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Prevalence of *Staphylococcus aureus* and its antimicrobial resistance and virulence genes in pet dogs of Durban: the risks of reservoir populations

By

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As the candidate's supervisor I have/have not approved this thesis/dissertation for submission.

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Abstract

The evolution of multidrug resistant bacteria is one of the greatest challenges to modern medicine to date, with cases of multidrug resistant, untreatable *Staphylococcus aureus* being reported all over the globe. *S. aureus* is often transmitted across species through contact. Therefore, the importance of surveying potential populations becomes apparent, especially in dogs, which are often close with humans and can carry virulent forms of *S. aureus* asymptomatically. The aim of this project was to gauge the extent of *S. aureus* colonisation in dog populations that is otherwise overlooked. This project tested this in two parts: by detecting prevalence rates of *S. aureus* and its pathogenic factors (virulence and antibