animals and capable of causing skin infections (WEESE and VAN DUIJKEREN, 2010). The implications for treating staphylococcal skin infections in dogs have become increasingly burdensome with a significant rise in the prevalence of methicillin resistant *S. pseudintermedius* (MRSP) antibacterial strains. In order to expand our knowledge of antibiotic resistance, many research efforts are underway globally to understand the prevalence of resistance amongst *S. pseudintermedius* isolates.

The most common form of methicillin resistance is conferred by the penicillin-binding protein 2a (PBP2a) encoded within the mobile genetic element by the gene *mecA*. Detection of *mecA* via PCR is the gold standard for the diagnosis of methicillin resistance *S. pseudintermedius* ioslates (Schissler *et al.*, 2009). No information is available on the prevalence of *mecA* in companion animals on the African continent.

Chapter 2: Literature Review

2.1. The Genus *Staphylococcus*

2.1.1. Taxonomy

Staphylococcus is classified as a Gram-positive bacteria from the Lactobacillales order in the family *Staphylococcaceae* (Bergey *et al.,* 1984). The term “Staphylococcus” comes from the Greek words “staphyle”, meaning grape bundle, and “kokkos”, meaning berry, which is representative of its morphology under the microscope as grapelike clusters or berries. The *Staphylococcus* genus includes 41 species and 24 subspecies (Bergey *et al.,* 2010). The classification of *Staphylococcus* is based on pigment production, pathogenicity and coagulase production. Interestingly, *Staphylococcus intermedius* from canine origin was first described in 1976 and renamed in 2005 as *S. pseudintermedius* **(figure 1)** (Devriese *et al.,* 2005)

2.1.2. Staphylococci of veterinary interest

Common staphylococcal bacteria include *S. aureus, S. schleiferi, S. epidermidis, S. hyicus, S. sciuri, S. simulans, S. chromogenes, S. pseudintermedius* and *S. delphini.* Important staphylococcal pathogens in livestock responsible for abscesses, mastitis and pyoderma include *S. aureus, S. epidermidis* and *S. pseudintermedius* (Oliver, 1984). *S. hyicus* is commonly reported in pigs for exudative epidermitis and polyarthritis (Wegener, 1994) *S. delphini* has been reported from skin lesions in pigeons and dolphins. *S. pseudintermedius* is identified as the most common pathogen of bacteria isolated from ear and canine skin infections (Griffeth *et al.*, 2008).

2.1.2.1. Staphylococcus intermedius and *Staphylococcus pseudintermedius*

Previously known as *S. intermedius,* which was first described in 1976, the bacterium was reclassified as *S. pseudintermedius* in 2005, and is an affiliate of the *S. intermedius* group (SIG). In 2007, members of the SIG were reclassified and consist of S*. pseudintermedius, S. intermedius* and *S. delphini* (Sasaki *et al.*, 2007). The basis of this reclassification was a study of rDNA-gene sequences of phenotypically similar stains from different animal species **(Figure 1).**

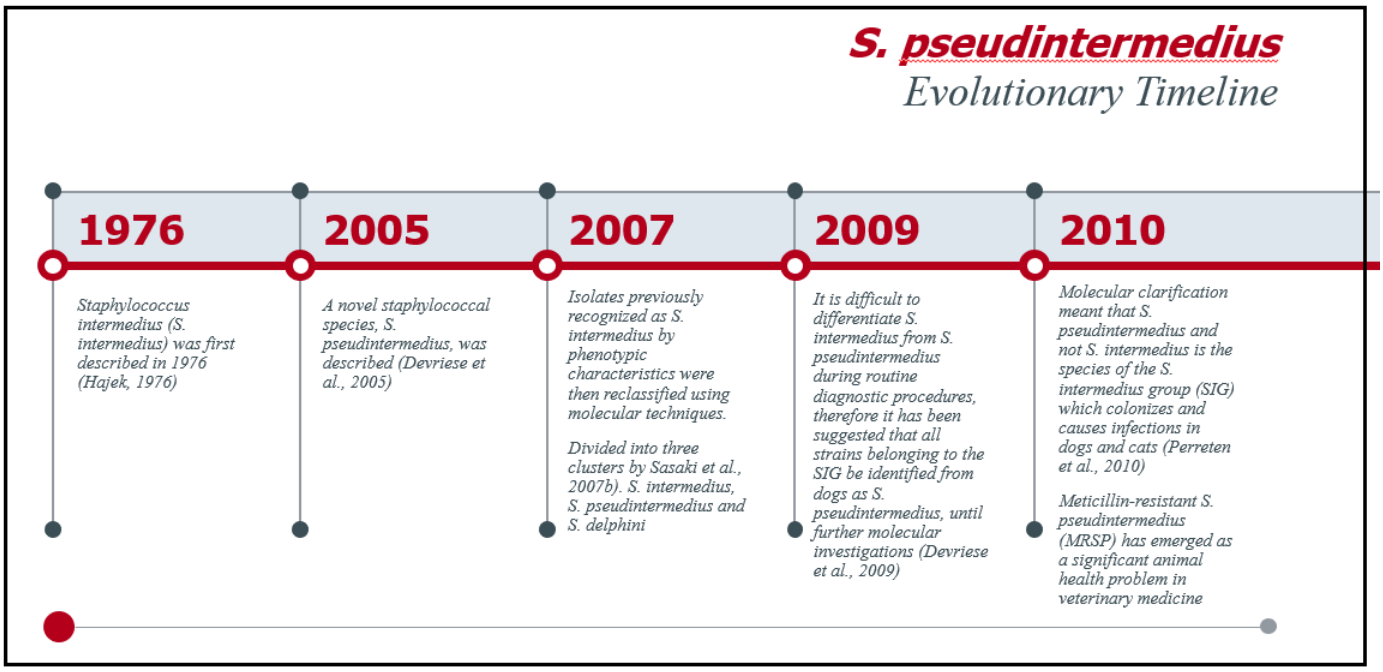

Figure 1. The timeline of the classification and reclassification of the *Staphylococcus pseudintermedius* group (Perreten *et al.*, 2010) from 1976 to 2010 through the advancement of molecular techniques.

Sequence analysis of different housekeeping genes namely *sod*A and *hsp*60 gene regions (Bannoehr *et al.*, 2009; Sasaki *et al.*, 2010). In isolates formerly recognized as *S. intermedius* from dogs led to the bacterium being renamed as *S. pseudintermedius*. The *hsp60* and *sodA* genes sequences established that the previously identified *S. intermedius* consisted of three genes known to be the SIG, which ultimately differentiated *S. intermedius*, *S. pseudintermedius* and *S. delphini*. This resulted in the presumptive identification of canine microbiological isolates fitting into the SIG cluster, to be *S. intermedius*, *S. pseudintermedius* and *S. delphini.* Two different research papers by Sasaki et al. and Bannoehr et al. verified this reclassification independently by gene sequencing of canine isolates (Sasaki *et al.*, 2010; Bannoehr *et al.*, 2009). As a result of these new findings, it has been recommended that all of *S. intermedius* isolates originating from pyoderma should be classified as *S. pseudintermedius* until gene sequencing was done.

S. pseudintermedius is a common resident organism on the dermis and mucosa of dogs (Griffeth *et al.*, 2008). Changes in the microenvironment on the surface of the skin can disrupt the equilibrium of the cutaneous ecosystem, allowing *S. pseudintermedius* to proliferate and become pathogenic. As such, pyoderma is usually a secondary disease. This disruption is most commonly

associated with allergic dermatitis (Schroeder, 2010). The most common pathogen in pyoderma is the dog-specific coagulase-positive *S. pseudintermedius* (Bryan *et al.*, 2012; Scott DW 2001). Occasionally other important pathogens are described, including *S. schleiferi, S. schleiferi* subsp. *schleiferi* and *S. schleiferi* subsp. *coagulans*. Other bacteria may also be involved, such as Gram-negative bacteria, *Pseudomonas aeruginosa*, *Escherichia coli* – usually secondary to *S. pseudintermedius* infections (Bryan *et al.*, 2012; Rantala *et al.*, 2004; Scott DW 2001).

2.1.2.2. Staphylococcus aureus

S. aureus in dogs is commonly found on locations of the body such as mucous membranes and moist areas (Shaw, Stitt And Cowan, 1951; Leonard and Markey, 2008). *S. aureus* has the ability to form disease in a non-invasive manner, through food poisoning and enterotoxins. It is the organism responsible for cutaneous infections such as impetigo, folliculitis and furunculosis. *S. aureus* is a common hospital pathogen associated with nosocomial infection and can result in systemic infections with consequent endocarditis, pneumonia, meningitis or osteomyelitis. The risk of morbidity, mortality and hospitalization is enormous for this bacterial disease (Leonard and Markey, 2008).

S. aureus has been cultured in healthy canine and feline patients from the skin and mucosa; there is also evidence of colonisation of the skin and ear in cases of pyoderma and otitis. However, *S. aureus* has a lower risk of infection compared with *S. pseudintermedius* (Lilenbaum *et al.*, 2000) in animals.

2.2. Staphylococcal Colonisation of Dogs

2.2.1. Pyoderma and Ear Infections in Dogs

Pyoderma is a surface infection that affects the skin and hair follicles (Loeffler and Lloyd, 2018; Lloyd and Garthwaite, 1982). It is caused by the proliferation of bacteria on the surface of the epidermis with dissemination into deeper layers of skin, leading to the spread of infection (Loeffler and Lloyd, 2018). Bacterial pyoderma is the second most common dermatosis in dogs following flea allergic dermatitis (Schroeder, 2010).

Otitis externa refers to the irritation of the external ear canal (tip of the pinna to the tympanic membrane) and is commonly considered a syndrome rather than a diagnosis (Jacobson, 2002). Otitis may also be classified according to the type of exudate. Changes in the outer ear canal presents as either unilateral or bilateral, either acute or chronic, and with mild to severe clinical signs. As a reaction to chronic inflammation, changes in the external ear canal may include glandular hyperplasia, glandular dilation, epithelial hyperplasia, and hyperkeratosis (Jacobson, 2002).

2.2.2. Pathogenesis of Pyoderma

The pathogenesis of pyoderma is divided into two phases. Firstly, the pathogenic commensal colonises regions of the body surface resulting in surface pyoderma. This is classically represented by skin fold pyodermas (intertrigo) and pyotraumatic dermatitis, the most common examples of surface pyoderma (Loeffler and Lloyd, 2018). The second phase occurs when the skin's most superficial layer (the stratum corneum) is invaded causing impetigo and/or hair follicle invasion resulting in folliculitis. Deep pyoderma results when the infection spreads into the dermis via hair follicles (resulting in furunculosis) or it spreads more widely along tissue planes (resulting in cellulitis) **(Figure 2).** As infection evolves, the body employs non-specific (innate) immune defences (represented mainly by neutrophils and macrophages) and specific (acquired) immune defences in the form of cell-mediated immunity and antibodies. This inflammatory reaction results in the clinical signs of pyoderma (Loeffler and Lloyd, 2018; Schroeder, 2010).

2.2.3. Pyoderma Classification

Several criteria can be used to categorize canine pyoderma. The most widely accepted classification is centred around the depth of infection namely, surface, superficial or deep pyoderma (Paterson, 2017; Loeffler and Lloyd, 2018). The type of therapeutic intervention depends on this classification. Other factors that play a role in management include the underlying cause (e.g. allergic dermatitis), the identity and susceptibility of the pathogen and the presence of any co-morbidity (Schroeder, 2010).

2.2.4. Surface Pyodermas

Surface pyodermas are typified by superficial erosions of the skin such as pyotraumatic dermatitis, intertrigo and mucocutaneous pyoderma (Schroeder, 2010). Pyotraumatic dermatitis **(Figure 2B)**, commonly known as “hot spots”, is a common infection on the surface of the skin of dogs, particularly those with long coats. Hot spots are usually self-inflicted skin excoriations secondary to an allergic reaction and are most common in the summer months (Schroeder, 2010). Intertrigo is common in dogs with anatomical abnormalities, such as spaniels with lip folds, facial folds of bulldogs and the vulva folds of sterilized obese bitches **(Figure 2A)**. These infections are characterized by a purulent exudation in moist and macerated skin folds, with malodour as a consequence of local bacterial overgrowth (Loeffler and Lloyd, 2018). Mucocutaneous pyoderma (**Figure 3**) is an infection of unknown cause, which mainly affects the skin and lips (Schroeder, 2010) resulting in swelling, erythema and crusting, sometimes associated with fissuring and erosion (Loeffler and Lloyd, 2018).

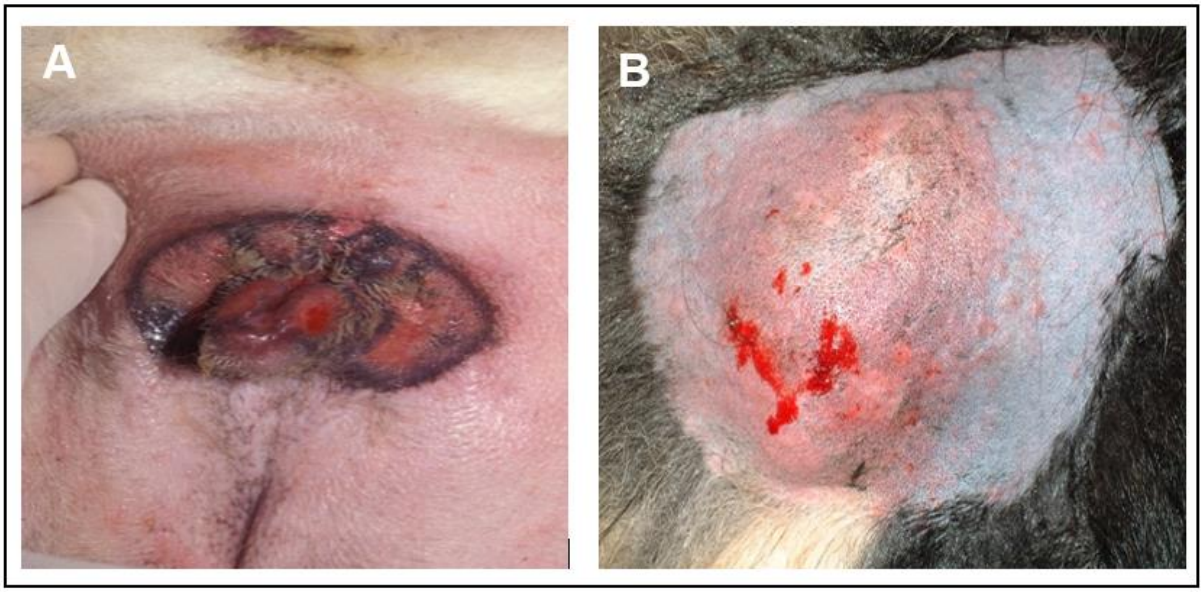

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Figure 2. Staphylococcal surface pyoderma infections in dogs. **(A)** Skin fold pyoderma infection in a spayed female dog. Sterilization had resulted in weight gain and vulval atrophy causing urine pooling in the skin folds that macerated the skin and predisposed it to a surface bacterial infection. **(B)** Acute superficial pyotraumatic dermatitis (the lesion has been closely shaved and cleaned) (University of Pretoria, Onderstepoort, Andrew Leisewitz).

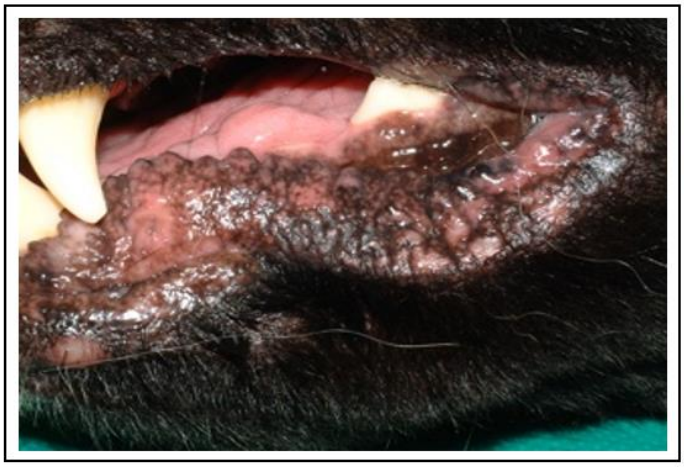

Figure 3. Staphylococcal mucocutaneous pyoderma in a German shepherd dog resulting in light crusting of the lips with some depigmentation (University of Pretoria, Onderstepoort, Andrew Leisewitz).

2.2.5. Superficial Pyoderma

Superficial pyoderma is likely the most frequently found form of pyoderma in dogs and is a result of a bacterial invasion of the epidermis (**Figure 4**). If a follicle becomes infected, inflammation spreads into the follicular ostium and surrounding epidermal tissue. The clinical presentation is characterized by papules, pustules or epidermal collarettes, generally presenting on the ventral abdomen, medial thighs or the tail (**Figure 5**). Pruritus and subsequent alopecia caused by self-trauma are commonly associated findings in cases of surface pyoderma (Loeffler and Lloyd, 2018).

Impetigo, superficial folliculitis and superficial spreading pyoderma are typical examples of a superficial pyoderma (Schroeder, 2010). Impetigo (**Figure 6**) is a subcorneal pustular disease, which is a non-follicular bacterial abscessation affecting the epidermal surface layers and which occurs mostly in puppies (Loeffler and Lloyd, 2018; Schroeder, 2010). Superficial folliculitis is, by definition, an infection of the hair follicle and is characterized macroscopically by a wide range of lesions usually beginning as a papule, followed briefly by a fragile pustule which then ruptures and develops into a crust. Patchy and focal alopecia are frequently observed as consequences of folliculitis (Bajwa, 2016; Schroeder, 2010). Lastly, superficial spreading pyoderma is characterized by the absence of pustules and, rather, broad, spreading epidermal collarettes with

an erythematous, moist leading edge are found. This results in large areas of erythema and exfoliation (Loeffler and Lloyd, 2018; Schroeder, 2010).

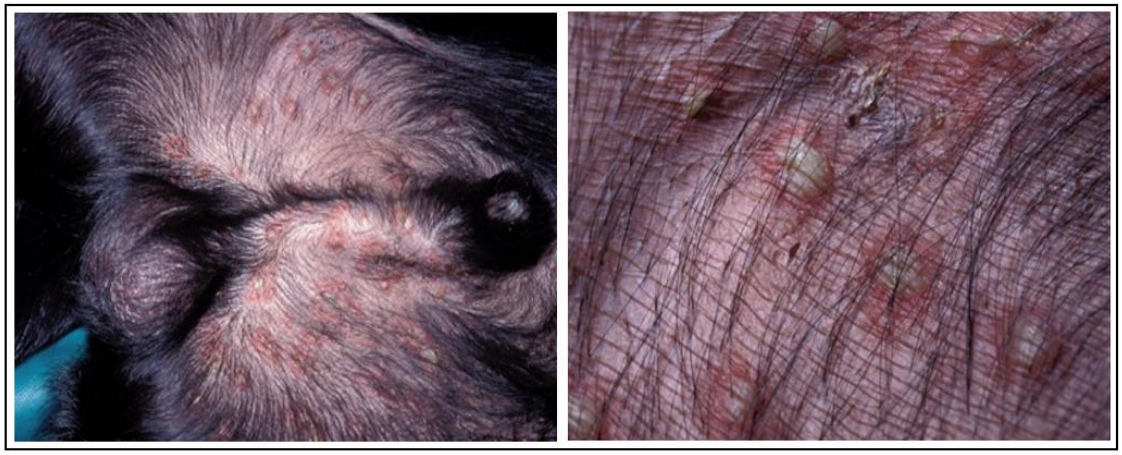

Figure 4. Superficial pustular disease due to bacterial infection in a dog (University of Pretoria, Onderstepoort, Andrew Leisewitz).

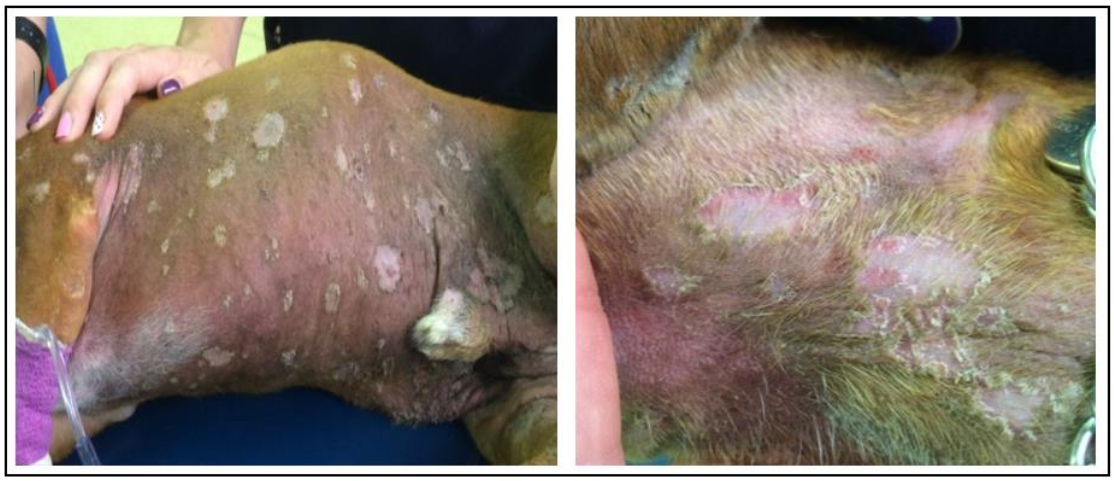

Figure 5. Superficial pyoderma with classic focal lesions, light crusting and epidermal collarette formation caused by staphylococcal infection (University of Pretoria, Onderstepoort, Andrew Leisewitz).

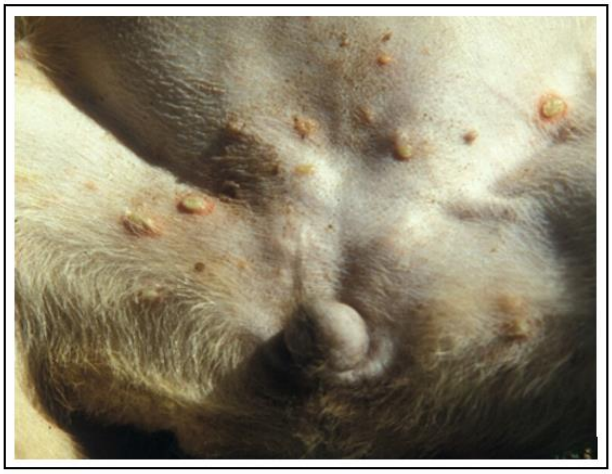

Figure 6. Impetigo caused by staphylococcal infection in a puppy subjected to poor husbandry (University of Pretoria, Onderstepoort, Andrew Leisewitz).

2.2.6. Deep Pyoderma

Although deep pyoderma is a less common condition, it is more serious as it spreads to the dermis, therefore having an increased risk of haematogenous spread and bacteraemia, due to the proximity to blood vessels. In deep pyoderma, the infection spreads below and beyond the boundaries of the hair follicle **(Figure 7)**. Follicular rupture (furunculosis) can result in a granulomatous tissue response directed against free keratin from the fragments of the root sheath and hair shaft (which act as microscopic foreign bodies), stimulating the formation of pyogranulomas scar tissue (Loeffler and Lloyd, 2018).

Deep pyoderma is divided into deep folliculitis, cellulitis and furunculosis. Macroscopically, the characteristics of deep pyoderma are lesions that ulcerate, leading to the leakage of fistulas, pain or pruritus, and regional or generalized lymphadenopathy. Deep pyoderma occurs secondary to allergic, parasitic (particularly demodicosis (**Figure 8**)), endocrine, autoimmune, actinic, neoplastic, pressure point, post-grooming or self-traumatic conditions (Loeffler and Lloyd, 2018; Schroeder, 2010).

Certain large breed dogs are more susceptible to deep pyoderma. For example, German shepherd dogs have breed associated folliculitis / furunculosis, which can result in severe pain, draining sinuses, fistulae and varying degrees of erythema and swelling (Loeffler and Lloyd,

2018). Bull terrier dogs (especially white animals) frequently present with a breed specific deep pyoderma / cellulitis. Other underlying causes such as autoimmune, allergic, actinic and neoplastic dermatoses could also appear as deep pyoderma (Ettinger, Feldman and Cote, 2010).

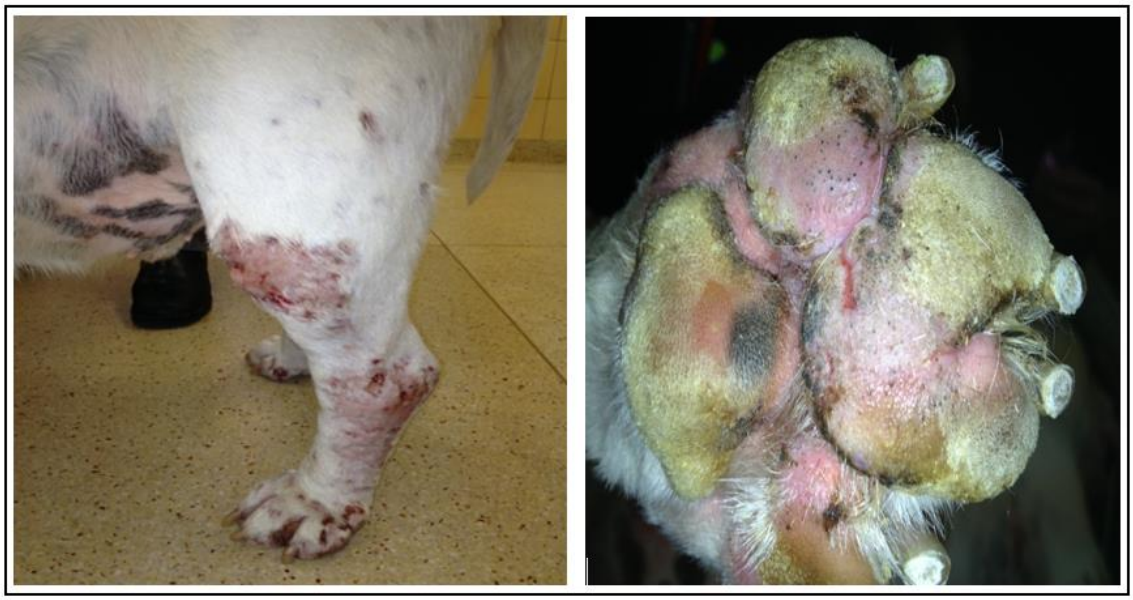

Figure 7. Deep pyoderma caused by staphylococcal infection associated with Bull terrier dogs manifesting as a deep scarring cellulitis of the hind legs and pododermatitis. (University of Pretoria, Onderstepoort, Andrew Leisewitz).

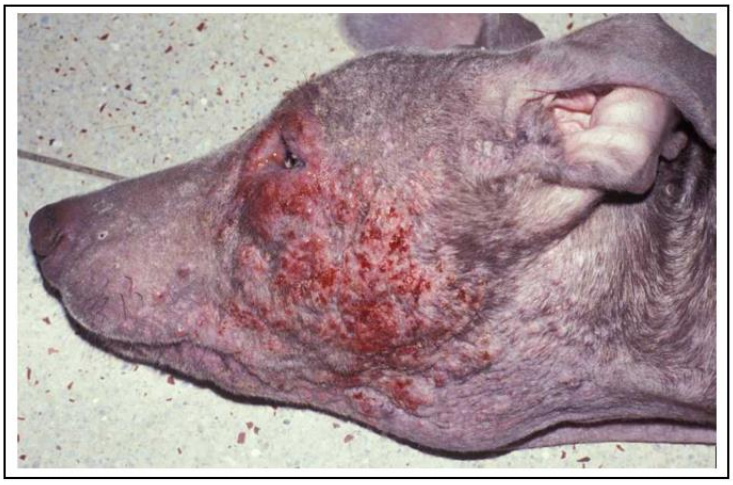

Figure 8. Deep pyoderma associated with generalized demodicosis in a Doberman with colour dilution alopecia caused by bacterial infection (University of Pretoria, Onderstepoort, Andrew Leisewitz).

2.2.7. Otitis

Otitis is a frequent call for small animal practice consultations. Although sometimes considered to be part of dermatology, it should be emphasized that otitis is often the consequence of an underlying disease. The investigation of otitis must be cognisant of the primary, perpetuating and predisposing causes and requires a logical and ordered approach, that cannot involve only the management of infectious agents (Ettinger, Feldman and Cote, 2010). Bacterial causes of ear disease are not considered primary and, as such, simply applying antimicrobial treatment is usually unsuccessful. Regardless of the cause or clinical diagnosis, otitis is regarded as any inflammation of the ear canal. Such cases are typically multifactorial and are therefore classified as primary and secondary causes which are induced by predisposing and perpetuating factors (Ettinger, Feldman and Cote, 2010).

The most common organisms responsible for middle ear disease are *Pseudomonas aeruginosa, S. pseudintermedius, Proteus spp., Escherichia coli* and the yeast *Malassezia pachydermatis*, which are all able to form biofilms. Biofilms can lead to infection persistence despite adequate therapy, since it is necessary to disrupt this biofilm in order to facilitate effective antimicrobial therapy (Bajwa, 2019).

2.2.7.1. Pathogenesis and Mechanism of Infection

Primary factors are ailments that directly impact the external auditory canal and can contribute to inflammation. These include otic parasites such as *Otodectes cyanotis,* hypersensitivity disease (such as food allergy and atopic dermatitis), endocrine disease (such as hypothyroidism), otic neoplasia and foreign bodies. Underlying hypersensitivity disease is the most common primary factor leading to the development of otitis in dogs (Ettinger, Feldman and Cote, 2010). Predisposing factors are responsible for changing the environment of the ear canal and thus increasing the risk of otitis. Predisposing factors typically include increased hair growth in the auditory canal, excessive swimming, congenitally stenotic canals, otic masses and inappropriate ear cleaning (Ettinger, Feldman and Cote, 2010). Perpetuating factors are responsible for chronicity, which typically includes bacteria such as *Staphylococcus* and *Pseudomonas*, and the yeast *Malassezia* (Paterson and Matyskiewicz, 2018).

Recurring inflammation and infection can lead to secondary changes in the ear canal that can lead to further difficulties in the management of otitis and possible end-stage ear disease, characterized by irreversible anatomical change. Severe glandular modifications, fibrosis, stenosis, and calcification along the outer ear canal are typical irreversible changes that persist and worsen, even if the primary inciting cause is removed.

Erythematous otitis externa (erythematous OE) is clinically characterized as inflammation of the outer ear canal without secretion (**Figure 9A**), whereas inflammation with copious amounts of ceruminous exudate is characteristic of erythroceruminous otitis externa (erythroceruminous OE) (**Figure 9B**). Suppurative otitis externa has erosions in the ear canal along with a purulent exudate and stenotic otitis is characterized by hyperplastic changes of the ear canal (Ettinger, Feldman and Cote, 2010).

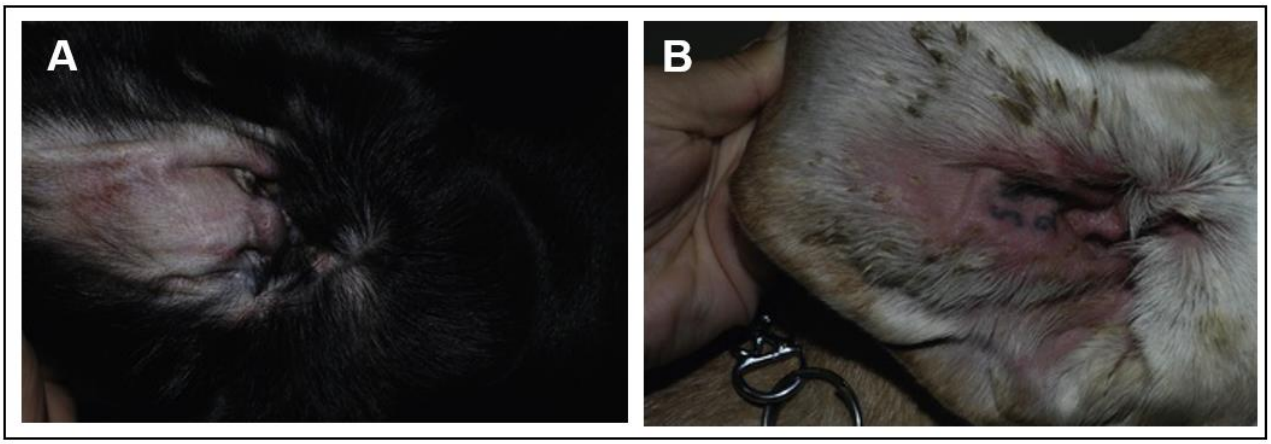

Figure 9. (A) Erythematous otitis externa (OE) is clinically characterized as inflammation of the outer ear canal without secretion. (**B)** Erythroceruminous OE is characterized by inflammation of the outer ear canal with copious amounts of ceruminous exudate. Images taken from Ettinger and Feldman (2010).

2.2.7.2. Incidence of Otitis

Otitis is classified anatomically as otitis externa (OE), otitis media (OM) and otitis interna (OI). OE affects the external auditory canal. OE in dogs and cats in the veterinary practice has a recorded prevalence of 5–12% of dog and 2% of cat consultations (Ettinger, Feldman and Cote, 2010). OM

is localized to the middle ear canal and is usually secondary to OE. Primary OM is more frequently recognized in cats. The prevalence of OM ranges widely and is more frequently associated with chronic OE (Ettinger and Feldman; Lorek *et al.*, 2020). OI is an inflammation of the inner auditory canal and is generally resultant of OM, clinically represented by the development of vestibular signs (Ettinger, Feldman and Cote, 2010).

A study done by Lorek *et al.* (14), highlights the importance of managing perpetuating factors such as OM as an important element in the successful management of canine OE. In this thesis, 123 magnetic resonance imaging (MRI) studies were analysed. OM was observed in 41/197 ears (21%) that also had chronic OE. These MRI findings suggest that it is not uncommon to find occult OM in dogs with chronic OE.

2.3. Identification of *Staphylococcus pseudintermedius* species

2.3.1. Phenotypic Identification

The ideal phenotypic identification of *S. pseudintermedius* requires a battery of tests. Key differences amongst the phenotypic and biochemical tests utilised in the identification of *S. pseudintermedius* are displayed in **Table 1** below. Current standard operating procedures in the laboratory to detect *S. pseudintermedius* phenotypically include the following:

2.3.1.1. Morphology

Staphylococcal species are typically spherical cocci and form grapelike clusters that are 3 planes and 1 micrometer diameter in size. Staphylococci are nonmotile, halotolerant, catalase positive and oxidase negative (Mahon, Lehman and Manuselis, 2014; Paharik and Horswill, 2016).

S. pseudintermedius colonies are grown on Columbia blood agar with 5% horse blood (CBA) and MacConkey medium (McC) at a temperature of 37ºC for a period of 24 hours. Significant colonies are sub-cultured. These colonies are then placed into an incubator overnight in a CO_2 environment at 37ºC forming a purified culture. Morphologically, *S. pseudintermedius* colonies are small, round, entire and white with a narrow zone of beta-haemolysis on CBA (**Figure 10**) (Fitzgerald, 2009), while on McC the colonies are small, round and pink (Murray and Baron, 2003).

Figure 10. Blood agar plate with colonies of *Staphylococcus pseudintermedius*. Image taken from Department of Veterinary Disease Biology (2011), Faculty of Health and Medical Sciences, University of Copenhagen, Denmark (Copenhagen, 2011).

2.3.1.2. Gram Stain

The purified culture described above is used for primary identification. A colony smear for Gram staining is made. S. *pseudintermedius* are Gram-positive organisms. The staining process requires a primary stain (crystal violet), iodide (that fixes the crystal violet), a decolourizer with ethanol or acetone and finally a counterstain with safranin. The cell wall envelope of S. *pseudintermedius* is a complex surface of peptidoglycan polysaccharides with a low lipid content, which causes retention of the crystal violet. Gram-positive species stain a violet colour whilst Gram-negative species do not retain the crystal violet, due to a high lipid composition in their cell walls, and thus stain red (Tighe and Brown, 2002; Jorgensen, Pfaller and Carroll, 2015). Following the Gram stain, coagulase and catalase tests are performed.

2.3.1.3. Coagulase

Staphylococcal species express an enzyme coagulase, which help propagate its pathogenic effects in vivo. A tube is used with plasma, which is inoculated with staphylococcal. Upon mixing the bacterial sample with the plasma, conversion of fibrinogen to fibrin occurs and clumping takes place. The bacteria are coagulase-positive if the fluid is fully clotted or partially clotted with visible

clumps. Coagulase-negative species will remain in the fluid phase and not form clots (MacFaddin, 2000).

2.3.1.4. Catalase

Catalase is an enzyme found within aerobic species including aerobic organsims. Catalase forms a reaction to break down products of oxygen metabolism. Catalase breaks down hydrogen peroxide, which helps protect cells from oxidative damage. Oxidative damage damages the DNA of the bacteria. The catalase test is a diagnostic tool used in laboratories to help distinguish different types of bacteria that may appear morphologically similar under microscopes (Jorgensen, Pfaller and Carroll, 2015).

The catalase test is rapid and relatively inexpensive. Bacterial colonies that are not grown on blood culture are mixed with hydrogen peroxide on a slide. Rapid formation of bubbles occurs from oxygen release, which indicates a catalase positive organism.

2.3.1.4. Biochemical Tests

Following the basic identification, the purified culture undergoes secondary identification. A suspension is made by transferring a colony to a tube of 5% saline that was used to inoculate the following biochemical tests as displayed in **Table 1** (Bergey *et al.*, 2010): Aesculin hydrolysis, mannose, trehalose, urease, xylose, DNAse, mannitol salt agar, purple maltose agar. Finally, the purified culture is inoculated at 37ºC overnight in normal air on an antibiogram on Mueller–Hinton agar with novobiocin 5 µg and polymixin B 300 disk.

The final phenotypic diagnosis of S. *pseudintermedius* is made on the following results of the above test: positive on DNAse, purple maltose agar (weak positive), mannose, trehalose and urease; positive or negative on mannitol salt agar; negative on aesculin and xylose; and sensitive to both novobiocin and polymixin B (Murray and Baron, 2003; Winn and Koneman, 2005). Other commonly used phenotypic tests include VP (Voges Proskauer), Vitek or Staph API for identification.

Table 1. Key differences amongst the phenotypic and biochemical tests utilised in the identification of *Staphylococcus pseudintermedius* (Jorgensen, Pfaller and Carroll, 2009; Baker, 1984; Perry *et al.*, 2003; Kluytmans *et al.*, 2002; Stefani *et al.*, 2012; Sewid *et al.*, 2018; Layer *et al.*, 2006).

Phenotypic Assay (Primary identification)	**Advantages**	**Disadvantages**
Gram stain	• The purified culture is used for primary identification • Rapid and simplest test to distinguish microorganisms and class bacteria as either Gram-positive or -negative	• Results do not match with the final identification of microorganisms Due to technical uncertainty and misinterpretation, false results in a small percentage of cases can occur
Catalase	• Used together with oxidase and gram stain in primary identification. • Rapid and relatively inexpensive	• Hydrogen peroxide is unstable • Growth for catalase testing must be done within a 24-hour culture • False negatives can result from delay in interpreting the test, as organisms lose their catalase activity over time
Oxidase	• Used together with catalase and gram stain in primary identification • *S. pseudintermedius* is oxidase negative	• Reagents have a short shelf life • Limited to certain types of growth media • Bacteria that are grown on dyes may give abnormal results

Phenotypic Assay (Secondary identification)	Advantages	Disadvantages
Mannitol Salt Agar	• In mannitol salt agar, staphylococci thrives at high salt concentrations • Only a pure culture is used for secondary identification • When mannitol is fermented, the acid produced turns the phenol red to yellow pH indicator • Little chance of contamination	• Other salt-tolerant bacteria can grow on this medium too
Production of deoxyribonuclease (DNase) on DNase agar	• Only a pure culture is used for secondary identification • Positive organism hydrolyzes deoxyribonucleic acid • Used to differentiate *S. pseudintermedius* from other staphylococci which do not produce DNase • Advantageous if plasma is not available to perform coagulase test or when coagulase tests are difficult to interpret	• Some MRSP strain do not give positive result
Purple Maltose agar	• Maltose purple agar is used for distinguishing *S. aureus* from *S. pseudintermedius*	• Further biochemical tests required for complete identification
Esculin hydrolysis	• Mainly used for the identification of enterococci and streptococci • *S. pseudintermedius* always negative	• Further biochemical tests required for complete identification

Phenotypic Assay (Secondary identification)	Advantages	Disadvantages
Trehalose	• Accurate, rapid, and economical presumptive test. • Demonstrates the difference of *S. epidermidis* from other coagulase-negative staphylococcal species. • *S. pseudintermedius* positive.	• Further biochemical tests required for complete identification.
Urease test	• *S. pseudintermedius* positive.	• Light exposure can develop peroxide inhibiting the urease test. • Can undergo auto-hydrolysis.
Voges-Proskauer	• Voges-Proskauer positive-for *S. pseudintermedius.*	• Less reliable than PCR-based identification. • Additional biochemical testing using pure culture is recommended.
API Staph system (API ID 32 STAPH)	• Rapid and easy identification. • The API can be evaluated separately.	• The API Staph system only recognizes species included in the database. • Current phenotypical studies cannot accurately distinguish between *S. aureus* and *S. pseudintermedius* veterinary strains. • Only pure cultures can be used.

2.3.1.5. Other bacterial colonisation of dogs

In dogs with otitis externa and pyoderma, recent studies of canine ear canal microbiota have been evaluated (Korbelik *et al.*, 2019; Bradley *et al.*, 2020; Tang *et al.*, 2020; Kasai *et al.*, 2020). These studies demonstrate a decline in bacterial diversity in otitis externa and pyoderma. Endemic canine ear and skin organisms, which are primarily non initiative in the disease process, may become opportunists when pathological changes occur (Pye, 2018).

In the laboratory search of S. *pseudintermedius*, other organisms are often found as well. These organisms are normally located in the canine ear and skin and do not have a primary role in initiating the disease process, but rather become opportunists when pathological changes occur (Miller *et al.*, 2013). Otitis infections are commonly polymicrobial in nature **(Table 2)** – some of these organisms are considered an incidental finding, however most are secondary opportunistic invaders in dogs with otitis (Lamm *et al.*, 2010).

Table 2. Common organisms found in otitis externa and their key characteristics

Organism	Organism characteristics	Blood Agar	MacConkey Agar	Gram stain	Oxidase	Catalase
Pseudomonas aeruginosa	Gram-negative, rod-shaped bacterium that can be found in canine skin and ears. Produces destructive enzymes such as collagenases and proteases that cause epithelial destruction in the skin and ear.	Grey, green, haemolytic	Colourless	Negative rods	Positive	NA

	Damages the internal ear canal causing ulceration and erosion, and ultimately destruction of the ear drum. Commonly found in otitis media. The body in turn mounts a severe immune response, which results in severe inflammation, erythema and excessive purulent exudate (Hillier *et al.*, 2006). Produces a biofilm protecting itself from the antibiotics administered topically (Mekić, Matanović and Šeol, 2011).					
Staphylococcus schleiferi subsp. *coagulans*	Found in the normal canine ear and dogs with canine otitis (Loeffler and Lloyd, 2018; Paterson, 2017). *Staphylococcus schleiferi* can become methicillin resistant (MRSS). *Staphylococcus schleiferi* possesses virulence factors to help proliferate soft tissue infections. They produce a biofilm, are resistant to cationic antimicrobial peptides (CAMPs), and carry genes that encode for the production of enterotoxins (Lee *et al.*, 2019).	White, haemolytic	Scanty-no growth	Positive cocci	NA	Positive

Streptococcus canis	Found in dermatitis, pneumonia, adult septicaemia, foetal/neonatal septicaemia. Considered an incidental finding or a secondary opportunistic invader (Lamm *et al.*, 2010).	Small, white, B haemolytic	Scanty-no growth	Positive cocci	NA	Negative
Proteus mirabilis	Gram-negative and anaerobic Found in the normal canine ear and dogs with canine otitis (Loeffler and Lloyd, 2018; Paterson, 2017). *Proteus mirabilis* are opportunists and not primary pathogens causing solely otitis externa, but will grow if a favorable medium for growth is provided, such as otitis (Oliveira *et al.*, 2008).	Swarms, no discrete colonies	No colour	Negative rods	Negative	NA
Escherichia coli	Gram-negative rod-shaped bacterium that can be found in canine skin and ears. Most common bacterial pathogen in cases of infectious otitis externa. In dogs with otitis or enteritis, *E. coli* is the most commonly found opportunistic invader causing secondary infection.	Grey	Pink	Negative rods	Negative	NA

Malassezia pachydermatis	Yeast that is found in the normal canine ear and dogs with canine otitis (Loeffler and Lloyd, 2018; Paterson, 2017). Can localise in the tympanic cavity. Secondary opportunistic invader in most dogs with otitis/atopy/food allergy.	No growth	No growth	Budding yeast	NA	NA

2.3.1.6. Challenge with the Current Phenotypic and Biochemical Methods

Current phenotypic and biochemical methods are fallible. Discrepancies in colour change and variation in the expression of key characteristics in biochemical testing result in the misidentification of bacterial species (Speers, Olma and Gilbert, 1998; Kasela and Malm, 2018; Schissler *et al.*, 2009). Whilst current phenotypic biochemical tests such as the API ID 32 Staph (API System, BioMe´rieux, Paris, France) and BBL crystal identification systems Gram-positive ID kit (Becton Dickinson Microbiology Systems, Cockeysville, Md.) are rapid and user-friendly, the reproducibility of the individual substrate reactions ranges varies and has a low level of accuracy (Schissler *et al.*, 2009). As a result, these tests cannot accurately distinguish between *S. aureus* and *S. pseudintermedius* veterinary strains (Couto *et al.*, 2001). Furthermore, supplementary kits and further testing are needed for the differentiation of *S. schleiferi, S. schleiferi* subsp. *coagulans* and *S. schleiferi subsp. schleiferi* (Layer *et al.*, 2006; Zdovc *et al.*, 2004).

Furthermore, the ability of current biochemical methods to discern *S. pseudintermedius* from other staphylococci is inadequate as these species can be interchangeably misidentified and most tests for *S. pseudintermedius* are heavily biased in the *S. aureus* direction (Bond and Loeffler, 2012). For example, most tube coagulase tests used by laboratories rely on human plasma. Human plasma has been reported to have coagulase-reacting factor and anti-staphylococcal antibodies, which result in aberrant results (Bello and Qahtani, 2005). Rabbit plasma is a preferable medium, however it is expensive, and the shelf life of the reagents is relatively short (Kateete *et al.*, 2010).

Another key challenge with current phenotypic tests, is the bacterial misidentification amongst species. The production of coagulase is an important feature in the identification and description of staphylococci. Currently, there are two procedures for the assessment of coagulase: the slide test and the tube test (Cunha, Sinzato and Silveira, 2004). The tube test detects both bound and free coagulase and it must be assessed at both 4 and 24 hours. The slide test detects bound coagulase, also termed “clumping factor”. Importantly, some rare strains of coagulase positive staphylococci will test negative in both tube and slide coagulase tests. Certain laboratories use a rapid latex agglutination test to detect coagulase formation in these rare cases.

As the species of interest in this study, *S. pseudintermedius,* produces only free coagulase, the slide test is an unsuitable method of coagulase detection and require tube testing. Other species

that produce only free coagulase are *S. schleiferi subsp. coagulans* and *S. intermedius.* Problematically, a negative slide test if paired with a positive urease result can lead to misidentification of *S. schleiferi,* *S. schleiferi* subsp. *coagulans* and *S. schleiferi* subsp. *schleiferi* or *S. chromogenes.* Additionally, since *S. pseudintermedius* is similar to *S. aureus*, based on biochemical characteristics such as coagulase positivity and haemolysis on blood agar, clinical isolates from canine origin are often misidentified as *S. aureus* (Börjesson *et al.*, 2015). Although mannitol salt agar can distinguish *S. pseudintermedius* from *S. aureus,* some strains of *S. pseudintermedius* can be positive or negative on MSA (Procop and Koneman, 2016).

Only 90% of the strains are positive for the phenotypic characteristics displayed in **Table 1** which means that one can never rely completely on any single characteristic (Bergey *et al.*, 2010). Thus, the more tests that are done, the more accurate the identification of the bacteria will be.

2.3.2. Genotypic Identification

2.3.2.1. PCR for the Detection of *S. pseudintermedius*

Polymerase Chain reaction (PCR) has revolutionized molecular biology, allowing rapid and exponential amplification of specific target DNA sequences. The basic steps of PCR are as follows:

- Step 1: Denaturation of the DNA template.
- Step 2: Annealing of different sequences of oligonucleotides.
- Step 3: DNA polymerase extension from the oligonucleotides (primers) clones the template DNA.

Deoxyribonucleotide triphosphate (dNTP) must be included in the PCR reaction and the amplification of the DNA template is achieved by repeating the above steps for several cycles (Schwarz *et al.*, 2018).

To date, an extensive number of conventional PCR tests have been described to identify *S. pseudintermedius*. Different target regions using PCR to identify staphylococcal species specific thermonuclease (*nuc*) gene and sequence analysis, heat shock protein (*hsp60*) gene region (Sasaki *et al.*, 2010; Bond and Loeffler, 2012), or *MboI* restriction of the *pta* gene have been utilised to discriminate *S. pseudintermedius* from additional staphylococcal bacteria

included within the SIG (Bannoehr *et al.*, 2009). However, these approaches are costly, time consuming and not suitable for day-to-day use in laboratories with high throughputs as the assay make use of PCR and sequencing or restriction digest analysis. While at the molecular level SIG's taxonomy can be easily distinguished, phenotypically, confusion still exists as a result of varying biochemical properties expressed among and inside SIG species (Bond and Loeffler, 2012).

2.4. Methicillin Resistant *Staphylococcus pseudintermedius* (MRSP in Veterinary Medicine

Currently, the main treatment for *S. pseudintermedius* in skin and ear infections is systemic antibiotic administration or topical antiseptic application (Loeffler and Lloyd, 2018). However, with the emergence of methicillin- and multidrug-resistant staphylococcal organisms (MRSP) that are responsible for the increase in frequently encountered refractory skin infections, a drastic change in the philosophy of oral antibiotic treatment is urgently needed (Jeffers, 2013). These infections are typically treated with ß-lactam antibiotics. However, the emergence and worldwide increase of MRSP has resulted in resistance to all ß-lactam antibiotics, complicating effective treatment (Loeffler and Lloyd, 2018).

Staphylococcal infections that are associated with canine and feline skin anomalies are typically treated with penicillin or amoxicillin clavulanic acid. Due to the high levels of resistance to penicillin, other popular choices for treatment include first generation cephalosporins (e.g. cephalexin), the combination sulphonamide-trimethoprim, clindamycin or erythromycin (Rota *et al.*, 2013). *S. pseudintermedius* strains with increasing resistance to these antibiotics are clinically more severe compared to methicillin sensitive *S. pseudintermedius* (MSSP) and this has generated significant concern over the last few decades (Morris *et al.*, 2017).

The World Association for Veterinary Dermatology (WAVD) has published guidelines on the recommendations for approaches and treatment of MRSP. These guidelines deliver succinct protocols to veterinarians on the management, therapeutic considerations and preventative measures related to MRSP (Morris *et al.*, 2017). Thus, South African veterinarians should endorse these treatment guidelines for the optimum therapeutic treatment of canine *S. pseudintermedius* infections in South Africa. Yet, antibiotic treatment regimens that still exist in South Africa often go unchallenged, become accepted practice, and evolve into unproven dogmas that go against

what are now regarded as core principles of antibiotic stewardship as stipulated by the WAVD. Elsewhere in the world, similar situations have resulted in a surge in the populations of multidrug resistant *S. pseudintermedius* (MDRSP) strains (Bourély *et al.*, 2019; Grönthal *et al.*, 2017; Kadlec and Schwarz, 2012).

2.4.1. Epidemiology and Ecology

Methicillin was originally introduced in 1959 to treat staphylococci that produced β-lactamase and were, as a result, resistant to penicillin (Knox, 1960). Methicillin-resistant *S. aureus* (MRSA) was isolated in hospital settings soon after the introduction of methicillin. The incidence of such findings has since increased exponentially, as bacteria continue to evolve and acquire various mobile genetic elements responsible for antibiotic resistance (Grönthal *et al.*, 2017). Methicillin resistance is characterized by the expression of *mecA*. This gene sequence encodes a modified penicillin-binding cell wall protein, PBP2a (Bond and Loeffler, 2012). Although *mecA* is important for the resistance to methicillin / oxacillin, its expression is heterogeneous. Oxacillin is used as a surrogate in laboratories in place of methicillin. Phenotypically, methicillin resistance is categorized as homotypic or heterotypic based on the morphology of the plate. A heterotypic phenotype has small colony sizes whose growth is uninhibited by 10 μg of oxacillin per ml, while the remaining colonies are killed by this and lower concentrations. These resistant subpopulations that exist in small proportions can, under the selective pressure of antibiotics, provide clinical resistance. In contrast, homotypic resistance is characterized by a uniform oxacillin resistance. For populations that exhibit this phenotype, there is little change in colony size on agar (Finan *et al.*, 2002). These subpopulations arise from heterotypic populations following exposure to β-lactam antibiotics (Hartman and Tomasz, 1986).

The *mecA* gene is either induced (in response to an external stimuli) or constitutively expressed (expressed continually). Transcription is mediated via a two component regulatory system, consisting of a repressor (*mecI*) and a sensor / inducer (*mecR1*) (Santiago *et al.*, 2015). The genetic element responsible for the carriage and potential transfer of the *mecA* gene is the staphylococcal chromosome cassette *mec* (SCC*mec*), bonwhich is a mobile element and thus transferrable between bacteria (Bond and Loeffler, 2012; Bannoehr *et al.*, 2009; Sasaki *et al.*, 2010). The attainment of SCC*mec* in *S. pseudintermedius* strains has resulted in an increased incidence of MRSP globally (Moodley *et al.*, 2009; Bond and Loeffler, 2012).

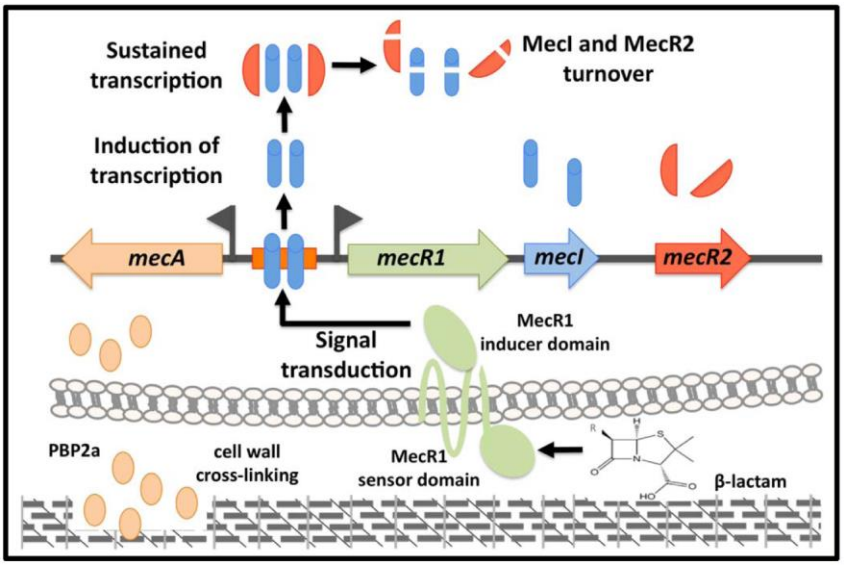

Figure 11. Staphylococcal chromosomal cassette mec (SCCmec). The MRSA phenotype is due to the mecA gene-coded extra penicillin-binding protein (PBP2a), which has a decreased affinity for many b-lactams. *MecR1* (Methicillin resistance mecR1 protein) is activated in response to β-lactam antibiotic exposure. MecR1 subsequently induces the expression of *mecA* (Methicillin resistance gene) and *mecR1-mecI-mecR2.* *MecR2*'s (Methicillin resistance mecR2 protein) anti-repressor activity is important for maintaining *mecA* induction, as it promotes proteolytic cleavage inactivation of *mec.* Flags denote the entry and exit point through the cell membrane. Figure 13 depicts a form of inducible resistance, since the regulatory genes are intact; as opposed to constitutive expression, in which deletions in the regulatory gene(s) is found. Adapted Copied from Arêde et al. (Arêde *et al.*, 2012).

Phenotypic methicillin resistance was first described in the mid-1980s in France, in both diseased and healthy dogs (Pellerin *et al.*, 1998). The first detection of *mecA* in *S. pseudintermedius* was in a strain isolated from canine pyoderma in the USA in 1999 and later in Europe in 2007 (Loeffler *et al.*, 2007; Gortel *et al.*, 1999). Since these early reports, MRSP has now been reported globally, and is a major canine health hazard (van Duijkeren *et al.*, 2011), making empiric antimicrobial treatment choice difficult.

A 2006 study at a dermatology clinic in northern Germany found that 23% of isolates from dogs and cats were MRSP (**Figure 12**) (Loeffler *et al.*, 2007). A 2009 review of 901 coagulase-positive staphylococcal (CoPS) from dogs in Germany demonstrated that the total number of clinical MRSP isolates (n=61) was found to have been substantially higher than MRSA isolates (n=15) (**Figure 12**) (Ruscher *et al.*, 2009). Methicillin resistance was identified in 14% of *S. pseudintermedius* clinical isolates from Finland in 2017 ($n = 1958$; 98% of which were from dogs) (**Figure 12**) (Grönthal *et al.*, 2017b).

Research conducted in southern China found that out of 144 clinical isolates, 69 *S. pseudintermedius* isolates collected from dogs and cats were classified as MRSP. Amongst these, resistance was shown to four or more antimicrobials at the same time (**Figure 12**) (Feng *et al.*, 2012). In another study conducted in North China, it was demonstrated that 33 out of 260 dogs (12.7%) with pyoderma were positive for MRSP (**Figure 12**) (Wang *et al.*, 2012). In a survey of 590 SIG isolates submitted to an Italian veterinary diagnostic laboratory over a period of two months in 2008, MRSP represented 10 out of 48 true SIG isolates (21%). In addition to being methicillin-resistant, these isolates were also resistant to non ß-lactams (mainly, lincosamides, fluoroquinolones, gentamicin and tetracyclines) (**Figure 12)** (De Lucia *et al.*, 2011).

Former studies in South Africa indicated that *S. pseudintermedius* isolates are resistant to second line antibiotics such as lincosamides, fluoroquinolones and trimethoprime-sulphamethoxazole (**Figure 12)** (Qekwana, Oguttu and Odoi, 2019). Other South African studies demonstrated high levels of ampicillin and doxycycline resistance amongst *S. pseudintermedius* isolates obtained from cases of canine pyoderma (**Figure 12)** (Qekwana, Oguttu and Odoi, 2019; Blunt, van Vuuren and Picard, 2013).

The studies described above highlight that treating staphylococcal skin infections in dogs is increasingly challenging. Furthermore, the data highlights that resistance in antimicrobials is a global, urgent danger to the health of both animals and their owners respectively, as animals can harbour resistant bacteria (Fitzgerald, 2009) and potential horizontal transmission of resistant genes between individuals can occur (Scott Weese, 2008).

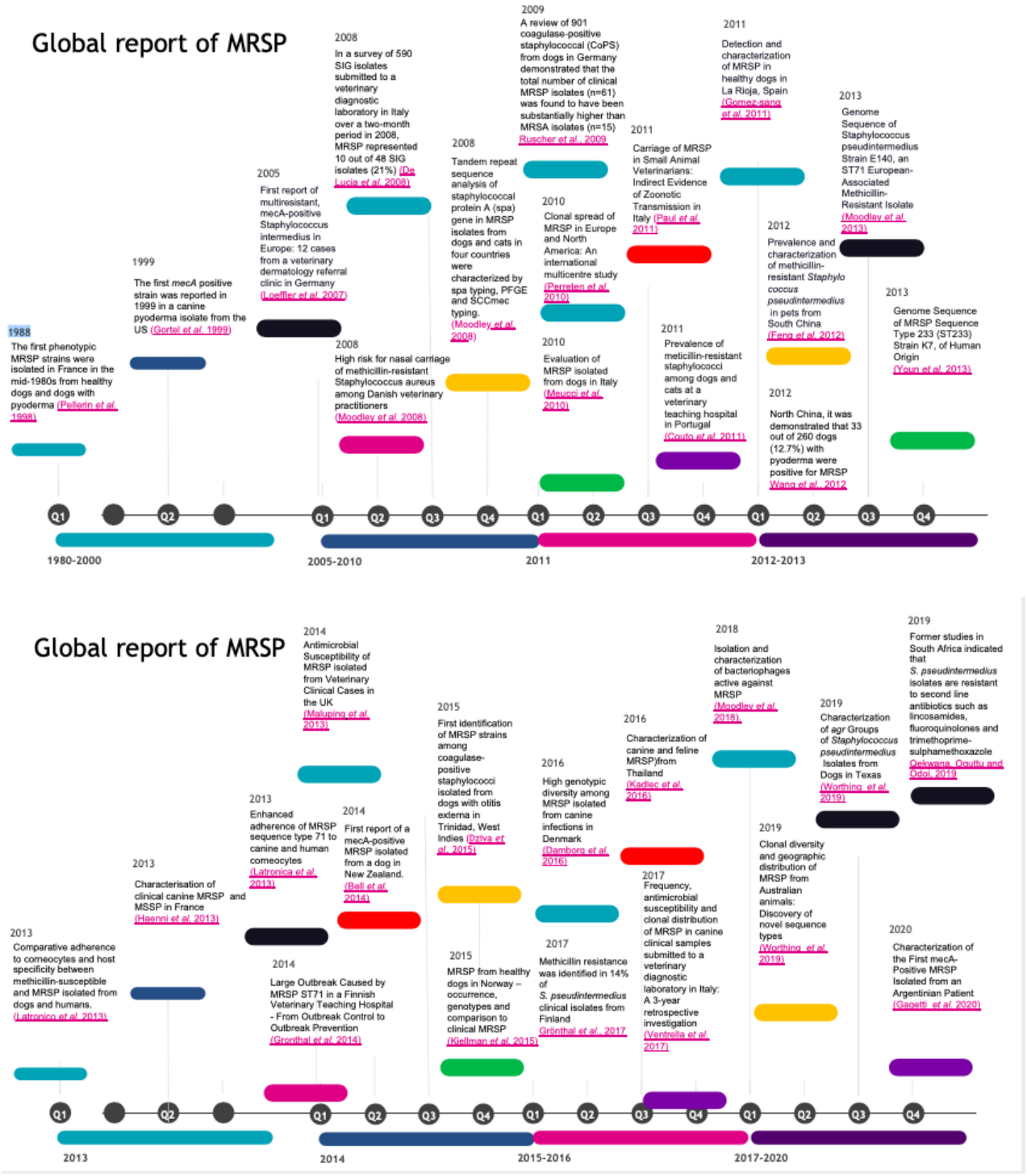

Figure 12. Global timeline of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) in animals and humans from 1988-2020.

2.5. Phenotypic Approaches Used for Recognition and Identification

Despite the increasing prevalence of multi-drug resistant organisms, misuse of antimicrobials persists. This promotes the advancement and selection of MRSP strains in healthy dogs, creating a huge challenge to effective veterinary treatment (Rota *et al.*, 2013). This increasing problem of antimicrobial resistance has forced veterinarians to restrict the general use of these drugs. Thus, prescription of the most appropriate treatment agent is vital, especially for the preservation of currently available antibiotics for future use.

There is no single test that can pick up all resistant strains of *Staphylococcus*, as the optimum conditions for phenotypic susceptibility testing differ between strains (Brown, 2001). Variations exist in:

- agent tested (methicillin / oxacillin);
- culture medium (agars such as Mueller–Hinton and Columbia are better discriminators of susceptible strains and MRSA than agars such as IsoSensitest and DST);
- NaCl concentration (5% NaCl is set at a selected figure of growth of non- staphylococcal organisms, but encourages growth of MRSA, which is set to grow at a higher NaCl concentration);
- inoculum (using greater volumes improves the detection rate of small resistant sub-populations); and
- incubation conditions (decreasing the incubation temperature, increases the rate of detection of resistance) (Brown, 2001).

Methicillin resistance is commonly detected using oxacillin disk diffusion, oxacillin broth microdilution, and oxacillin salt agar screen. In diagnostic microbiology, oxacillin is the penicillinase-stable β-lactam of choice, as it is more stable than methicillin. Though staphylococci may be better classified as oxacillin-resistant, the conventional standard specifies a methicillin-resistant label (Schissler *et al.*, 2009). Horstmann *et al.* (Horstmann *et al.*, 2012) evaluated five commercial selective media currently used for the detection of MRSP. ChromagaiTM MRSA (BD Diagnostics); chromIDTm MRSA agar (BioMerieux); oxacillin resistance screening agar base (ORSAB); and Brilliance MRSA agar (Oxoid) have been tested for MRSP detection. The findings showed that the most practical and accurate for detecting and isolating MRSP from clinical material phenotypically is ORSAB (Oxoid) and Brilliance MRSA agar (Oxoid) (Horstmann *et al.*, 2012).

2.5.1 Oxacillin Salt Agar Screen (OSA)

Many commercial OSA products, (ORSAB (Oxoid); Brilliance MRSA agar (Oxoid)) consist of 6.0 µg/ml oxacillin and 4% NaCl, augmented by Mueller-Hinton agar and are the most widely used tests in the detection of MRSA (Schissler *et al.*, 2009). Any growth observed on the plate shows that the organism is resistant to methicillin; no growth indicates susceptibility. Growth on OSA is an indicator of resistance to the entire drug class that is β-lactam antibiotics (Horstmann *et al.*, 2012).

Due to heterogeneous phenotypes amongst MRSP strains, detection in methicillin resistance is complicated, and the National Committee for Clinical Laboratory Standards has thus recommended that the oxacillin salt agar screen (OSA) test be performed. The OSA test is recognized as both sensitive and specific, but between different investigators it varies in sensitivity and specificity. Kali *et al.* (Kali, Stephen and Umadevi, 2014) assessed conventional phenotypic screening studies compared to PCR of the *mecA* gene. 102 clinical MRSA isolates identified by the oxacillin and cefoxitin disk diffusion were subjected to PCR for the *mecA* detection and culture on OSA media. Although all 102 isolates were resistant in oxacillin and cefoxitin disk diffusion, 92 (90.1%) isolates were positive for the mecA gene. Out of the 92 mecA PCR-positive isolates, 91 strains were correctly identified as MRSA on OSA. The sensitivity of the OSA test was shown to be 98.91% in accurately identifying methicillin resistance. Thus, in resource short settings in which molecular methods are not available, the oxacillin screen agar can be used to validate methicillin resistance (Kali, Stephen and Umadevi, 2014).

2.5.2 Antimicobial susceptinbility tests

The purpose of antimicrobial susceptibility tests (ASTs) is to guide antibiotic choice in clinical practice and improve clinical outcomes. Furthermore, ASTs are considered an essential part of surveillance of the responsible use of antimicrobials, in all antimicrobial stewardship programs (Schwarz *et al.*, 2018).

ASTs are typically performed on isolates where the pathogenic organism has already been identified and/or where poor clinical response predicts bacterial resistance. In general, clinically infected areas of an animal are sampled in the form of swabs, these swabs are placed into a growth medium, which are then sent to the laboratory. The swabs are inoculated onto agar plates

so that a pure bacterial culture can be obtained. In order to test sensitivity, antimicrobial-impregnated paper disks are added to the plate, with the tested outcome being bacterial growth around the disk. Veterinary diagnostic laboratories perform either agar disc diffusion (ADD), Etest (agar dilution) or microbroth dilution. Notwithstanding the diagnostic test used, the objective is to predict the clinical response to the antibiotics tested, so as to guide the clinician in appropriate drug selection. To ensure accurate, reproducible results, laboratories should comply with standards established by authorities on antimicrobial susceptibility testing of veterinary pathogens. These authorities include the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). In addition to providing a guideline for the care of a specific patient, susceptibility testing also has a more general, global function in the tracking of resistance patterns of a specific species over the course of time (Córdova-Guerrero *et al.*, 2014).

The setting of antibiotic breakpoints includes factors such as (1) pharmacokinetics, the absorption, distribution, serum and tissue levels elimination of the antibiotic, therapeutic levels in certain parts of the body; (2) pharmacodynamics of antibiotics, i.e. how the antibiotic interacts with the bacteria; (3) minimum inhibitory concentration (MIC), which determines the concentration of antibiotic required to inhibit growth of a pathogen; and (4) clinical efficacy trials (Kahlmeter *et al.*, 2019). A bacteria is defined as either susceptible, intermediate susceptible or resistant, which predicts therapeutic success (susceptible organisms) or treatment failure (when resistant) (Humphries, Abbott and Hindler, 2019). Hence, both EUCAST and CLSI report AST results as “susceptible” (S), “intermediate” (I), or “resistant” (R), but each organization deals with these factors differently and thus precludes them from having comparable breakpoints.

Cusack *et al.* report on practical differences between CLSI and EUCAST methodology and the implications on each methodology in laboratories. Cusack *et al.* highlight key differences such as disc diffusion methodologies where CLSI uses sheep blood for MHA supplementation while EUCAST uses horse blood. In addition, increased expense was encountered when adopting EUCAST methodologies and important differences amongst published zone diameters for certain bacteria, e.g. *Staphylococcus* and Enterobacteriaceae, were noted. Perhaps the greatest difference is the stringent and transparent behaviour of EUCAST breakpoint-setting processes for antimicrobials. The discrepancies in clinical breakpoints between CLSI and EUCAST substantially affect the perception of susceptibility of clinical isolates and impact wider

antimicrobial resistance (AMR) monitoring initiatives, making data comparison between different systems within and between countries difficult (Cusack *et al.*, 2019).

While it is easy for most clinicians and laboratories to interpret and apply results categorized as “S” or “R”, there can be uncertainty about the therapeutic efficacy of an antimicrobial classified as “I”. EUCAST has modified the intermediate category due to the therapeutic uncertainty and uncontrolled technical laboratory factors causing discrepancies in the category. As a result, EUCAST has adopted a “susceptible, increased exposure” category instead, which allows for increased likelihood of therapeutic success by adjusting the dosing regimen to raise the antibiotic concentration at the infection site (EUCAST, 2017).

In addition, EUCAST and CLSI have different classifications for clinical breakpoints. The CLSI has established “epidemiological cut-off values” (ECVs), which are MICs or disk diffusion zone diameters that guide physicians in handling bacterial infections by organisms but cannot be used to predict clinical outcome to therapy (EUCAST, 2017). EUCAST has a similar definition of epidemiological cut-off (ECOFF) values. ECVs and ECOFF values can be useful to assist clinicians to know whether the isolate has a presumed or acquired mutation that might make it less likely to respond to an antimicrobial (EUCAST, 2017). As pharmacokinetic-pharmacodynamic data, as well as clinical outcome data becomes increasingly available for different antimicrobials, this information, when combined with *in vitro* susceptibility test results, can be used to define clinical breakpoints (McAdam, 2019).

In conclusion, the method selected by a laboratory is based on a number of factors, including the cost, the availability of suitable antimicrobial agents and the sample volume to be examined in the laboratory (Schwarz *et al.,* 2018). Most veterinary laboratories in South Africa use either disc diffusion or MIC testing according to either CLSI or EUCAST clinical breakpoint tables (Brink *et al.*, 2007). The selected method will also be influenced by the output of the laboratory – the ADD approach is used by smaller laboratories with relatively low case volumes, whereas larger laboratories will use semi-automated MIC methods such as broth microdilution (BMD).

2.5.3 Cefoxitin / Oxacillin Disc Diffusion

Disc diffusion is one of the oldest AST strategies, and continues to be a commonly used method in microbiology laboratories for testing of antimicrobial sensitivity (Matuschek, Brown and Kahlmeter, 2014). This is due to the lower cost per test compared to other ASTs, antimicrobial selection flexibility, and easy-to-assemble assays. The EUCAST disc diffusion method uses two media: Mueller–Hinton (MH) agar and MH agar with 5% defibrinated horse blood (MH-F). MH is the agar of choice for non-fastidious organisms such as *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus* and *Enterococcus*. MH-F is used for fastidious organisms such as *Streptococcus*, *Pasteurella* and several others (EUCAST, 2017).

Agar plates are prepared and kept at room temperature prior to inoculation and must be dried prior to use to remove excess moisture on the plate surface. Using a freshly prepared bacterial suspension (in saline equivalent to 0.5 MacFarland), plates are inoculated by taking a sample of the suspension with a sterile cotton swab and applying this to the prepared agar plate – either by streaking the swab in three directions or by use of a plate rotator. Antimicrobial disks must warm to room temperature prior to use. Disks are applied within 15 minutes of streaking the plates and are placed firmly onto the surface of the medium. Disks are spaced evenly to ensure zones of inhibition of susceptible isolates do not overlap, as this would impede the measurement of zone diameters. The disks are filter-paper impregnated with an antibiotic at a particular concentration (Jorgensen and Ferraro, 2009).

Inhibition zones are measured after a predetermined period of incubation. The inhibition zone is taken as the point where there is no obvious growth on the plate when examined with the naked eye from 30 cm away. The diameters of the inhibition zone can then be determined by using a calliper, ruler, or an automated zone reader. MH agar plates must be viewed from the back, whilst reflecting light onto the plate, which is held against a dark background. MH-F agar plates are viewed with reflected light from the front, lid removed. The outcome is the “zone of inhibition”, and the results are measured to the nearest millimetre, which are indicative of “clinical breakpoints” – namely the classification of the organism as S, I or R to each of the antibiotics tested (**Figure 13**).

EUCAST uses the ECOFF as one of many tools in the formulation of clinical breakpoints. These breakpoints are calibrated to the EUCAST clinical MIC breakpoints, which have been established by analysis of MIC-zone diameters, as well as inhibition zone diameter and MIC distributions

(EUCAST, 2017). Based on the cut-off values, an antibiogram profile is generated which represents specific antimicrobial drugs that are effective in killing the organism (Jorgensen and Ferraro, 2009).

Figure 13. A suspension of the organism is inserted into an agar plate (usually: Mueller–Hinton agar) containing filter-paper disk impregnated with a certain antibiotic at a particular concentration (Jorgensen and Ferraro, 2009). The outcome of the assay is a "zone of inhibition," and the results are measured to the nearest millimetre, which are indicative of "clinical breakpoints" – namely classification of the organism as susceptible, intermediate, or resistant to each of the antibiotics tested.

2.5.3.1 Cefoxitin / Oxacillin Minimum Inhibitory Concentration by Broth Microdilution

The MIC is defined as the lowest concentration of an antibiotic that prevents visible growth of the bacteria. A number of MIC methods are used in diagnostic laboratories, such as the (1) agar dilution method, (2) the gradient strip method, which combines an agar-based diffusion method with a dilution method to determine MIC and (3) semiautomated broth microdilution methods (Procop and Koneman, 2016). These techniques determine the *in vitro* activity of an antibiotic against a bacterial culture of interest. The isolate of interest must first be cultured, so that a pure culture can be obtained – an inoculum from this pure culture is then added to a series of containers (tubes, agar plates, or wells) with either a broth or agar medium and increasing concentrations of an antimicrobial agent (**Figure 14).** This test method is particularly useful for species for which there are no conditions for disk diffusion, or for certain fastidious species that do not grow on MH agar (Lainhart, Yarbrough and Burnham, 2018).

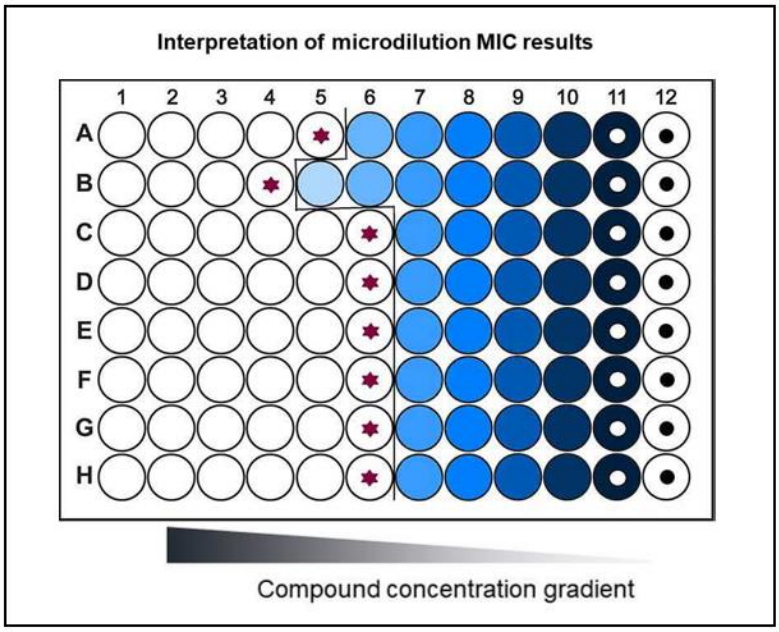

Figure 14. Interpretation of microdilution results with one strain and different antibiotics, where "no growth" is represented by the white circles and "growth" is represented by blue circles. Reading the plate from left to right indicates the declining concentration of the anti-microbial and the lowest concentration of the antimicrobial where no growth is observed is termed minimum inhibitory concentration (Andrews, 2001)

2.5.3.2 Oxacillin / Cefoxitin Clinical Breakpoints

Disc diffusion and MIC have been used to determine oxacillin resistance in *S. pseudintermedius* in commercial laboratories. In *S. aureus*, cefoxitin is endorsed by both EUCAST and CLSI as the preferred agent for detecting MRSA and methicillin-resistant coagulase-negative *Staphylococcus* (MRCoNS) isolates by disc diffusion (Clinical and Laboratory Standards Institute, 2015; EUCAST, 2017). However, for *S. pseudintermedius*, EUCAST advocates the use of cefoxitin, whereas CLSI recommends oxacillin for detection of MRSP as cefoxitin is not a good inducer of the *mecA* in *S. pseudintermedius* (Clinical and Laboratory Standards Institute, 2015; EUCAST, 2017).

Moreover, the cefoxitin breakpoints recommended for the detection of MRSA are not reliable for prediction of MRSP (Bemis *et al.*, 2009). This is illustrated in a study by Siak *et al.*, which compared the accuracy of methicillin resistance detection among MRSP isolates using *mec*A PCR with the phenotypic resistance to oxacillin and cefoxitin. The results of this study emphasized that higher breakpoints of ≤30 mm for oxacillin should be used in the definition of MRSP. Oxacillin was also more suitable for the identification of MRSP compared to cefoxitin (Siak and Burrows,

2013). A more recent study by Skov *et al.* also confirmed that the oxacillin disk produced a better result than the cefoxitin disk with respect to *mecA*-mediated methicillin resistance in *S. pseudintermedius* (Skov *et al.*, 2020). Wu *et al.* (Wu *et al.*, 2016) tested oxacillin and cefoxitin disk zones and MICs for a series of 115 SIG isolates (from both human and veterinary samples) and correlated these findings to *mecA* PCR. An interesting outcome of this study was the clear division between oxacillin MICs for *mecA*-positive versus *mec*A-negative isolates – all *mecA*-positive isolates had MICs that were ≥0.5 µg/ml whilst MICs all measured ≤0.25 µg/ml for all *mecA*-negative isolates (Wu *et al.*, 2016). This data should encourage veterinary laboratories to comply with CLSI requirements and to validate extrapolations to veterinary isolates from human isolates prior to use.

2.5.3.3 Direct Detection of Penicillin Binding Protein 2a (PBP2alpha) by Latex Agglutination Test

The intention of the latex agglutination assay is to rapidly detect the expressed protein product of *mecA*, as an indirect means of detecting *mecA* in MRSA and MRSP. The PBP2a latex agglutination test (produced by ThermoFisher) is a 5-minute test. The test is reported to be faster, more user-friendly and less labour-intensive than other genotypic modalities like PCR for *mecA* and oxacillin screening agar tests. This test has been reviewed worldwide and demonstrates a high sensitivity and specificity (Hussain *et al.*, 2000; Bemis *et al.*, 2006; Maluping, Paul and Moodley, 2014). A high level of agreement has been shown between PBP2a results and conventional *mecA* PCR in canine staphylococcal isolates (Bemis *et al.*, 2006).

It is important that the bacteria are incubated with an oxacillin disc to induce the expression of *mecA*. If the incubation is not adhered to, the protein will not be detected in bacteria that express the gene in a constitutive form. Generally, the PBP2a distribution test includes a latex reagent consisting of an antibody against PBP2a that reacts with exposed PBP2 from the MRSA bacterial membranes. Preparation of extracts entails boiling a suspension of the inoculum under alkaline conditions, neutralization and then centrifugation. The supernatant can then be mixed with the latex reagent on a test card. A positive test, suggestive of PBP2a presence, occurs when there is agglutination on the test cards within 3 minutes of mixing. The main limitation of the PBP2a latex agglutination test is its inability to discriminate between *Staphylococcus* species (Akcam *et al.*, 2009).

2.6 Genotypic Identification

2.6.1.1 PCR for Detection of *mecA*

To date, an extensive number of PCR tests have been described to identify MRSP (Haenni *et al.*, 2014; Maluping, Paul and Moodley, 2014). A literature search on PUBMED revealed 21 studies using PCR testing of isolates, which identified the presence of *mecA* (the gene conferring resistance to β-lactam antimicrobials). While PCR for the *mecA* gene is the gold standard for defining MRSP, chait fails to provide any further information, such as the DNA sequence polymorphism in order to assess MRSP species, or clonal identity of MRSP in order to study the genome composition from *mec*A sequence analyses. Furthermore, false negatives can occur, in which true MRSP organisms are not identified because the gene is not expressed or absent (Chambers, 1997; Brown *et al.*, 2005).

2.6.1.2 Molecular Typing Methods of Methicillin resistant *S. pseudintermedius*

Once the characterization of bacterial species has been performed phenotypically, molecular typing can be used for studying the bacterial genome composition. Molecular analysis may be used directly if the phenotypic findings are inconclusive, or not available, thereby offering immediate support for an optimal treatment or control strategy. Molecular typing offers insight into the behaviour and acquisition of resistant genes. This information allows clinicians to track the occurrence of drug-resistant strains in a health care system or to determine whether a cluster of infections is related or unrelated in an outbreak (Coleman and Tsongalis, 2016). As a result, molecular characterization of the resistant genes is an important part of clinical investigations of bacterial infections (Schwarz *et al.,* 2018). Some of the molecular methods, such as PCR and hybridization techniques, are used routinely in South Africa for the typing of MRSA, although new methods such as whole genome sequencing (WGS) are also used.

To date, there is limited molecular epidemiological data available for MRSP. Different typing methods continue to be used with no agreement on the optimal approach. A variety of molecular methods have been used to accurately identify and characterize *S. pseudintermedius* isolates so as to better describe their distribution and study transmission. A standardized sequence-based

technology would be the preferred method for typing MRSP, as this is the most robust means of identifying and tracing MRSP (Kadlec *et al.*, 2015). This would allow a worldwide comparison in the study of hospital outbreaks and surveillance studies, evaluating molecular changes in MRSP.

2.7 Epidemiology and Zoonotic Potential

The implications of the zoonotic potential of canine staphylococcal organisms have increased the need for more research. As a non-commensal human organism, MRSP should be considered an emerging zoonotic agent, especially as the number of cat and dog MRSP infections continues to increase (Bond and Loeffler, 2012). MRSP shares similar characteristics with the human MRSA, including the risk factors for acquisition – repetitive antimicrobial therapy, frequent hospital visits and invasive procedures. This poses a risk in multi-pet households or dogs living with immune-compromised owners. Other zoonotic species of the SIG group are uncommon, however a recent publication showed the first human isolation of *S. delphini* (Magleby *et al.*, 2019). The first clinical report of a human *S. pseudintermedius* infection was reported in 2006 (Van Hoovels *et al.*, 2006).

Globally, prevalence of MRSP associated infections reported in human health care facilities has increased. A retrospective review conducted over two years (2013–2015) by Somayaji *et al.* (84) in a human hospital, isolated 27 *S. pseudintermedius* isolates from 24 human cases. *S. pseudintermedius* was isolated in 75% (18/24) of skin and soft tissue infections and methicillin resistance was found in 22,2% of those. Amongst the patients audited in this study, 92,1% of those who were infected with *S. pseudintermedius* were either living with a dog or had continual contact with a dog (Somayaji *et al.*, 2016).

More recently, Kronbichler *et al*. (Kronbichler *et al.*, 2019) provided the first evidence of chronic *S. pseudintermedius* nasal carriage in humans with polyangiitis granulomatosis. All isolates were genotypically *mecA*-negative and responsive to methicillin (Kronbichler *et al.*, 2019). A similar finding was made in patients who suffered from chronic sinonasal infections that were otherwise refractory to standard medical management. Findings indicated a correlation between *S. pseudintermedius* in the human patients who owned a dog ($p<0.01$) and most isolates displaying multidrug resistance, making the human infections difficult to treat (Ference *et al.*, 2019).

MRSP has been shown to survive in the environment for extended periods of time and therefore there is an opportunity to contract MRSP-related infections unknowingly through indirect forms of contact (Laarhoven *et al.*, 2011). A study done in 2011 in Norway, showed that 2,1% of rats residing in impoverished neighbourhoods with high levels of unemployment, intravenous drugs use and HIV infection, were colonised with MRSP. Although isolation of MRSP from rats does not necessarily prove causation, rats are a well-documented source of other zoonotic pathogens and have become increasingly popular as pets, thus increasing the risk of *S. pseudintermedius* transmission to humans. Since MRSP has a long-term survivability in the environment, bacteria may pass from rats to humans and dogs or vice versa (Himsworth *et al.*, 2013).

A small number of studies shows a high prevalence of MRSP shared between owners and their pets, although not all studies have demonstrated human colonisation of MRSP. The similarity of *Staphylococcus* isolates between 119 dogs and 107 owners was assessed by means of a contrasting cross-sectional trial by Han *et al*. (Han, Yang and Park, 2016). They found no association between the bacteria shared between healthy dogs and their owners (Han, Yang and Park, 2016). A 2019 study conducted in 303 dogs and 80 cats in six communities in New South Wales (NSW), Australia characterized MRSP and MRSA from indigenous people who are disproportionately affected by these organisms in community-acquired infections. This study did not isolate MRSP from communities in NSW (Ma *et al.*, 2019a). The absence of MRSPs in NSW cats and dogs, along with the low overall levels of methicillin resistance, is probably associated with limited antibiotic use as a result of limited veterinary care (Ma *et al.*, 2019a).

2.8 Veterinary Environment

MRSP can spread in veterinary facilities and the community at large through indirect means such as equipment, infected veterinary workers, and colonised or contaminated patients. Risk factors that make MRSP a danger in the veterinary setting include antimicrobial use, animals with existing co-morbidities predisposing them to infection, a lack of appropriate hygiene protocols, and ineffective disinfection. MRSP infected dogs, in contrast to dogs with methicillin-susceptible *S. pseudintermedius* infections, have typically been exposed to antimicrobials within the 30 days prior to MRSP detection. This perhaps suggests that antimicrobial use predisposes dogs to MRSP infections (Weese, 2013).

A year-long MRSA active surveillance program, conducted at the veterinary medical centre of Ohio State University, demonstrated causal factors for the acquisition of methicillin-resistant diseases. These included recent veterinary hospital release, prolonged hospital stay, recent surgery (preceding 90 days), history of antibiotic use, and owners who had a veterinary-related occupation (van Balen *et al.*, 2013).

Due to the close association between veterinarians and animals with MRSP, veterinary professionals account for a high percentage of carriers of MRSP. Paul *et al.* studied MRSP and MRSA prevalence amongst small animal dermatologists at an Italian National Veterinary Conference where 128 veterinarians had nasal swabs collected. Seven were carrying MRSP (n = 5, 3.9%) or MRSA (n = 2, 1.6%). Amongst the 128 veterinarians, five carried MRSP clones that had recently emerged in cats and dogs (Paul *et al.*, 2011).

Contrary to growing reports showing an overall increase in the prevalence of MRSP and methicillin-susceptible *S. pseudintermedius*, a study carried out in Australia by Worthing *et al*. (Worthing *et al.*, 2018). in two veterinary hospitals showed limited methicillin-resistant Staphylococci transmission between veterinary staff and pets or among members of the veterinary community (Worthing *et al.*, 2018). The variability in the levels of *Staphylococcus* colonisation and the decreased antimicrobial resistance of the *Staphylococcus* isolates in this study may reflect environmental factors at host and local level or simply reflect stricter sanitary precautions.

A nosocomial MRSP outbreak in a Finnish veterinary hospital in November 2010–January 2012 led to the implementation of strict hygiene policies and control measures in order to regulate the outbreak. These policies included the frequent use of alcohol-based hand rubs (before and after every patient) and the use of personal protective equipment when handling MRSP patients. Furthermore, a strict “search and isolate” process of all patients entering the hospital for the first time that were potentially colonised with multidrug resistant organisms was implemented to avoid further contamination. All these policies were crucial in the cessation of the MRSP outbreak (Grönthal *et al.*, 2014).

One of the characteristics of *S. pseudintermedius* which allows it to adapt to the veterinary environment, is the production of a biofilm. Biofilm production is an important virulence factor, as it enhances the adherence of the bacterial cells to surfaces. Infections that produce a biofilm are

significantly more pathogenic, because sessile bacteria are typically more resistant to antibiotics and surface disinfectants than non-sessile phenotypes, and can withstand host immune responses more easily (Meroni *et al.*, 2019). Strict hygiene practices must be followed to monitor and prevent nosocomial infections. Reports of resistance to disinfectant are rare, but some bacteria have shown tolerance that builds up over time, mediated either by exogenous mobile genetic elements or through intrinsic genetic adaptation (Mc Carlie, Boucher and Bragg, 2020).

2.8.1 Diagnostic Surveillance of MRSP and Antimicrobial Usage Behaviour

Before the emergence of MRSP, most *S. pseudintermedius* infections were treated with antimicrobials empirically, with relative success. More recently, the bacterium has undergone mutations or acquired genes that has resulted in β-lactam resistance. As MRSP prevalence increases, the surveillance of MRSP in clinical practice should increase too, as it influences the antimicrobial prescribing behaviour of veterinarians.

Conceivably, veterinarians continue to see resistant infections in the skin and ear. It highlights the deficiency in veterinarians addressing underlying causes, as well as perpetual prescribing of antibiotics for bacterial infections that leads to resistance. Factors that drive antimicrobial usage include individual prescribing behaviour and practice norms. Other factors that lead to resistance apart from pharmacokinetic and distribution, include lack of owner compliance, clients who indiscriminately use leftover antibiotics without prior consultation with their veterinarians and inappropriate usage of antibiotics in the face of fungal infections.

King *et al.* (King *et al.*, 2018) evaluated the antimicrobial driving behaviour of veterinarians and owners who had received antimicrobials during consultations. This behavioural study concluded that increased communication about antimicrobial resistance resulted in less antimicrobial usage amongst owners and veterinarians. Interestingly, there was a perceived or real client demand for antibiotics amongst veterinarians especially in time-limited consultations, which led to “just in case” antimicrobial use to avoid treatment failure. Upon questioning owners in this study, most owners reported that antimicrobials were given to them by the veterinarian because they trusted the clinician to make the best judgement and assumed it was thus appropriate. These findings indicate that this client demand for antibiotics is perhaps perceived rather than real. Both parties agreed that better communication would enhance a decrease in antimicrobial usage but would be hindered by short consultation time. Moreover, practice culture was also found to influence overall

antimicrobial usage; veterinarians over 40 years of age were more inclined to prescribe antimicrobials as opposed to younger veterinarians who were more conscious of antimicrobial stewardship and resistance. However, in practices where antimicrobial policies had been unchanged for the past decade, younger veterinarians were more likely to adapt themselves to these polices to avoid conflict with their superiors, which represented a barrier to change to conserve antimicrobials (King *et al.*, 2018). Knowledge of these factors are necessary to inform and address intervention measures amongst the general public and clinicians, and ultimately reduce overall use.

Perhaps the greatest limitation in achieving appropriate diagnostic surveillance in veterinary practice is the cost implications associated with culture and antibiotic sensitivity for owners. Clinicians often encounter skin infections and most first occurrences are treated empirically. However, these treatment plans can enhance the risk for further resistance of an MRSP strain that does not respond to the empirical treatment. In refractory cases, an MRSP infection should be suspected and culture and antibiotic sensitivity should be implemented. Culture and antibiotic sensitivity should guide clinicians into making evidence-based decisions, which is a practical approach to antimicrobial stewardship in small animal medicine (Norris *et al.*, 2019).

Key steps in the appropriate usage of antimicrobials include the accurate diagnosis of bacterial infections, selection of the appropriate antibiotic, administration at the correct dose until clinical cure and the diagnosis and treatment of the underlying disease. Chipangura *et al.* (Chipangura *et al.*, 2017) investigated antimicrobial usage patterns by small animal veterinarians in South Africa. Findings in this study showed a lack of antimicrobial prudence according to usage guidelines within South Africa, with irrational use amongst many veterinarians (Chipangura *et al.*, 2017).

2.9 Control options

Antibiotic resistance has thus made the treatment of staphylococcal skin infection in dogs increasingly difficult. An important consideration is the potential for a global threat to human health, as these animals can become reservoirs of such strains, with potential horizontal transmission between animals and humans. Thus, there is a need to thwart and control *S. pseudintermedius* infection in dogs. In order to elicit the required change, non-antimicrobial options are needed to decrease the prevalence of MRSP.

2.9.1 Non-Antimicrobial Control Options

There is currently insufficient research on signs of animal MRSP decolonisation. Moreover, there is little evidence of the efficacy of daily application of steps to decolonise these animals, such as the application of disinfecting shampoos. While studies of the precipitating factors associated with MRSP infections are uncommon, it can be concluded that animals colonised with MRSP have a higher risk of developing an MRSP related infection in the case of injuries (surgical or non-surgical) and antimicrobial exposure (van Duijkeren *et al.*, 2011).

Non-antimicrobial treatment may involve washing the animal with products containing chlorhexidine, for example, which may aid skin decontamination. A longitudinal study conducted by Windahl *et al.* (Windahl *et al.*, 2012) demonstrated that long-term animal colonisation with MRSP can persist as long as one year after clinical infection (Windahl *et al.*, 2012). House cleaning and disinfection is likely to help avoid re-colonisation (van Duijkeren *et al.*, 2011).

Surgical wound infections are a common source of MRSP. Improving wound care and control without using antimicrobial drugs is necessary and preferable. Reducing the use of antimicrobials involves proper wound cleaning and debriding of the contaminated wounds with topical wound care antiseptics, including chlorhexidine and iodine products (e.g. povidone-iodine) (van Duijkeren *et al.*, 2011). Topical therapy with chlorhexidine digluconate products may be as effective as systemic therapy (Borio *et al.*, 2015). Other studies have demonstrated that sodium hypochlorite (6.15%) is an effective agent for decontamination, and may be sufficient as sole treatment for cases of superficial pyoderma (Pariser *et al.*, 2013).

In otitis cases, cleaning the ear is vital as it facilitates inspection of the ear canals, allows for removal of materials containing microorganisms, inactivation of the biofilm and removal of small foreign cells, toxins and damaged degenerated cells (Nelson and Couto, 2009). A commercially available ear antiseptic containing a combination of chlorhexidine and Tris-EDTA demonstrated strong bactericidal activity against MRSP *in vitro*. Infections that are localized to the middle and inner ear canal are likely to be less controlled with antiseptic solutions (van Duijkeren *et al.*, 2011).

New approaches to canine pyoderma prevention, such as vaccines, may help boost the control options. An alternative management option for MRSP infections is the use of lytic-active

bacteriophages to MRSP**.** Moodley *et al.* (Moodley *et al.*, 2019) described bacteriophages as a treatment option for complicated staphylococcal infections. A bacteriophage is a virus, which infects and reproduces inside the bacterium. Since some staphylococcal infections are highly resistant, bacteriophages against MRSP may be developed for topical treatment of MRSP in skin and wound infections (Moodley *et al.*, 2019). To date, evidence on the efficacy of bacteriophages or lysins in the management of MRSP infections (prevention or treatment) has not been published, nor are there approved products containing bacteriophages or lysins (Moodley *et al.*, 2019; van Duijkeren *et al.*, 2011). Other drugs, such as oxyclozanide, have demonstrated in vitro antibacterial activity against MRSP (Levinson *et al.*, 2019).

2.9.2 Antimicrobial Control Options

The effectiveness of antimicrobials in decolonizing animals is not well demonstrated and thus the use of antimicrobials simply for this purpose would potentially increase the likelihood of further resistance selection. To date, local or systemic application of antimicrobials for decolonizing MRSP carrier animals has not been studied or approved (van Duijkeren *et al.*, 2011).

Since the clinical outcome of MRSP infections is variable, there is no standardized management plan. Individualising patient care is therefore essential. When deciding on the appropriate treatment for the patient that is clinically infected with MRSP, consideration should be given to the susceptibility profile of MRSP isolation from animals, the extent and location of the infection, the occurrence of systemic or other underlying disease and any pre-existing co-morbidity. Knowledge on the effectiveness of antimicrobial treatment of MRSP-infected animals is scarce; the only data on MRSP-infected patients' outcomes that is available, involves case reports on a limited number of patients (Wettstein *et al.*, 2008) (van Duijkeren *et al.*, 2011). Furthermore, the possible use of antimicrobials in animals that are essential for human MRSA care is controversial because of the possibility of developing resistance to such agents. As a result, legal limits on the use of particular antimicrobial agents in animals are already in place in certain European countries (van Duijkeren *et al.*, 2011). Additional information is required on the efficacy of different therapeutic approaches in MRSP-infected animals, and work should therefore concentrate on non-antimicrobial approaches for wound care, pyoderma and OE (van Duijkeren *et al.*, 2011).

2.10 Prevention of Transmission

The British Small Animal Veterinary Association (BSAVA), amongst others, has developed guidelines for the management of MRSA in veterinary practices. These practice standards extend to MRSP as well. In a veterinary environment, patients with confirmed or suspected MRSP should be isolated to reduce the risk of transmission, in accordance with normal infection control guidelines. The use of barrier safety procedures and restricting contact with staff involves wearing protective tags, protective gear and gloves in veterinary centres. The spread of MRSA and MRSP would be decreased by proper care and cleaning of contaminated areas. Obviously, proper hygiene is the key to reducing the spread of MRSP amongst animals and between humans and animals (van Duijkeren *et al.*, 2011).

2.11 Mechanisms of Antimicrobial Resistance

Antimicrobial resistance is an urgent, global threat to human health and animals may become reservoirs and sources of horizontal transmission of such resistant strains to humans (Fitzgerald, 2009; Weese, 2013)**.** The prevalence of MRSP in healthy dogs, from the results of various global studies, ranges from 0–4.5% (Griffeth *et al.*, 2008; Schmidt *et al.*, 2014; Wedley *et al.*, 2014; Hanselman, Kruth and Weese, 2008; Vengust *et al.*, 2006; Murphy *et al.*, 2009), to 8–34% (Beck *et al.*, 2012; Sasaki *et al.*, 2007b), to up to 66% (Kawakami *et al.*, 2010). There are a number of molecular mechanisms that lead to drug resistance in MRSP. These include mutations, acquisition of mobile genetic elements, phenotypic alterations, the presence of drug efflux pumps, and the alteration of the antimicrobial target. There are three forms of acquired resistance, conjugation, transduction and transformation.

2.11.1 Non beta-lactam Resistance in MRSP

Schmidt *et al.* (Schmidt *et al.*, 2018) evaluated the impact of frequently used antimicrobial agents on the selection and persistence of antimicrobial resistance. This study, conducted in the United Kingdom using staphylococci samples isolated from canine mucosa, demonstrated that levels of resistance to most antimicrobials increased in staphylococci immediately post-treatment, with an overall increase in the prevalence of MRSP (Schmidt *et al.*, 2018).

2.11.2 Macrolides and Lincosamides

Tylosin and Erythromycin are two commonly used first-line macrolide antimicrobials in the systemic treatment of pyoderma. Both have a narrow Gram-positive spectrum and are relatively inexpensive. One disadvantage of Erythromycin is that cross-resistance with lincosamides can occur. Commonly utilized lincosamides include Clindamycin and Lincomycin, which have a narrow Gram-positive and anaerobic spectrum. Both have excellent first-line antibacterial treatment properties for pyoderma (Leclercq, 2002; Patel, Forsythe and Smith, 2008). Resistance to macrolide and lincosamide antibiotics is caused by ribosomal methylation or mutation modification at the ribosomal target site of antibiotic attachment, which inhibits the antibiotic action. There are two additional forms of resistance:

- Antibiotic flux
- Drug inactivation (Leclercq, 2002; Patel, Forsythe and Smith, 2008)

2.11.3 Resistance to Tetracyclines

Doxycycline is the most commonly used tetracycline in small animals due to its lipid soluble composition in comparison to other tetracyclines. Furthermore, it is strongly protein-bound in canine plasma, which enhances its absorption and increases its overall effectivity (Maaland *et al.*, 2013). Doxycycline is often used in the management of respiratory tract infections and staphylococci skin infections (Maaland *et al.*, 2013).

Tetracyclines bind to the 30S ribosomal subunit of susceptible organisms, thereby interfering with the binding of aminoacyl-tRNA to the messenger RNA molecule/ribosome complex. This impedes protein synthesis in multiplying bacteria. Antibiotic resistance in tetracyclines is mediated by a number of mechanisms, namely (Grossman, 2016):

1. Tetracycline-specific resistance genes carried and transferred by mobile genetic elements.
2. Ribosomal binding site mutations.
3. Increased expression of intrinsic resistance mechanisms following mutations in the chromosomes (Grossman, 2016)

The CLSI breakpoints for doxycycline effectiveness in *Staphylococcus* in canines are currently based on human clinical breakpoints. Maaland *et al.* (Maaland *et al.*, 2013) aimed at defining doxycycline susceptibility breakpoints amongst canine *S. pseudintermedius*. Following

pharmacokinetic/pharmacodynamic analysis, the researchers confirmed that human breakpoints were unsuitable for canine *S. pseudintermedius* isolates and instituted new breakpoints for disk diffusion testing specific to dogs (Maaland *et al.*, 2013).

2.11.4 Resistance to Fluoroquinolones

Enrofloxacin and marbofloxacin are two antibiotics commonly used in the treatment of MRSP. Fluoroquinolones have broad spectrum coverage and are effective against Gram-negative *Pseudomonas* infections. Due to the wide spectrum of activity, ease of administration and low levels of toxicity, fluoroquinolones have become widely used for treating skin and urinary tract infections. These drugs have good oral absorption and become widely distributed into the surrounding tissues (Martinez, McDermott and Walker, 2006). Fluoroquinolones are, however, expensive and over-usage has resulted in the emergence of resistant *Staphylococcus* and *Pseudomonas* infections (Hooper, 2001; Patel, Forsythe and Smith, 2008). Fluoroquinolones inhibit bacterial DNA gyrase, an enzyme that is responsible for DNA supercoiling during replication of the separating strands. This inhibition results in degradation of chromosomal DNA at the replicating fork (Hooper, 2001).

Oral absorption varies amongst the various classes of fluoroquinolones. Respectively, marbofloxacin, enrofloxacin, difloxacin, and orbifloxacin have an 80% absorption rate from the gastrointestinal tract with 100% oral bioavailability (Boothe, 2012)· Ciprofloxacin, by comparison, has been shown to have about 80% absorption but only 40% bioavailability for dogs and 33% bioavailability for cats (Boothe *et al.*, 2006; Albarellos, Kreil and Landoni, 2004). For dogs and cats, 10–40% of enrofloxacin that is consumed is converted into ciprofloxacin (Albarellos, Kreil and Landoni, 2004).

The MIC for fluoroquinolones is low when treating canine pyoderma infections and thus higher dosages are recommended. Nseir *et al*. (Nseir *et al.*, 2005) demonstrated that low dosages of fluoroquinolones may upregulate the genes responsible for methicillin resistance (Nseir *et al.*, 2005) associated with the *mecA* gene. Therefore, exposure to suboptimal doses of fluoroquinolones increases the risk of not only fluoroquinolone resistance but also methicillin resistance amongst MRSP.

A fourth-generation fluoroquinolone, pradofloxacin, is the most recent antibiotic for use in small animals. Pradofloxacin was developed for the treatment of wound sepsis, superficial and deep pyoderma, as well as urinary tract, gingival, periodontal, and acute upper respiratory infections (Arslan *et al.*, 2005). Pradofloxacin's efficacy in the treatment of deep pyoderma was recently compared with an amoxycillin, clavulanic acid combination agent. The results established that 86% of dogs and cats in the clinical trial on pradofloxacin achieved clinical remission, while 73% on amoxycillin/clavulanic acid achieved clinical remission. These results show that pradofloxacin for deep bacterial pyoderma is an effective therapy equivalent to amoxycillin with clavulanic acid (Mueller and Stephan, 2007).

2.11.5 Resistance to Aminoglycosides

The most commonly used aminoglycoside in small animal practice is gentamicin, which is applied topically into the ear canal. Amikacin can also be used systemically. Aminoglycoside's mechanism of action is to alter the genetic code. It achieves this through interruption of protein synthesis by attaching to the ribosomal subunit 30S (Ida *et al.*, 2001). Disadvantages of these agents that preclude them from routine use in staphylococcal infections are the potential nephrotoxic effects and the inconvenient route of administration. However, the increase in MRSP has left clinicians with little choice in antimicrobial treatment, making aminoglycosides the last option available (Gold, Cohen and Lawhon, 2014).

Resistance to aminoglycosides in staphylococci is mediated through cellular aminoglycoside-modifying enzymes (AMEs). Aminoglycoside resistance in MRSP has been identified in various studies (Fitzgerald, 2009; van Duijkeren *et al.*, 2011; Hensel, Zabel and Hensel, 2016; Rota *et al.*, 2013; Gold, Cohen and Lawhon, 2014). Gold *et al.* (Gold, Cohen and Lawhon, 2014) evaluated the prevalence of amikacin resistance in *S. pseudintermedius* in 422 dogs. The results demonstrated that methicillin-resistant isolates were more likely to exhibit amikacin resistance as well, (37%, 31 of the 84 samples) when compared to methicillin-susceptible isolates (7%, 22 of the 338 samples) (Gold, Cohen and Lawhon, 2014). The increase in amikacin resistance has implications for the management of life-threatening infections in veterinary medicine and underlines the need for prudent antimicrobial use.

2.12 Summary

The presence of MRSP has rapidly increased in cats and dogs, primarily because of clonal spread. Due to these bacteria's multi-resistant properties, they represent a large challenge to animal and human healthcare. The considerations surrounding the management of staphylococcal skin infections in dogs have thus become increasingly burdensome. Furthermore, antibiotic resistance is an urgent, global threat to human health, as animals can become reservoirs of resistant strains (Fitzgerald, 2009) with potential horizontal transmission between animals and humans (Weese, 2013)**.** Therefore, there is urgent need for efficacious measures to thwart and control *S. pseudintermedius* infection and spread in dogs. In order to elicit the required change, a comprehensive understanding of the ecology of this microorganism, along with background knowledge on the bacterial and host factors involved in its pathogenesis are needed. Various methods exist to identify MRSP, phenotypically and genotypically. However, with the increasing emergence of multiple bacterial resistance mechanisms, effective antibiotic treatment of identified MRSP infections becomes ever more difficult.

Chapter 3: Rationale & Objectives of the Study

3.1. Background

Staphylococci are normal mucosal and skin commensals found in humans and animals. *S. pseudintermedius* is the main coagulase positive *Staphylococcus* (CoPS) species in dogs (Patel, Forsythe and Smith, 2008). Currently, the main treatment for *S. pseudintermedius* is primarily through the use of systemic or topical antibiotics. However, the recent emergence of methicillin- and multidrug-resistant staphylococcal skin infections has necessitated a dramatic change in philosophy of oral antibiotic treatment (Jeffers, 2013).

No information is available on the prevalence and genetic characteristics of these antibiotic-resistant organisms in companion animals on the African continent. This information is crucial for understanding the ecology and epidemiology of MRSP. Therefore, this study reports on the isolation of MRSP strains from dogs with pyoderma and otitis from various provinces in South Africa and their preliminary genomic characterization.

3.2. Problem Statement

There is currently no literature describing the presence of MRSP from canine pyoderma and OE in South Africa and there is no description of the presence of the known antimicrobial resistance gene in this microorganism in circulation in the country.

3.3. Research Questions Related to this Study

- Is there a good association between the laboratory diagnosis of *S. pseudintermedius* and the PCR confirmation of *S. pseudintermedius*?
- Is there an association between the disc diffusion result of antibiotic resistance on each isolate and the presence of *mecA*?
- Is there an association between the presence of *mecA* and previous antibiotic use?
- Is there an association between the presence of *mecA* and a specific antibiotic class used previously?

- Is there an association between the presence of *mecA* and the number of antibiotic classes used previously?
- Is there an association between geographic origin of the sample or dog breed and the presence of *mecA*?

3.4. Hypothesis

The following is hypothesized for this study:

- There an acceptable coordination between the laboratory diagnosis of *S. pseudintermedius* and the PCR confirmation of *S. pseudintermedius*
- There is an association between the disc diffusion result of antibiotic resistance on each isolate and the presence of *mecA*
- Isolates that are identified as presumptive MRSP by conventional culture and antibiogram will carry *mecA.*
- The prevalence of MRSP (*mecA* positive) will be low (<5% of cases) in isolates from dogs with pyoderma or OE from the major centers in South Africa.
- All cases identified as MRSP at a molecular level will have a history of second tier antibiotic usage.

3.5. Research Objectives

3.5.1 Primary Objectives

- To provide preliminary point prevalence data for MRSP in South Africa.

3.5.2 Secondary Objectives

- To provide preliminary data on the presence of the antimicrobial resistance gene by geographical centre in South Africa.
- To provide preliminary data on the antimicrobial susceptibility trends of MRSP in South Africa and to draw a comparison between the susceptibility profile of the isolate and historic antibiotic treatment given to the dog.

3.6. Study Outcomes

- The research was undertaken by the author, Cameron David Prior, as part of the requirements towards fulfilment of a postgraduate MSc Veterinary Science degree.
- The study provides the first molecular description of MRSP amongst clinical isolates from dogs in South Africa.

Chapter 4: Methods

4.1. Introduction

S. pseudintermedius is the most frequent bacterial pathogen isolated from canine skin and ear infections, (Ma *et al.*, 2019; Bourély *et al.*, 2019) and is a leading cause of pyoderma, which accounts for 20% of ear infections in dogs presenting to veterinarians (Cole *et al.*, 2006). The worldwide spread of MRSP has become a significant animal health problem (Hensel, Zabel and Hensel, 2016). In South Africa alone, there is an estimated 9.2 million dogs living in households (Pet insurance has become a must have for all pet owners, 2020) and given the close interaction between humans and their pets, there is an increased risk for communal spread of multidrug resistant bacteria (MDR), particularly in animals that are treated for life threatening diseases, which can become zoonotic (Hartantyo *et al.*, 2018). As the prevalence of MDR bacteria continues to increase, misuse, abuse and overuse of antimicrobials remains the key factor for selection of MRSP strains in healthy dogs, representing a huge challenge for effective veterinary treatment (Rota *et al.*, 2013).

Otitis externa and pyoderma are common diseases in dogs (Mathie *et al.*, 2010). Atopy, adverse food reactions, flea bite hypersensitivity and bacteria are proven predisposing factors of otitis externa and pyoderma (Schroeder, 2010). In South Africa, first line antimicrobial treatment is based on clinical evaluation of the skin and ears including otoscopy, cytology and culture results (Jacobson, 2002; Schroeder, 2010). A number of different antimicrobials are commonly used for the treatment of otitis including fusidic acid, aminoglycosides, polymyxin B, fluoroquinolones, silver sulfadiazine, Tris-EDTA, oxytetracycline and off-licensed topical preparations (Jacobson, 2002). Guidelines have been provided from the WAVD referring to recommendations for the management, therapeutic considerations and preventative measures of MRSP infections in dogs (Morris *et al.*, 2017) that should be applied by veterinarians to accomplish the optimum therapeutic management of canine *S. pseudintermedius* infections in South Africa. Prior to these published guidelines, recommendations in South Africa for the management of otitis externa or pyoderma were lacking. The result of this has been suboptimal or inappropriate treatment decisions in the management of MRSP infections. Prior literature evaluations for antimicrobial resistance patterns of *Staphylococcus* species in South Africa illustrated a significantly increased proportion of *S.*

aureus and *S. pseudintermedius* isolates resistant to second and third line antibiotics such as lincosamides, fluroquinolones and trimethoprim-sulphaemethoxazole (Qekwana, Oguttu and Odoi, 2019). Furthermore, Blunt *et al.* (2013) (Qekwana, Oguttu and Odoi, 2019; Blunt, van Vuuren and Picard, 2013), illustrated high rates of resistance to ampicillin and doxycycline among dogs with *S. pseudintermedius* associated pyoderma in South Africa (Qekwana, Oguttu and Odoi, 2019; Blunt, van Vuuren and Picard, 2013).

Antibiotic resistance is an urgent, global threat to human health, as animals can become reservoirs of resistant strains (Fitzgerald, 2009). Currently, information on *S. pseudintermedius* associated pyoderma or OE, related antimicrobial resistance patterns and risk factors for SP carriage is not available in South Africa. The aim of this study is to determine the 1) prevalence of MRSP in dogs with pyoderma or otitis externa, 2) antimicrobial resistance patterns of MRSP and 3) the risk factors or predictors of MRSP-associated pyoderma or OE in dogs in South Africa.

Permission to perform this study was granted by the Research Ethics Committee (REC104-18; V094-18) of the University of Pretoria at Onderstepoort.

4.2. Materials and Methods

4.2.1. Study Design and Sample Collection

A total of 68 samples including 49 skin and 16 ear samples were collected by veterinarians from 64 dogs that presented with pyoderma and/or OE at 28 veterinary clinics, six veterinary specialist centres and one academic veterinary hospital in South Africa. Samples were collected over 24 months, between November 2017 and December 2019. Participating laboratories included IDEXX (Gauteng), Pathcare (Western Cape), Wemmershoek Diagnostic Laboratory (Western Cape), Vetdiagnostix (KwaZulu-Natal), and the Onderstepoort Bacteriology Laboratory (Gauteng).

Veterinarians who consented to participate in this study completed a questionnaire for each dog diagnosed with pyoderma and/or OE. Information on each questionnaire included the dog identification number, sampling site, clinical signs, gender, age (in months), breed, sterilization status and treatments used, namely glucocorticoids and topical and/or systemic antimicrobials.

Questionnaires were returned and cases were coded. To ensure confidentiality, identifying owner details on questionnaires were deleted prior to being sent to the researcher.

Samples were collected using Amies transport swabs and transported on Amies Transport medium (Thermofisher Scientific, Oxoid Limited, Basingstoke, UK) to five participating veterinary diagnostic laboratories in the provinces of Western Cape, KwaZulu-Natal and Gauteng for bacterial identification and antimicrobial resistance testing using a combination of culture and standard/classical biochemical methods. Antimicrobial susceptibility results for *S. pseudintermedius* isolates were electronically submitted by participating laboratories. Further molecular identification and characterization of genetic resistance in *S. pseudintermedius* isolates were carried out at the Veterinary Public Health Laboratory, Faculty of Veterinary Science, University of Pretoria.

4.2.2. *Staphylooccus pseudintermedius* Isolation and identification

Isolates were obtained by streaking the swabs across the surface of Columbia blood agar (CBA) with 5% horse blood agar plates with subsequent streaking of the bacterial culture using the quadrant method. Suspect *S. pseudintermedius* colonies that appeared small, round, entire and white with a narrow zone of beta-haemolysis on CBA were grown on CBA for 24 hours at 37ºC. Single colonies were subcultured on CBA and then incubated overnight at 37ºC, in order to obtain a purified culture.

To identify *S. pseudintermedius*, pure colonies were tested by Gram staining, catalase and oxidase tests. Gram-positive cocci that were catalase positive and oxidase negative were identified as suspected *S. pseudintermedius*. Secondary identification was performed using a series of biochemical tests, which were all conducted by referring laboratories. Purified colony cultures were suspended in 5% saline and three to five drops of the suspension inoculum were tested for aesculin, trehalose, urease, and xylose production. The purified culture was also used to spot inoculate the following solid medium: DNAse, Mannitol Salt Agar, and purple maltose agar. Presumptive identification of *S. pseudintermedius* was done based on the phenotypic characteristics of the organism as outlined in **Table 3.** Furthermore, the *S. pseudintermedius* and *Staphylococcus* spp isolates identification were confirmed with PCR.

Table 3. Phenotypic criteria used for the preliminary identification of *Staphylococcus pseudintermedius*-presumptive samples.

Measurements	Outcome
Cell morphology	Cocci
Gram Stain	Positive
Catalase test	Positive
Oxidase test	Negative
MacConkey Agar	Colonies are small, convex, light pink
Maltose Agar	Negative
Aesculin	Negative
DNAse	Positive
Mannitol Salt Agar	(Mannitol fermentation) Positive or negative
Purple maltose agar	Positive
Trehalose	Positive
Urease	Positive
Xylose	Negative

4.2.3. Antimicrobial Susceptibility Testing

S. pseudintermedius isolates were tested for antimicrobial resistance against a panel of 15 antimicrobials using the disk diffusion method as previously described (INSTITUTE and Testing, 2018). The panel of 15 antimicrobials consisted of the following antimicrobials: penicillin (10 µg), ampicillin/amoxycillin (10 µg), amoxycillin/clavulanic acid (30 µg), cephalosporin 1st [cephalothin (30 µg)], cephamycin [cefoxitin (1 µg)], cephalosporin 3rd [cefazidime (30 µg), ceftriaxone (30 µg)], tetracycline/doxycycline (30 µg), fluoroquinolones 3rd and 4th (5µg), erythromycin (15 µg), clindamycin/ Lincomycin (2 µg), gentamicin (10 µg), amikacin (30 µg), kanamycin (30 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (25 µg).

Antimicrobial disks were obtained from Becton Dickinson (BD) (USA) and Oxoid Thermo Scientifc (UK). Pure *S. pseudintermedius* colonies were inoculated on Mueller–Hinton (MH) agar (Thermo Fisher Scientific, Oxoid, UK) and incubated overnight at 37°C. Bacterial suspensions (0.5 McFarland) of overnight cultures were prepared in 0.85% physiological saline. A sterile cotton swab was used to inoculate MH agar plates. Antimicrobial discs were placed on inoculated MH agar plates by a BBL Sensi-disk or Oxoid disk dispenser and incubated aerobically at 37°C for 18 hours. *S. pseudintermedius* (ATCC®49051™) was used as the positive control strain. Initially, each isolate was assigned to the susceptible (S), intermediate (I), or resistant (R) category. This classification was done according to the CLSI interpretative criteria (**Table 4**). However, in the final analysis, intermediate readings were assigned to the resistant (R) category. Multidrug resistance (MDR) was defined as an isolate that is not susceptible to at least one agent in three or more antimicrobial classes (Sweeney *et al.*, 2018). Depending on the referring laboratory, presumptive *S. pseudintermedius* isolates were classified as MRSP, if they were resistant to cefoxitin.

Table 4. Antimicrobial resistance zone diameter (in mm) interpretative criteria for *Staphylococcus* species (Clinical and Laboratory Standards Institute, 2015). **CONC**-concentration; **RES**- Resistance (mm); **SEN**-Sensitivity (mm)

GENERAL 1 ANTIBIOTICS	**CODE**	**TARGET BACTERIA**	**CONC.**	**RES**	**SEN**
Penicillin	**P**	Broad	10 µg	14	20
Ampicillin, Amoxycillin	**AMP**	Broad	10 µg	13	17
Amoxycillin/ Clavulanic acid	**AMC**	Broad	30 µg	13	18
Oxacillin	**OX**	(Methicillin resistance detection) *S. pseudintermedius*	1 µg	17	18
Cephalosporin 1st	**KF**	Broad	30 µg	14	18
Cephalosporin 2nd (Cefoxitin	**FOX**	(Methicillin resistance detection) *S. pseudintermedius*	1 µg	10	13
Cephalosporin 3rd	**CTX**	Broad	30 µg	14	18
Tetracycline/ Doxycycline	**TE**	Broad	30 µg	14	19
Fluoroquinolones 3rd & 4th	**ENR**	Broad	5 µg	16	22
GENERAL 2 ANTIBIOTICS					
Erythromycin (Macrolides)	**E**	Gram positive	15 µg	13	23
Clindamycin/ Lincomycin	**DA**	Gram positive	2 µg	14	21
Gentamicin	**CN**	Broad	10 µg	12	15
Amikacin	**AK**	Broad	30 µg	14	17
Kanamycin	**K**	Broad	30 µg	13	18
Chloramphenicol (Florfenicol)	**C**	Broad	30 µg	12	18
Sulphamethoxazole/ Trimethoprim	**SXT**	Broad	25 µg	10	16

4.2.4. DNA Extraction

The S*. pseudintermedius* isolates were propagated aerobically overnight at 37°C on horse blood agar (Becton, Dickinson and Company Sparks, USA). Bacterial DNA was extracted using the boiling method (Mainga *et al.*, 2018). Briefly, a loopful of bacterial cells was suspended in an Eppendorf microcentrifuge tube containing 1 ml of sterile Fatty Acid (FA) buffer medium (BD, USA), mixed by vortexing, and centrifuged at 12000 rpm for 5 minutes at room temperature. The supernatant was discarded, and the pelleted bacterial cells were re-suspended in 1 ml sterile FA buffer (Becton, Dickinson and Company Sparks, USA). Suspension of the pellet in FA buffer, vortexing and centrifugation were repeated twice, and each time the supernatant was discarded. Finally, the pellet was resuspended in 500 µl of sterile water, boiled in a heating block for 20 minutes and cooled on ice for 10 minutes. The suspension was centrifuged at 12 000 rpm for 5 minutes and the supernatant stored at -20°C for later use in PCR reactions.

4.2.5. PCR Confirmation of *Staphylococcus pseudintermedius*

The *S. pseudintermedius* isolates were tested by PCR-restriction fragment length polymorphism (PCR-RFLP) according to Bannoehr *et al.* on a 320-bp fragment of the *pta* gene (Bannoehr *et al.*, 2009) and PCR protocol by Sasaki *et al.* on a 926-bp fragment of the *nuc* gene (Sasaki *et al.*, 2010) to confirm their *S. pseudintermedius* status.

Briefly, the Bannoehr PCR protocol included a final volume of 25 µl, containing 2.5 µl 10X Thermopol reaction buffer (New England BioLabs, USA), 0.25 µl of 100mM $MgCl_2$, 2.0 µl of 2.5 mM dNTPs, 0.3 µM of each primer, 1U of Taq DNA Polymerase and 5 µl of DNA template. Sterile H_2O was used to make up the reaction to a final volume of 25 µl. Cycling parameters included initial denaturation at 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The RFLP protocol of Bannoehr *et al.* (Bannoehr *et al.*, 2009) involved mixing and incubating 25 µl of the PCR with 5U of *MboI* and 5 µl of 5X digestion buffer for 2 hours at room temperature.

The Sasaki *et al.* (Sasaki *et al.*, 2010) protocol consisted of a 25 µl final volume PCR reaction mixture containing 2.5 µl of 10X Thermopol reaction buffer, 0.25 µl 100 mM $MgCl_2$, 2 µl 2.5 mM dNTPs, 0.3 µM of each primer, 1U of Taq DNA Polymerase, 5 µl of the DNA template and sterile

H_2O to make up the reaction's final volumes. Cycling parameters consisted of initial denaturation at 95°C for 2 minutes; 30 cycles of denaturation at 95°C for 30 seconds; annealing at 56°C for 35 seconds; extension at 72°C for 1 minute; and final extension at 72°C for 2 minutes.

S. pseudintermedius ATCC® 49051™ strain and sterile water were used as positive and negative controls, respectively. All PCR reagents were purchased from New England BioLabs (NEB, USA) except for primers, which were obtained from Inqaba Biotec (South Africa). PCR reactions were carried out in a C1000 Touch™ (Bio-Rad, Hercules, USA) or Veriti 96-well Thermal Cycler (Applied Biosystems, Singapore). PCR and/or digested products were resolved in 2% (wt/vol) agarose by electrophoresis in TAE (Tris–acetate-ethylenediamine tetraacetic acid) buffer, stained with ethidium bromide (EthBr) and visualized under ultraviolet (UV) light in a Gel Doc system (Bio-Rad laboratories, USA).

4.2.6. PCR Identification of *Staphylococcus* species

All isolates that were PCR negative for *S. pseudintermedius* were tested by multiplex PCR to verify their *Staphylococcus* status (Morot-Bizot, Talon and Leroy, 2004). Briefly, frozen cultures were propagated aerobically on horse blood agar overnight at 37°C (Difco™, USA). DNA was extracted from overnight cultures as described above. A multiplex PCR consisting of five primer pairs that target *S. aureus*, *S. saprophyticus*, *S. epidermidis* and *S. xylosus* was used to speciate the isolates (Morot-Bizot, Talon and Leroy, 2004) (**Table 5**). PCR reactions consisted of 2.5µl of 10X Thermopol reaction buffer, 0.25 µl of 100 mM $MgCl_2$, 2µl 2.5 mM dNTPs, 0.4 µM of each primer, 1U of Taq DNA polymerase and 5 µl of DNA template. Sterile H_2O was used to make up the reaction to a final volume of 25 µl. PCR cycling conditions were performed as follows: initial denaturation at 94°C for 3 minutes; followed by 40 cycles of denaturation at 95°C for 1 second; annealing at 55°C for 30 seconds; extension at 72°C for 30 seconds; and a final extension at 72°C for 3 minutes. All reactions were carried out in a C1000 Touch™ (Bio-Rad, USA) or Veriti 96-well Thermal Cycler (Applied Biosystems, Singapore) machine. Five microlitres of the PCR reaction mixture were analysed in 2% gel electrophoresis in TAE (Tris–acetate-ethylenediamine tetraacetic acid) buffer. Amplicon sizes were estimated by comparison with a 100-bp molecular size ladder (NEB, USA). The gel was stained with EtBr (Inqaba Biotec, South Africa) and visualized under UV light in a Gel Doc system (Bio-Rad laboratories, USA).

4.2.7. PCR detection of *mecA*

All isolates including confirmed *S. pseudintermedius* and other *Staphylococci* isolates were screened for the presence of the *mecA* gene by PCR amplification of a 162 bp fragment of the *mecA* gene, according to Haenni *et al*. (Haenni *et al.*, 2014). Primers used for the multiplex PCR are described in **Table 5**. The PCR reaction mixture of 25 µl contained 2.5 µl 10X Thermopol reaction buffer, 0.25 µl 100 mM $MgCl_2$, 2 µl 2.5 mM dNTPs, 0.3 µM of each primer, 1U of Taq DNA Polymerase and 5 µl of DNA template. Sterile H_2O was used to make up the reaction to a final volume of 25 µl. PCR conditions consisted of an initial denaturation at 94°C for 5 minutes; 30 cycles at 94°C for 30 seconds; at 59°C for 60 seconds; at 72°C for 60 seconds; and a final extension at 72°C for 5 minutes (Stegger *et al.*, 2012). DNA from *S. pseudintermedius* (MRSP ST71 E140™ strain) isolated from a dog bite wound infection in Denmark (Moodley *et al.*, 2013) and sterile water were used as positive and negative controls, respectively. Positive controls from other *Staphylococcus* species were not included.

Table 5. Primers as described in Bannoehr *et al*. (Bannoehr *et al.*, 2009), Sasaki *et al*. (Sasaki *et al.*, 2010) and Haenni *et al*. (Haenni *et al.*, 2014) for the identification of *Staphylococcus* species and antimicrobial resistance (Martineau *et al.*, 1996; Martineau *et al.*, 1998; Martineau *et al.*, 2001; Morot-Bizot, Talon and Leroy-Setrin, 2003; Moodley *et al.*, 2013; Paul *et al.*, 2011; Couto *et al.*, 2001)

Primer	Sequence	Size (bp)	Reference
TStaG422	5′-GGC CGT GTT GAA CGT GGT CAA ATC A-3′	370	
TStag765	5′-TIA CCA TTT CAG TAC CTT CTG GTA A-3′		(Martineau *et al.*, 2001)
Sa442-1	5’- AATCTTTGTCGGTACACGATATTCTTCACG -3’	108	(Martineau *et al.*, 1998)
Sa442-2	CGTAATGAGATTTCAGTAGATAATACAACA		
Se705-1	ATCAAAAAGTTGGCGAACCTTTTCA	124	(Martineau *et al.*, 1996)
Se705-2	CAAAAGAGCGTGGAGAAAAGTATCA		
Xyl F	AACGCGCAACGTGATAAAATTAATG	539	(Morot-Bizot, Talon and Leroy-Setrin, 2003)
Xyl R	AACGCGCAACAGCAATTACG		
pta_f1	**5’-** AAA GAC AAA CTT TCA GGT AA **-3’**	320	(Bannoehr *et al.*, 2009)
pta_r1	**5’-** GCA TAA ACA AGC ATT GTA CCG **-3’**		
pse-F2	**5’-** TRG GCA GTA GGA TTC GTT AA **-3’**	926	(Sasaki *et al.*, 2010)
pse-R5	**5’-** CTT TTG TGC TYC MTT TTG G **-3’**		
mecA P4	**5’-** TCC AGA TTA CAA CTT CAC CAG G **-3’**	162	(Couto *et al.*, 2001; Paul *et al.*, 2011)
mecA P7	**5’-** CCA CTT CAT ATC TTG TAA CG **-3’**		

4.2.8. Statistical Analysis

Data was collected for 14 variables (sex, age, gender, hospital visits, referral centre visits, admission to hospital, surgery, wounds, pruritus, antimicrobial route, systemic glucocorticoids, 1st and 2nd tier antimicrobial use, antimicrobial failure resulting in culture and ear drops) and put into Microsoft Excel for Mac 2019, Version 16.3.5 spread sheets. All descriptive statistical analyses were performed using SPSS 17.0 software for Windows, whereas exact logistic regression analyses were done using Stata/IC 11.2. Variables listed in **Table 6** were analysed by exact logistic regression model for risk factors from animals with *mecA* positive and *mecA* negative isolates. Statistical analysis was performed using Fisher's exact test to determine non-random associations between categorical variables. The variables were placed into a univariate analysis in the form of 2x2 tables with Fisher's exact p-values. Those with $p<0.25$ were entered into a multiple exact logistic regression model and non-significant variables ($p>0.05$) were eliminated until only significant ones remained.

4.3. Results

4.3.1. *Staphylococcus pseudintermedius* and other bacteria

Of the 68 samples received from participating laboratories, 83.8% (57/68) of isolates were true *S. pseudintermedius* based on standard bacteriology and both the Sasaki *et al.* PCR and Bannoehr *et al.* PCR-RFLP protocols. The remaining 11/68 sampled isolates included seven that were *Staphylococcus* spp. positive and four that were *Staphylococcus* spp. negative on PCR. However, the 68 samples were collected from 65 dogs as 3 dogs had more than one ear and skin sample. Thus 83% (54/65) of the sampled dogs were infected with *S. pseudintermedius*.

Of the true *S. pseudintermedius* culture samples confirmed on PCR (n=57), it was found that a number of these samples (n=57) were positive for other bacterial species on culture in addition to *S. pseudintermedius*, namely *Streptococcus canis* was detected in 17.5% (10/57), *Enterococcus* spp was found in 15.8% (9/57), *Pseudomonas aeruginosa* in 8.8% (5/57), *Enterobacter* spp. in 5.2% (3/57), *Neisseria animaloris, Staphylococcus epidermides*, *Proteus mirabilis* and

Staphylococcus aureus which were present in 2/57 (3.5%) and *Escherichia coli* which was present in 1/57 (1.8%) of samples as displayed in **Table 6**.

Only isolates that were confirmed to be S. pseudintermedius by PCR (Sasaki et al., 2010) and PCR-RFLP (Bannoehr et al., 2009) were considered true S. pseudintermedius and included in the study hereafter.

Table 6. Other micro-organisms detected in culture samples of dogs with pyoderma, otitis, wound, urinary tract and nasal infections positive for *Staphylococcus pseudintermedius*

Patient Identifier	Sample Site	PCR Result	Other bacterial species detected in culture	PCR Agreement	*mecA*
1 (7911719)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannoehr**	**+**
2 (7911719)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannoehr**	**+**
3 (7778319)	Ears	***s.pseudintermidus***	***Streptococcus canis***	**sasaki and bannoehr**	**-**
4 (7778319)	Ears	***s.pseudintermidus***	***Streptococcus canis***	**sasaki and bannoehr**	**-**
5 (WC17/05)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannoehr**	**-**
6 (VMG1842)	Ears	***s.pseudintermidus***	***streptococcus canis***	**sasaki and bannhoehr**	**+**
7 (VMG1917)	Skin	***s.pseudintermidus***	***Proteus mirabilis***	**sasaki and bannhoehr**	**+**
8 (VMG0015)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
9 (VMG0054)	Skin	***s.pseudintermidus***	***Staph epidermidis; Enterococcus***	**sasaki and bannhoehr**	**+**
10 (VMG0115)	Skin	***s.pseudintermidus***	***Enterococcus***	**sasaki and bannhoehr**	**+**
11 (VMG0147)	Skin	***s.pseudintermidus***	***Enterococcus***	**sasaki and bannhoehr**	**+**
12 (VMG0459)	Skin	**negative**	**Pseudomonas stutzeri**	**Morot-Bizot**	**+**
13 (VMG0684)	Skin	***s.pseudintermidus***	***Streptococcus canis; Enterobacter***	**sasaki and bannhoehr**	**+**
14 (VMG1283)	Ears	***s.pseudintermidus***	***Enterococcus***	**sasaki and bannhoehr**	**+**
15 (VMG1549)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
16 (VMG1814)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
17 (VMG1984)	Skin	***s.pseudintermidus***	***Pseudomonas aeruginosa; Enterococcus***	**sasaki and bannhoehr**	**+**
18 (VMG0929)	Nasal	***s.aureus***	***s.aureus***	**Morot-Bizot**	**+**
19 (VMK1543)	Ears	**negative**	***Enterococcus; E.coli***	**Morot-Bizot**	**+**
20 (VMK1619)	Ears	***s.pseudintermidus***	***Enterococcus; Pseudomonas aeruginosa***	**sasaki and bannoehr**	**+**

21 (VMK0557)	Skin	***s.pseudintermidus***	***Enterococcus***	**sasaki and bannoehr**	**+**
22 (VMK1011)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannoehr**	**+**
23 (VMK1354)	Skin	***s.pseudintermidus***	***E.coli***	**sasaki and bannhoehr**	**+**
24 (VMK1596)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
25 (VMK0698)	Skin	***s.aureus***	***Pseudomonas aeruginosa; Acinetobacter***	**corbier**	**+**
26 (VMK0756)	Ears	***s.pseudintermidus***	***Enterobacter; Streptococcus canis***	**sasaki and bannhoehr**	**+**
27 (VMC1305)	Skin	***s.pseudintermidus***	***Enterococcus***	**sasaki and bannhoehr**	**+**
28 (CT-13008)	Ears	***staph.spp***	***Enterococcus***	**Morot-Bizot**	**+**
29 (CT-15270)	Skin	***negative***	***Pseudomonas aeruginosa; Enterococcus***	**Morot-Bizot**	**-**
30 (CT-15872)	Skin	***s.pseudintermidus***	***Staph epidermidis***	**sasaki and bannoehr**	**-**
31 (VMG-1642)	Urinary	***s.pseudintermidus***	**None**	**sasaki and bannoehr**	**+**
32 (VMK-1326)	Skin	***s.pseudintermidus***	***Neisseria animaloris***	**sasaki and bannhoehr**	**+**
33 (VMK-1343)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
34 (WC-20-6-19)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**-**
35 (WC-20-6-19)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**-**
36 (7963219)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
37 (BO-1557-19)	Ears	***staph.spp***	***Streptococcus canis; Pseudomonas aeruginosa***	**Morot-Bizot**	**+**
38 (BO-1557-19)	Ears	***staph.spp***	***Streptococcus canis; Pseudomonas aeruginosa***	**Morot-Bizot**	**+**
39 (B2025/19) 7830119	Skin	***s.pseudintermidus***	***Streptococcus canis***	**sasaki and bannhoehr**	**+**
40 (B2146/19) 270013	Ears	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
41 (B2169/19) 3414515	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**-**
42 (B2159/19) 5061717	Ears	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**-**
43 (JB580427)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
44 (JB579854)	Ears	***s.pseudintermidus***	***Proteus mirabilis***	**sasaki and bannhoehr**	**+**
45 (JB575596)	Skin	***staph.spp***	***None***	**Morot-Bizot**	**-**
46 (JB575359)	Ears	***staph.spp***	***Proteus mirabilis***	**Morot-Bizot**	**+**

47 (BA BIRKENTOCK)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
48 (BA 2444/19)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
49 (BA RAMBO PYODERM)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**-**
50 (8272719)	Ears	***s.pseudintermidus***	***Streptococcus canis; S.aureus***	**sasaki and bannhoehr**	**+**
51 (VDG1746)	Skin	***s.pseudintermidus***	***Streptococcus canis; Enterobacter***	**sasaki and bannhoehr**	**+**
52 (VDK1273)	Ears	***s.pseudintermidus***	***Streptococcus canis; Enterococcus***	**sasaki and bannhoehr**	**+**
53 (VDK2936)	Skin	***s.pseudintermidus***	***None***	**sasaki and bannhoehr**	**+**
54 (VDK0055)	Skin	***s.pseudintermidus***	***Neisseria animaloris; S.aureus***	**sasaki and bannhoehr**	**+**
55 (VDK4473)	Skin	***s.pseudintermidus***	***Pseudomonas aeruginosa***	**sasaki and bannhoehr**	**+**
56 (VDK4484)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
57 (VDC5187)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
58 (19-22896)	Skin	***s.pseudintermidus***	***Pseudomonas aeruginosa***	**sasaki and bannhoehr**	**+**
59 (19-23291)	Skin	***s.pseudintermidus***	***Streptococcus canis; Pseudomonas aeruginosa***	**sasaki and bannhoehr**	**+**
60 (19-23286)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
61 (19-VDG: 4671)	Nasal	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
62 (19-VDG: 4549)	Urinary	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
63 (19-VDG: 4467)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
64 (19-VDK: 4745)	Skin	**negative**	**None**	**Morot-Bizot**	**+**
65 (19-VDK: 4730)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
66 (19-VDK: 4728)	Nasal	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
67 (19-VDK: 4612)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**
68 (19-22896)	Skin	***s.pseudintermidus***	**None**	**sasaki and bannhoehr**	**+**

4.3.2. Antimicrobial Resistance

According to the disc diffusion test, the following rates of resistance were observed: 93.0% (53/57) of *S. pseudintermedius* isolates were resistant to ampicillin, 84.2% (48/57) to penicillin, 72% (41/57) to cephalothin, 70.2% (40/57) to amoxicillin/clavulanic acid, 60.0% (34/57) to ceftiofur, and 50.9% (29/57) to cefoxitin. Resistance to non ß-lactams included**:** 63.2% (36/57) to doxycycline, 61.4% (35/57) to clindamycin and lincomycin, 56.1% (32/57) to sulphamethoxazole/trimethoprim, and 55.4% (31/57) to enrofloxacin, 49.1% (28/57) to erythromycin, 42.1% (24/57) to gentamycin and 38.6% (22/57) to tilmicosin, 36.8% (21/57) to kanamycin, 12.3% (7/57) to amikacin and 3.5% (2/57) to Chloramphenicol (**Table 7**). The *mecA* gene was found in 86.0% (49/57) of *S. pseudintermedius* isolates, 3.0% (2/68) of *S. aureus and* 6.0% (4/68) in other Staphylococcal isolates. Multi-antimicobial resistance were observed in 49.1% (28/57) of *S. pseudintermedius* that were all *mecA* positive.

Table 7. Antimicrobial resistance in *Staphylococcus pseudintermedius* clinical isolates S=Sensitivity; S%=Sensitivity percentage; R= Resistance; R%= Resistance percentage.

Class of Antimicrobial	Antimicrobial	S	S %	R	R %
Penicillins	Penicillins	6	10.5	48	84.2
	Ampicillin	2	3.5	53	93.0
	Amoxycillin/ Clavulanic acid	15	26.3	40	70.2
Cephalosporins	Cephalothin (Cephalosporin 1st)	14	25.0	41	72.0
	cefoxitin (Cephalosporin 2nd) (MRSP detection)	20	35.1	29	50.9
	ceftiofur (Cephalosporin 3rd	36	63.2	34	60.0
Tetracyclines	Doxycycline	19	33.3	36	63.2
Macrolides, Lincosamides	Erythromycin	27	47.4	28	49.1
	Clindamycin/ Lincomycin	20	35.1	35	61.4
	Tilmicosin	18	31.2	22	38.6
DFR (dihydrofolate reductase) Inhibitors	Sulphamethoxazole/ Trimethoprim	23	40.4	32	56.1
Fluoroquinolones	Enrofloxacin	24	42.1	31	54.4
Aminoglycosides	Gentamicin	31	54.4	24	42.1
	Amikacin	51	89.5	4	7.0
	Kanamycin (Neomycin, framycetin)	23	40.4	21	36.8

4.3.3 Demographics of samples

The highest number of clinical *S. pseudintermedius* isolates were obtained from the Gauteng province, 47.4% (27/57); followed by KwaZulu-Natal 28.1% (16/57); and Western Cape 21% (12/57) **(Table 8).**

The average age of the dogs in the study was 65 months (SD= 36 months). The 68 samples were collected from 65 dogs as 3 dogs had more than one ear and skin sample, thus 54/57 dogs were truly infected with *S. pseudintermedius.* Of the 57 *S. pseudintermedius* isolates, 18.5% (10/54) were detected in samples from africanus and 18.5% (10/54) from Bull Terrier dogs, while 12.97% (7/54) were from unknown breeds. A total of 5.5% (3/54) were detected in samples from German Shepherds, 7.4% (4/54) from Boerboel, 5.5% (3/54) from Jack Russel Terrier, 3.7% (2/54) from Border Collie, 3.7% (2/54) from Dachshund, 3.7% (2/54) from Rottweiler, 3.7% (2/54) Weimaraner, 3.7% (2/54) from Yorkshire Terrier, 1.8% (1/54) from Bulldog, 1.8% (1/54) from Staffordshire Bull Terrier, 1.8% (1/54) from Beagle, Miniature Pincher 1.8% (1/54), Spaniel 1.8% (1/54), Chow Chow 1.8% (1/54), Doberman 1.8% (1/54), Great Dane 1.8% (1/54), Pekingese 1.8% (1/54), Poodle 1.8% (1/54) and 1.8% (1/54) were from Rhodesian Ridgeback **(Table 8).**

4.3.4. Risk Factors

Of the variables investigated in **Table 8** through univariate analysis, only hospital admission, pruritus and antibiotic failure increased the odds of recovering a *mecA* positive *S. pseudintermedius* isolate from an isolate ($p<0.1$). The multivariable logistic regression model is displayed in **Table 9**. Risk factors with $p<0.25$ were entered into a multiple exact logistic regression model and non-significant variables ($p>0.05$) were eliminated until only significant ones remained. In doing so, the final model showed that pruritus was a significant risk factor in isolates for the carriage of *mecA*.

In the final model, some parts of the questionnaires were lacking in information and the researcher was thus unable to account for the total population number in 2 variables, mainly sex and age. These were still included in the final model on account of the small population in this study.

Table 8. Univariable analysis of risk factor variables from animal with *Staphylococcus pseudintermedius* *mecA* positive isolates (n=49) and *mecA* negative isolates (n=8). Final calculation done according to number of clinical isolates. n=Number; n%=Number percentage.

Variable level	mecA +ve (PCR)		mecA -ve (PCR)		p-value
	n	n%	n	n%	
Sex					
Male, Neutered	14	77.78	4	22.22	0.862
Male, Not Neutered	15	88.24	2	11.76	
Female, Spayed	10	90.01	1	9.09	
Female, Not Spayed	7	87.50	1	12.50	
Age					
0-3 years	12	80.00	3	20.00	0.631
4-6 years	14	82.35	3	17.65	
7-12 years	20	90.91	2	9.09	
Hospital Visits					
0 visits	24	82.76	5	17.24	0.808
1-5 visits	14	82.35	3	17.65	
6-10 visits	4	100.00	0	0	
>10 visits	7	100.00	0	0	
Seen at Referral Centre					
No	33	86.84	5	13.16	1.000
Yes	16	84.21	3	15.79	
Admission to Hospital					
No	33	80.49	8	19.51	0.090

Yes	16	0	0	0	
Surgery					
No	44	84.62	8	15.38	1.000
Yes	5	100	0	0	
Wounds					
No	45	84.91	8	15.09	1.000
Yes	4	100	0	0	
Pruritus					
No	21	72.41	8	27.59	0.004
Yes	28	100	0	0	
Antimicrobial route					
Oral	22	88.00	3	12.00	0.452
Topical	13	92.86	1	7.14	
Topical and Oral	7	87.50	1	12.50	
No Antimicrobial usage	7	70.00	3	30.00	
Systemic glucocorticoids					
No	23	82.14	5	17.86	0.47
Yes	26	89.66	3	10.34	
1st and 2nd Tier Antimicrobial use					
No	24	82.76	5	17.24	0.706
Yes	25	89.29	3	10.71	

Antimicrobial failure resulting in culture					
No	24	77.42	7	22.58	0.059
Yes	25	96.15	1	3.85	
Ear drop					
No	42	84	8	16	0.577
Yes	7	100	0	0	

Table 10. Sixty-eight (68) presumptive methicillin-resistant staphylococci bacterial samples recovered from cases of pyoderma, otitis, wound infections, urinary tract infections and nasal infections from dogs of various breeds and ages were collected from various collaborating veterinary microbiology laboratories in South Africa over 24 months. Dogs were examined by veterinarians for the clinical diagnosis of either otitis externa or pyoderma. *Staphylococcus pseudintermedius* identification confirmed with PCR (n=57). Sample 1+2; 3+4 and 37+38 originate from the same dog.

Patient Identifier	Sample Site	Symptoms	Gender	Sterilized	Breed	Age (Months)	*S. pseudintermedius* Presence
1 (7911719)	Skin	Rash	Male	Yes	Bull Terrier	48	Positive
2 (7911719)	Skin	Rash	Male	Yes	Bull Terrier	48	Positive
3 (7778319)	Ears	Rash	Male	Yes	Bull Terrier	60	Positive
4 (7778319)	Ears	Rash	Male	Yes	Bull Terrier	60	Positive
5 (WC17/05)	Skin	Rash	Male	Yes	Dachshund	24	Positive
6 (VMG1842)	Ears	Ear Infection	Male	Yes	Yorkshire Terrier	36	Positive
7 (VMG1917)	Skin	Rash	Male	Yes	Boerboel	84	Positive
8 (VMG0015)	Skin	Ear Infection	Male	No	Boerboel	12	Positive
9 (VMG0054)	Skin	Rash	Male	No	Jack Russell Terrier	48	Positive
10 (VMG0115)	Skin	Swelling or inflammation	Male	No	Boerboel	24	Negative
11 (VMG0147)	Skin	Rash	Female	Yes	German Shepherd	108	Negative
12 (VMG0459)	Skin	Rash	Female	No	Sussex Spaniel	24	Negative
13 (VMG0684)	Skin	Erythema or redness	Female	No	Rottweiler	84	Positive
14 (VMG1283)	Ears	Ear Infection	Female	Yes	Poodle	96	Negative
15 (VMG1549)	Skin	Rash	Male	No	Dachshund	120	Negative
16 (VMG1814)	Skin	Drainage or material	Male	Yes	German Shepherd	84	Positive
17 (VMG1984)	Skin	Drainage or material	Male	No	Jack Russell Terrier	96	Negative
18 (VMG0929)	Nasal	Rhinitis	Male	No	Hungarian Visla	36	Negative

Patient Identifier	Sample Site	Symptoms	Gender	Sterilized	Breed	Age (Months)	*S. pseudintermedius* Presence
19 (VMK1543)	Ears	Ear Infection	Male	Yes	Bulldog	84	Positive
20 (VMK1619)	Ears	Ear Infection	Female	No	Africanus	48	Positive
21 (VMK0557)	Skin	Rash	Female	No	Bull Terrier	108	Positive
22 (VMK1011)	Skin	Other	Unknown	Unknown	Unknown	Unknown	Positive
23 (VMK1354)	Skin	Erythema or redness	Female	No	Rottweiler	48	Positive
24 (VMK1596)	Skin	Other	Unknown	Unknown	Unknown	Unknown	Positive
25 (VMK0698)	Skin	Erythema or redness	Female	No	Africanus	36	Positive
26 (VMK0756)	Ears	Ear Infection	Female	Yes	Bull Terrier	108	Positive
27 (VMC1305)	Skin	Erythema or redness	Female	Yes	Rhodesian Ridgeback	108	Negative
28 (CT-13008)	Ears	Ear Infection	Male	No	Africanus	108	Negative
29 (CT-15270)	Skin	Erythema or redness	Male	Yes	Bulldog	24	Negative
30 (CT-15872)	Skin	Rash	Male	Yes	Yorkshire Terrier	144	Negative
31 (VMG-1642)	Urinary	Urinary tract symptoms	Male	No	Africanus	60	Positive
32 (VMK-1326)	Skin	Rash	Male	No	Africanus	144	Positive
33 (VMK-1343)	Skin	Rash	Male	No	Africanus	84	Positive
34 (WC-20-6-19)	Skin	Erythema or redness	Female	No	Border Collie	108	Positive
35 (WC-20-6-19)	Skin	Erythema or redness	Male	No	Africanus	12	Negative
36 (7963219)	Skin	Rash	Female	Yes	Cocker Spaniel	144	Positive
37 (BO-1557-19)	Ears	Ear Infection	Male	No	German Shepherd	84	Negative
38 (BO-1557-19)	Ears	Ear Infection	Male	No	German Shepherd	84	Negative

Patient Identifier	Sample Site	Symptoms	Gender	Sterilized	Breed	Age (Months)	*S. pseudintermedius* Presence
39 (B2025/19) 7830119	Skin	Drainage or material	Male	No	Boerboel	36	Positive
40 (B2146/19) 270013	Ears	Ear Infection	Female	No	Miniature Pinscher	84	Positive
41 (B2169/19) 3414515	Skin	Rash	Male	No	Pekingese	72	Positive
42 (B2159/19) 5061717	Ears	Ear Infection	Female	Yes	German Shepherd	36	Positive
43 (JB580427)	Skin	Rash	Male	Yes	Weimaraner	60	Positive
44 (JB579854)	Ears	Ear Infection	Female	Yes	Chow Chow	96	Positive
45 (JB575596)	Skin	Rash	Female	No	Doberman	12	Positive
46 (JB575359)	Ears	Ear Infection	Female	Yes	Staffordshire Bull Terrier	144	Positive
47 (BA BIRKENTOCK)	Skin	Rash	Male	No	Africanus	0.5	Positive
48 (BA 2444/19)	Skin	Other	Unknown	Unknown	Unknown	Unknown	Positive
49 (BA RAMBO PYODERM)	Skin	Rash	Male	No	Bull Terrier	24	Positive
50 (8272719)	Ears	Ear Infection	Male	No	Bulldog	84	Positive
51 (VDG1746)	Skin	Rash	Female	Yes	Unknown	84	Positive
52 (VDK1273)	Ears	Ear Infection	Male	Yes	Africanus	0	Positive
53 (VDK2936)	Skin	Rash	Male	Yes	Unknown	48	Positive
54 (VDK0055)	Skin	Rash	Male	Yes	Unknown	48	Positive

Patient Identifier	Sample Site	Symptoms	Gender	Sterilized	Breed	Age (Months)	*S. pseudintermedius* Presence
55 (VDK4473)	Skin	Other	Female	Yes	Border Collie	84	Positive
56 (VDK4484)	Skin	Rash	Female	Yes	Unknown	96	Negative
57 (VDC5187)	Skin	Ear Infection	Male	Yes	Great Dane	48	Positive
58 (19-22896)	Skin	Rash	Male	No	Bull Terrier	17	Positive
59 (19-23291)	Skin	Rash	Female	No	Staffordshire Bull Terrier	36	Positive
60 (19-23286)	Skin	Swelling or inflammation	Male	Yes	Bull Terrier	60	Positive
61 (19-VDG: 4671)	Nasal	Rhinitis	Male	Yes	Jack Russell Terrier	75	Positive
62 (19-VDG: 4549)	Urinary	Urinary tract symptoms	Male	Yes	Weimaraner	84	Positive
63 (19-VDG: 4467)	Skin	Drainage or material	Female	Yes	Beagle	60	Positive
64 (19-VDK: 4745)	Skin	Rash	Female	Yes	Africanus	84	Positive
65 (19-VDK: 4730)	Skin	Rash	Male	No	Africanus	60	Positive
66 (19-VDK: 4728)	Nasal	Urinary tract symptoms	Male	Yes	N/A	0	Positive
67 (19-VDK: 4612)	Skin	Rash	Female	No	Africanus	24	Positive
68 (19-22896)	Skin	Rash	Male	No	Bull Terrier	24	Positive

4.4. Discussion

To the researcher's knowledge, this is the first study conducted in South Africa reporting on the occurrence of antimicrobial resistant MRSP, carrying the *mecA* gene. The study seeks to examine resistance trends as well as risk factors associated with MRSP isolates, which were recovered from canine otitis and pyoderma cases. A total of 68 presumptive MRSP clinical isolates were collected from laboratories of which 83.8% (57/68) were identified as true *S. pseudintermedius*, using molecular methods. The remaining 16% (11/68) of isolates were classified as staphylococci, which comprised of *S. aureus* and *S. epidermides*.

4.4.1. *S. pseudintermedius* in Dogs with Pyoderma and Otitis

S. pseudintermedius was the most frequently isolated organism in canine pyoderma and otitis testing. Primary phenotypic identification of *S. pseudintermedius* by diagnostic laboratories concurred with PCR Sasaki *et al*. (Sasaki *et al.*, 2010) and PCR-RFLP (Bannoehr *et al*., (Bannoehr *et al.*, 2009) at 83.8% (57/68) and 82.35% (56/68) respectively. These results reinforce previous studies in regards to the genera and species of bacterial isolates from cases of canine otitis and pyoderma where *S. pseudintermedius* was the most frequently isolated organism (Bajwa, 2016; Bajwa, 2019; Grönthal *et al.*, 2017; Loeffler and Lloyd, 2018; Paul *et al.*, 2011; Maluping, Paul and Moodley, 2014; Perreten *et al.*, 2010).

4.4.2. *mecA* Carriage in Dogs with Pyoderma and Otitis

It is clinically necessary to recognise methicillin resistance in *Staphylococcus* spp. isolates, as all methicillin-resistant staphylococci are considered resistant to all β-lactam antibiotics *in vivo,* irrespective of the results of disc diffusion. The most common form of methicillin resistance is conferred by the penicillin-binding protein 2a (PBP2a) (Reynolds and Brown, 1985; Hartman and Tomasz, 1984) encoded within the mobile genetic element by the gene *mecA* (Matsuhashi *et al.*, 1986). Detection of *mecA* via PCR is the gold standard for the diagnosis of methicillin resistance (Schissler *et al.*, 2009). The disc diffusion method identified 77.9% (53/68) isolates to be methicillin resistant while *mecA* positive PCR identified 72.1% (49/68) of the methicillin resistant isolates. Methicillin resistance may also be acquired through hetero-resistance, alternative mechanisms or observed in *mecA*-negative isolates producing high levels of β-lactamase. Hetero-resistance represents a minor subpopulation of antibiotic-resistant bacteria, which may go undetected in phenotypic testing (Band and Weiss, 2019). Consequently, organisms that show sensitivity on antibiograms, may clinically exhibit

resistance with subsequent treatment failures. The alternative mechanisms of methicillin resistance necessitate the use of one or more phenotypic tests combined with *mecA* PCR to determine a phenotype and genotype, in order for antimicrobial selection to be clinically effective (Schissler *et al.*, 2009).

Analysis of other methicillin resistance genes in this study by PCR, such as *femA*, *hsp60* and *sod*a*,* may have demonstrated other genes that are perhaps responsible for inducing a state of antimicrobial resistance. Genetic resistance mechanisms cannot be identified by *in vitro* phenotypic methods and as such, genetic testing is necessary to classify molecular mechanisms of antibiotic resistance in bacteria. Since current biochemical techniques are sufficient to determine the susceptibility to non-β-lactam antimicrobials, such laboratory techniques are likely to remain common in veterinary diagnostic laboratories. Moreover, in developing countries with limited resources, these biochemical methods may still be considered adequate tests for guiding clinical decision making (Kali, Stephen and Umadevi, 2014). Clinically, this should always consider how treatment will differ with different diagnostics.

4.4.3. Mixed Infections: Implications of MRSP and Other Staphylococci in Dogs with Pyoderma and Otitis Externa

In dogs with otitis externa, recent studies of canine ear canal microbiota have been evaluated (Korbelik *et al.*, 2019; Bradley *et al.*, 2020; Tang *et al.*, 2020; Kasai *et al.*, 2020). These studies demonstrate a decline in bacterial diversity in otitis externa and pyoderma. Endemic canine ear and skin organisms, which are primarily non initiative in the disease process, may become opportunists when pathological changes occur (Pye, 2018). Otitis infections are commonly polymicrobial in nature with secondary opportunistic invaders (Lamm *et al.*, 2010). The most common primary aetiological pathogens are members of the genus *Staphylococcus* (Penna *et al.*, 2010).

S. pseudintermedius was the most frequently isolated organism in canine pyoderma and otitis cases – 83.8% (57/68). Primarily, the coagulase-positive species were the most common species of *Staphylococcus* isolate. Several staphylococcal species were identified, including coagulase-positive *S. aureus* and coagulase-negative *S. epidermides* – 7% (4/57). The high prevalence of coagulase-positive species in otitis and pyoderma is consistent with previous studies (Morris *et al.*, 2006; Bourély *et al.*, 2019; Bajwa, 2019). The elevated prevalence of

S. pseudintermedius over *S. aureus* is expected, as this species is documented to be the dominant staphylococcal species in canine infections (Lyskova, Vydrzalova and Mazurova, 2007; Penna *et al.*, 2010).

While staphylococci are a common resident organism on the dermis and mucosa of dogs, changes in the microenvironment on the surface of the skin can disrupt the equilibrium of the cutaneous ecosystem, allowing staphylococci to become pathogenic. As a result, other staphylococci are often cultured from dogs, suggesting that they play an important role in the pathogenesis of otitis and pyoderma respectively (Loeffler and Lloyd, 2018; Penna *et al.*, 2010; Lilenbaum *et al.*, 2000; Ma *et al.*, 2020). Both *S. aureus* and *S. epidermides* isolated in this study have been reported to develop methicillin resistance (Xu *et al.*, 2020; Ma *et al.*, 2020; Penna *et al.*, 2010). Resultant antimicrobial resistance occurs due to the high frequency of conjugation and exchange of plasmids between members of the *Staphylococcus* species. Thus, knowledge of species members and their respective pathological properties in otitis and pyoderma provides practical information for the appropriate management of canine otitis externa (Kasai *et al.*, 2020).

4.4.4. Mixed Infections and Implications in Dogs with Pyoderma and Otitis Externa

A smaller portion of isolates in this study from the labs were found to have mixed infections that included MRSP and other non-Staphylococci bacteria such as *Streptococcus canis*, *Enterococcus* spp, *Pseudomonas aeruginosa*, *Enterobacter* spp., *Neisseria animaloris*, *Proteus mirabilis* and *Escherichia coli*. The high prevalence of *Enterococcus* in this study is consistent with previous studies, which reported lower levels of Proteobacteria (*Pseudomonas aeruginosa* 8.8% (5/57) and *E. coli* 1.8% (1/57)) and a higher prevalence of Firmicutes organisms (*Enterococcus* 15.8% (9/57)) in otitis isolates (Ngo *et al.*, 2018; Kasai *et al.*, 2020).

The implications of *Pseudomonas aeruginosa* in the clinical isolates of this study are clinically significant for the treatment of otitis among dogs. *P. aeruginosa* is not typically a canine ear inhabitant and when present, it can be difficult to eradicate (Pye, 2018). *P. aeruginosa* is found in soil, water, and decaying organic matter in the environment and thus could potentially become opportunistic upon host factors to support its growth (Pye, 2018).

Pseudomonas aeruginosa produces biofilms protecting itself from antibiotics administered topically (Mekić, Matanović and Šeol, 2011). Due to the bacterium's resistance to several classes of antibiotics, choosing antibiotics for treatment can be troublesome. Treatment is

further complicated by the increasing number of multidrug resistant strains with concurrent MRSP infection (Mekić, Matanović and Šeol, 2011).

Whilst non-staphylococci bacteria are members of normal ear and skin flora, their significance in the pathogenesis of otitis and pyoderma may demonstrated by their presence in the cultures in this study. Predisposing factors in the current study that may have contributed to the occurrence of these opportunistic bacteria are not fully established. Further research into the link between altered microbiota and disease severity is necessary, as this information is important for the successful treatment of otitis among dogs.

4.4.5. Antimicrobial Resistance

Antimicrobial resistance was prevalent in this study. All isolates were resistant to at least one antimicrobial drug. The degree of multi-resistance in this study, 49.1% (28/57), has been far higher than in previous studies. This highlights the rapid development and spread of antimicrobial resistance in South Africa. This may be because the antimicrobials tested in this study are widely used in the key formulations available for the management of otitis externa. Overuse may contribute to the selection of resistant strains of canine staphylococci (Penna *et al.*, 2010).

The production of the enzyme β-lactamase is the major mechanism of staphylococci resistance (Weese and van Duijkeren, 2010). Therefore, the high incidence of amoxicillin resistance 70.1% (40/57) found in this study was not unexpected. In addition, methicillin-resistant isolates frequently displayed resistance to certain non β-lactams during disc diffusion testing; namely doxycycline, clindamycin, sulphamethoxazole/trimethoprim and enrofloxacin (**Table 7**).

The high levels of resistance of *S. pseudintermedius* to enrofloxacin is in agreement with findings from other studies (Grönthal *et al.*, 2017; Kadlec and Schwarz, 2012; Feng *et al.*, 2012). This is of great concern considering its use in both human and veterinary medicine. In keeping with Qekwana *et al.* (Hanselman *et al.*, 2009; Qekwana, Oguttu and Odoi, 2019; Qekwana *et al.*, 2017) and Blunt *et al*. (Qekwana, Oguttu and Odoi, 2019; Blunt, van Vuuren and Picard, 2013), levels of resistance to enrofloxacin were especially high amongst the *mecA* positive isolates at 53.1% (26/49). This is contrary to Eckholm *et al.* (Eckholm *et al.*, 2013; Fungwithaya *et al.*, 2017) and Fungwithaya *et al.* (Eckholm *et al.*, 2013; Fungwithaya *et al.*, 2017) who demonstrated that the initiation of second tier antibiotics, such as cefalexin or cefpodoxime, subsequently resulted in culture positive methicillin resistance isolates, which

were *mecA* positive. There was no statistically significant association in this study with only 31.6% (18/57) of the isolates having a history of second tier antibiotic use (p>0.1) compared to Eckholm *et al.* (2013) and Fungwithaya *et al*. (Eckholm *et al.*, 2013; Fungwithaya *et al.*, 2017).

4.4.6. Risk Factors for *mecA* Carriage in Dogs with Otitis Externa and Pyoderma

Regarding the different risk factors that were investigated in this study, findings showed that variations in *mecA* occurrence rates in dogs can be ascribed to a number of factors, namely, hospital admission, pruritis and antibiotic failure. Univariable analysis of risk factor variables from animals was based on *mecA* positive isolates from PCR as this is considered the gold standard for the defining MRSP **(Table 8).**

In the present study, based on the history provided by the referring veterinarian, 32.7% (16/49) of *mecA* positive isolates reported previous admission to hospital. Pruritis was found to have a significant association to *mecA* positive *S. pseudintermedius* carriage – 57.1% (28/49) of *mecA* positive isolates had a history of pruritis, whereas none of the dogs with *mecA* negative isolates (0/8) displayed this clinical sign (p=0.004). The majority of clinical isolates reported chronic pruritis as the main clinical sign. The acquisition of *mecA* may be increased in dogs with pruritis. Pruritis has been reported to alter the normal physiological barrier on the skin and in combination with inappropriate antimicorbial therapy, lack of diagnostics and a persistent underlying disease (usually hypersensitivity), (Bajwa, 2016).

Of the 26 clinical isolates in this study that reported antimicrobial failure by referring clinicians, 25 tested *mecA* positive. This highlights the importance of culture and sensitivity after failure of empirical treatment with first tier antibiotics. Consistent with previous studies, the present study found that gender, sterilization and age were not good predictors of *mecA* gene presence (Hanselman *et al.*, 2009; Qekwana, Oguttu and Odoi, 2019; Qekwana *et al.*, 2017).

While the true incidence of *S. pseudintermedius* is underreported as a human pathogen and likely underestimated (Limbago, 2016), humans who are in contact with dogs are the most likely candidates for *S. pseudintermedius* carriage. Dogs with chronic deep pyoderma and their owners often share the same strains of *S. pseudintermedius* (Guardabassi, Loeber and Jacobson, 2004). It has also been suggested that humans can act as a proxy for the *mec*A gene, thus distributing the bacteria geographically via human *S. aureus* (Fang, 2015). In the South African setting, the emergence of MRSP may have serious implications. The high

prevalence of MRSP (and most of these being *mecA* positive) isolates (86% (49/57)) in this study illustrates the concern about methicillin resistance in animal health and its effects on a One Health level. Longitudinal studies evaluating the prevalence of MRSP carriage in dogs are thus required. Humans are not natural hosts for *S. pseudintermedius.* However, with human carriage of *S. pseudintermedius,* there is the possibility that certain mobile genetic elements from MRSP could potentiate the spread of resistant genes to commensal skin flora (van Duijkeren *et al.*, 2011). Tuberculosis and HIV are common infections amongst impoverished South African communities. HIV is an important cause of immunosuppression. TB transmission is associated with low socio-economic status and frequently seen in HIV burdened communities (Tadokera *et al.*, 2020). Clustering of domestic animals in these settings thus remains a proxy for transmission of MDR bacteria, which could pose a risk to already immunocompromised individuals.

In conclusion, this study provides evidence on the high prevalence of *mecA* positive isolates in pyoderma and otitis clinical isolates taken from dogs in South Africa. Important risk factors for *mecA* positive carriage include hospital admission, pruritus and antimicrobial failure. Methicillin-resistant isolates were significantly more likely to exhibit non ß-lactam resistance, especially to doxycycline, clindamycin, sulphamethoxazole/trimethoprim and enrofloxacin. These findings have important zoonotic implications. A limitation of this study is that clinical isolates were not recruited randomly and the true prevalence of *mecA* carriage may therefore not be reflected. Additional limitations include the small number of isolates and the lack of a control group of methicillin sensitive organisms. The findings of this study have provided additional baseline data into this important canine pathogen, which has zoonotic potential. Further molecular epidemiological investigations will prove useful to better characterise MRSP.

Chapter 5: General Discussion

In dogs and cats, *S. pseudintermedius* is the most prevalent inhabitant of skin and mucosa. It is also one of the most common pathogens responsible for infections of the skin and/or ears. Recently, there has been an increasing incidence of MRSP infections and interest in these infections have been the focus of many studies. MRSP has been regarded as a One Health problem. One Health is an approach that combines the expertise of various disciplines that relate to animal, human or ecosystem health, to address complex health challenges based on specific principles (Destoumieux-Garzón *et al.*, 2018). While humans are not natural hosts for *S. pseudintermedius*, it is not known if *S. pseudintermedius* strains containing mobile genetic elements could present a reservoir for the spread of resistant genes to the human commensal skin flora and may thus have important zoonotic implications (van Duijkeren *et al.*, 2011). This illustrates the close systemic interaction of humans and animals and the possibility for acquiring antimicrobial resistant genes.

5.1. Phenotypic and Antimicrobial Susceptibility Tests used for the Detection and Identification of *S. pseudintermedius* and MRSP in South Africa

The primary identification of *S. pseudintermedius* by the diagnostic laboratories in South Africa makes use of phenotypic testing and this was found to have an acceptable concordance when compared to the PCR methods used in this study. Genetic resistance mechanisms are impossible to detect by in vitro phenotypic methods and as such genetic tests are required to identify the molecular mechanisms of antibiotic resistance in bacteria (Schwarz *et al.*, 2018). Despite the varied challenges presented by phenotypic identification of the bacterial species, a high percentage of clinical isolates in this study were identified as *S. pseudintermedius*.

The study made use of two molecular methods to identify *S. pseudintermedius* : a PCR (Bannoehr *et al.*, 2009) and a PCR-RFLP method (Sasaki *et al.*, 2010).

Both PCR methods yielded reliable results with a small margin of error. The margin of erroneous identification when used together is less than and substantially better than phenotypic methods of identification. The first molecular diagnostic test characterises the *pta* gene by the PCR-RFLP protocol according to Bannoehr *et al.* 83.82% (57/68 samples) (Bannoehr *et al.*, 2009). Slettemeås *et al.*, analysed 200 canine staphylococcal isolates using

PCR-RFLP and found that a small percentage (1%) of the *S. pseudintermedius* population have been shown to be incorrectly identified because of heterogeneity in the *MboI* restriction site (Slettemeås, Mikalsen and Sunde, 2010). This necessitated the use of a second PCR methodology by Sasaki *et al.* that targets the 926-bp *nuc* gene 82.3% (56/68 samples) (Sasaki *et al.*, 2010). The second PCR method has been determined not to be specific for the final identification of *S. pseudintermedius* as this gene has inadequate variation for the specific species detection of *S. pseudintermedius.* *S. pseudintermedius* isolates were deemed positive in the final model, based on positive identification using bacteriology methods, PCR and PCR-RFLP.

5.2. Antibiotic Resistance and MRSP Isolation in South Africa

In a previous study in South Africa done by Qekwana *et al.* (Qekwana, Oguttu and Odoi, 2019) on the antimicrobial susceptibility and resistance patterns of staphylococci isolated from dogs with pyoderma and otitis, records of a total of 334 clinical canine samples that were submitted to the bacteriology laboratory at Onderstepoort were assessed and predictors of staphylococcal infections were evaluated. Similar to Qekwana *et al.* (Qekwana, Oguttu and Odoi, 2019) the present study found that *S. pseudintermedius* was the most commonly isolated species and found a high level of fluoroquinolone resistance amongst the *Staphylococcus* spp. isolated (Qekwana, Oguttu and Odoi, 2019). These findings are in keeping with an international study conducted in Brazil, which evaluated the species distribution and antimicrobial susceptibility of staphylococci isolated from canine otitis externa (Penna *et al.*, 2010). Low levels of resistance towards aminoglycosides and macrolides were found in the present study, in keeping with the findings of Qekwana *et al.* (Qekwana, Oguttu and Odoi, 2019) and Penna *et al*. (Penna *et al.*, 2010). However, contrary to their results, multidrug resistance was higher in the present study (49.1%; 28/57) and more consistent with results reported by Morris *et al.* in 2006 (Morris *et al.*, 2006). This may represent a deterioration in the South African antimicrobial situation in a short period of time.

In the present study, 49.1% (28/57) of the MRSP isolates showed characteristics of multi drug resistance. These results reinforce the reports from private veterinarians that most dogs included in this study were exposed to multiple antibiotic classes and that many (47.4%, 27/57) were on concomitant immunomodulatory therapy, such as steroids, for underlying allergies, which may have resulted in an increased risk of acquiring MDR-MRSP.

5.3. *mecA* Status in South Africa

Epidemiologically, the findings of this study highlight the need to understand the prevalence of *mecA* in *S. pseudintermedius* infections and antibiotic resistance trends in South Africa. To the best of the researcher's knowledge, this is the first attempt to evaluate the prevalence of *mecA* in *S. pseudintermedius* isolates from dogs' skin and ear infections and to describe the associated antibiotic resistance patterns in canine otitis and pyoderma.

Whilst each of the PCR methods used in this study remain the gold standard for the detection of *mecA*, only 72.1% (49/68) of the methicillin resistant isolates (as identified by disc diffusion) corresponded with the PCR identification of the *mecA* gene. Some reports have shown that methicillin resistance can be encoded on genes other than *mecA*, especially in *mecA*-negative isolates that produce high levels of β-lactamase (Schissler *et al.*, 2009). Expanding screening of MRSP isolates for additional genes that encode methicillin resistance may have revealed the presence of other methicillin encoding genes. Other methicillin resistance encoding genes would have to be screened by PCR, such as *blaZ* (Milheiriço *et al.*, 2011), *sat4* (Perreten *et al.*, 2005), *tetK* (Tenover *et al.*, 2004), *tetM* (Ng *et al.*, 2001) and *ermB* (Novotna *et al.*, 2005), using primers specific for these genes. Given the preliminary nature of this study, the objective of this study is to provide the first molecular description of *mecA* in South Africa and thus these additional genes were not explored.

5.4. Risk Factors for *mecA* Carriage

Risk factors were investigated in this study. The findings showed that variations in *mecA* occurrence rates in dogs can be ascribed to a number of factors namely, hospital admission, pruritis and antibiotic failure.

Pruritis was found to be a significant predictor of dogs that carried *mecA* positive isolates – 57.1% (28/49) of the *mecA* positive isolates had a history of pruritis, while all dogs from which *mecA* negative (0/8) isolates were recovered did not display pruritis (p=0.004). Pruritus due to allergic dermatitis is the single most common reason for owners presenting dogs with skin disease to a veterinarian (Schroeder, 2010). Immunomodulation is almost always employed in treating dogs with pruritis and may alter normal immune defence mechanisms involved in skin barrier function. Immunomodulatory drugs, inappropriate therapy, lack of diagnostics and persistent underlying disease may predispose the acquisition of MRSP and *mecA* (Bajwa, 2016).

Systemic treatment with glucocorticoids in previous studies has been shown to predispose dogs to *mecA*-positive MRSP carriage. A study in Germany by Lehner *et al.* (Lehner *et al.*, 2014) assessed the epidemiological factors associated with *mecA* positive carriage in cats and dogs. Results by Lehner *et al.* (Lehner *et al.*, 2014) showed that animals had a higher risk of *mecA* carriage after receiving topical ear medication or glucocorticoids. Although 53% (26/49) of *mecA* positive isolates from dogs had exposure to glucocorticoids, 37.5% (3/8) tested *mecA* negative despite similar exposure. Despite the lack of statistical significance between glucocorticoid administration and *mecA* positive isolates in this study, atopic dermatitis is commonly managed with glucocorticoid treatment, which is known to cause immune suppression and could encourage *mecA* acquisition.

Similar to Hensel *et al.* (Hensel, Zabel and Hensel, 2016) *and* Lehner *et al.* (Lehner *et al.*, 2014), the use of multiple antibiotics was reported in 51% (25/49) of dogs that carried *mecA*-positive MRSP isolates. Previous antibacterial use and exposure to a variety of antibacterial classes were both common findings in dogs carrying *mecA* positive isolates. In contrast to Lehner *et al.,* the present study found that the risk of *mecA* positive carriage was higher in MRSP isolates from skin specimens (90.5%: 38/42) compared to those which were obtained from the ear canal (72.7%: 8/11). In addition, Lehner *et al.* showed that *S. pseudintermedius* isolates from ears were more likely to be *mecA* negative (27.3%) – only 3/11 in the present study. However, fewer *S. pseudintermedius* isolates in this study were recovered from ears, 19.3% (11/57), as opposed to 73.7% (42/57) that came from the skin, which may have influenced the final results.

From the present study's results, neither sex nor age played a role in the risk of *mecA* positive carriage. These results are consistent with Qekwana *et al.* (Hanselman *et al.*, 2009; Qekwana, Oguttu and Odoi, 2019; Qekwana *et al.*, 2017) who found that sex was not a significant predictor of staphylococcal infections in dogs in South Africa; this also correlates with the findings of Hanselman *et al.* (Hanselman *et al.*, 2009; Qekwana, Oguttu and Odoi, 2019; Qekwana *et al.*, 2017) who reported no significant association between sex and staphylococcal infections in dogs in Canada (Hanselman *et al.*, 2009). However, Boost *et al.* (Boost, O'Donoghue and Siu, 2007), described an association with age as a risk factor for *Staphylococcus* infections (Boost, O'Donoghue and Siu, 2007), which was similarly observed by Qekwana *et al.* (Hanselman *et al.*, 2009; Qekwana, Oguttu and Odoi, 2019; Qekwana *et al.*, 2017) with dogs between the ages of 2–4 years and 7–8 years being more likely to test positive for *S. pseudintermedius* than puppies or dogs older than 8 years.

Chapter 6: Conclusion

The findings in this study suggest that the antibiotic prescribing behaviour of South African veterinarians is similar to the conduct of veterinarians described in European companion animal studies (Gold, Cohen and Lawhon, 2014; Khodabandeh *et al.*, 2019; Liu *et al.*, 2017; Wegener *et al.*, 2018; Grönthal *et al.*, 2017; Wettstein *et al.*, 2008). Data on the incidence of resistance in South Africa indicates that recommendations regarding the cautious and conscientious use of antimicrobials and early microbiological diagnosis are essential for future control of bacterial drug resistance. This study's findings reinforce the need for a change in antibiotic prescribing habits in the treatment of *S. pseudintermedius* in the veterinary setting.

This study provides evidence that there is a high prevalence of *mecA* positive carriage in methicillin resistant SP pyoderma and otitis in dogs in South Africa. Important risk factors for *mecA* positive carriage are previous hospital admission, pruritis and previous antibacterial failure. Methicillin-resistant isolates were significantly more likely to be resistant to non ß-lactams, such as fluoroquinolones and tetracyclines. These findings have possible zoonotic implications. The findings of this study have provided some baseline data to justify further investigations into this important canine pathogen, but further molecular epidemiological investigations are required to further characterise MRSP in the South African pet population.

Based on the results of the final model in this study in **chapter 4**, the recommendations with respect to the empirical choice of an antibiotic for the management of pyoderma and otitis in dogs are in accordance with the clinical consensus guidelines of the WAVD and the PROTECT policy endorsed by the BSAVA. Antimicrobial selection is influenced by individual prescribing behaviours, patient factors (such as underlying cause and concurrent disease), client interaction and practice norms.

Despite the increasing importance of MRSP in veterinary medicine, there is a paucity of work describing MRSP in South Africa. The positive association between *mecA* positive carriage and antimicrobial therapy reported in the final model highlights the need for increased surveillance of antibiotic resistance within the veterinary environment in South Africa. An unexpected finding was the identification of such a high number of *mecA* positive methicillin resistant *S. pseudintermedius* organisms (84.2% (48/57)) submitted to veterinary laboratories. This represents a higher prevalence of *mecA* compared to findings reported in the rest of the

world where prevalence is usually reported to be between 5.88% (Rahmaniar *et al.*, 2020) and 77.78% (Ortiz-Díez *et al.*, 2020) of MRSP. The high prevalence of *mecA* confirms that this is in all likelihood an important molecular mechanism of methicillin resistance in circulation in South Africa.

6.1. Limitations

Limitations of this study include its small sample size and that samples were not collected randomly and hence included no non-methicillin resistant bacteria to allow for better assessment of the true incidence of MRSP. The researcher also did not evaluate isolates for other genes that are responsible for antimicrobial resistance.

The present study is limited by its retrospective nature, which means that key statistics such as MRSP prevalence amongst healthy and infected animals could not be measured. Furthermore, only methicillin resistant isolates were selected for study and this limited the ability to evaluate the incidence of MRSP in samples submitted to the laboratory for diagnosis. The small number of clinical isolates (n=68) made statistical evaluations impossible for many comparisons and associations. The clinical data provided by veterinarians with the samples they submitted were also insufficient and at times absent.

Whilst the methodology in this study touches on some of the molecular aspects in organism identification and the identification of *mecA* from isolates, the researcher did not assess the divergence between different MRSP strains. Additional molecular studies are required to further characterise the SP population from dogs in South Africa. This knowledge could be used to forecast the spread of methicillin resistance and to classify bacterial clones causing disease in South African otitis and pyoderma cases, compared to those circulating globally. Furthermore, virulent clonal populations of *S. pseudintermedius* could be identified, which would improve efforts to develop alternative therapeutic or control methods such as vaccines or phage therapies for major *S. pseudintermedius* clone groups associated with diseases (Solyman *et al.*, 2013). Defining the behaviour and polymorphism of resistance genes in South Africa has both molecular and clinical value, as it would assist with antibiotic use guidelines and infection control strategies.

6.2. Future Studies and Recommendations

Longitudinal studies in South Africa that evaluate the effect of routine antimicrobial therapy on resistance emergence or resolution are lacking. Thus, studies to evaluate the prevalence of methicillin-resistant staphylococci on skin and carriage sites in dogs with bacterial pyoderma and evaluation of the prevalence of MRSP colonisation after successful treatment of pyoderma and otitis are recommended. In addition, clinical studies that compare clinical resolution and duration of treatment in dogs with MSSP and MRSP pyoderma would be helpful to guide clinicians in deciding how long an antibiotic treatment course should be.

The findings of this study have provided additional baseline data for further investigations into this important canine zoonotic pathogen, but further molecular epidemiological investigations will prove useful to assess if there is a strong association between MRSP clonal types and geographical origin and human colonisation.

It would be advisable, but not necessarily economically feasible for all South African laboratories, to include complete speciation of staphylococci. Complete speciation can be used to forecast the spread of methicillin resistance and to classify bacterial clones involved in causing disease compared to those circulating globally. In addition, it would prevent the misdiagnosis of staphylococcal species, which has implications for the appropriate antimicrobial treatment. Lastly, speciation will enhance and strengthen global epidemiological data.

Local diagnostic laboratory use of molecular analysis could improve diagnostic accuracy and thus support the protection of antibiotics. Molecular insight into the behaviour and acquisition of resistant genes is key for the safeguarding of antimicrobials. This would however be an impractical suggestion in most laboratory settings (Schwarz *et al.* 2018).

In the South African setting, the emergence of MRSP may have serious implications. Whilst the findings of this study are limited, the high prevalence of *mecA* amongst canine isolates are useful in highlighting the risk it poses in animal health and its effects on a One Health level. A One Health collaborative effort, involving multiple disciplines, could be important to protect human health, animals and our environment (Destoumieux-Garzón *et al.*, 2018). Longitudinal studies evaluating the prevalence of MRSP in dog owners and their pets would be important. Tuberculosis and HIV are diseases that are prevalent amongst impoverished South African communities, and South Africa remains one of the world's top six TB and HIV burdened

countries (Tadokera *et al.*, 2020). Clustering of domestic animals in these settings may thus pose a risk to these immunocompromised individuals.

Globally, the adoption of antibacterial stewardship initiatives, such as those endorsed by the BSAVA, have discouraged the unnecessary use of antibiotics in the veterinary community. The lack of guidelines in South Africa has resulted in antibiotic treatment regimens that are often unchallenged, accepted practice and that have evolved into unproven dogmas that contravene the core principles of antibiotic stewardship. Thus, a restriction-of-use policy and guidelines are recommended in the South African setting.

The aim of this study is to provide the first molecular description of *mecA* positive carriage amongst methicillin resistant *S. pseudintermedius* clinical isolates from dogs in South Africa. Using bacterial isolates collected by collaborating laboratories, the researcher conducted a small-scale pilot study in order to evaluate the prevalence and levels of the *mecA* resistance gene in these isolates by geographical region in South Africa. Although the sample size was limited, the researcher provides preliminary data on the antimicrobial susceptibility trends of MRSP isolates and of the association between this status and the presence of *mecA* in South Africa. The researcher was also able to provide some data that demonstrates the association between hospitalization, previous antibiotic use and pruritus and this gene.

To conclude, the results support and highlight the need to endorse safe antimicrobial usage. The findings should encourage South African veterinarians to avoid polypharmacotherapy and encourage more careful antibiotic stewardship.

Chapter 7: References

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Chapter 8: Appendix

Antibiotic History Form

History Request Form **Submit with Samples**

Veterinarian Information	
Veterinarian name	
E-mail	
Region	
Phone	
Address	
Animal Information	
Animal ID	
Breed	
Gender	
Age	
Relevant Clinical Details/History	
Duration of treatment	
Based on the skin infection up until culture, what topical or systemic antimicrobial drugs have been used?	
Based on the ear infection up until culture, what topical or systemic antimicrobial drugs have been used?	

agriculture,
forestry & fisheries

Department:
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Dr Cameron David Prior
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
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Pretoria

Email: andrew.leisewitz@up.ac.za

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Dear Dr Prior

Your application, submitted on 17 October 2018, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him/her by any other Act of the Republic of South Africa;
2. All potentially infectious material utilised or collected during the study is to be destroyed at the completion of the study. Records must be kept for five years for audit purposes. A dispensation application may be made to the Director Animal Health in the event that any of the above is to be stored or distributed;
3. Bacterial isolates may be collected from various collaborating veterinary microbiology laboratories in South Africa, as stipulated in the application, and transported to Department of Veterinary Tropical Diseases, Onderstepoort Veterinary Faculty, University of Pretoria;

4. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and/or the National Road Traffic Act, 1996 (Act No. 93 of 1996);
5. Only a registered waste disposal company may be utilised for the removal of waste generated during the study.

Title of research/study: "Molecular characterization of Staphylococcus pseudintermedius from pyoderma and otitis externa in South African dogs"
Researcher: Dr Cameron David Prior
Institution: Onderstepoort Veterinary Faculty, University of Pretoria
Our reference: 12/11/1/1/6
Your ref.: -
Expiry date: 30 April 2019

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2018 -11- 0 6

SUBJECT: *PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)*

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

DECLARATION OF ORIGINALITY

This document must be signed and submitted with every essay, report, project, assignment, mini-dissertation, dissertation and/or thesis

Full names of student: .....Cameron David Prior.....

Student number: …….29072299……..

Declaration:

1. I understand what plagiarism is and am aware of the University's policy in this regard.

2. I declare that this …… dissertation …… (e.g. essay, report, project, assignment, mini-dissertation, dissertation, thesis, etc.) is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

3. I have not used work previously produced by another student or any other person to hand in as my own.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature of student: .....*Cameron Prior*.....

Signature of supervisor: ……

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UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Molecular characterization of *Staphylococcus pseudintermedius* from pyoderma and otitis externa in South African Dogs**
PROJECT NUMBER	**V094-18**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Dr. C Prior**

STUDENT NUMBER (where applicable)	**U_29072299**
DISSERTATION/THESIS SUBMITTED FOR	**MSc**

ANIMAL SPECIES/SAMPLES	**Canine**
NUMBER OF ANIMALS	**100**
Approval period to use animals for research/testing purposes	**November 2018 – November 2019**
SUPERVISOR	**Prof. A Leisewitz**

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 26 November 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

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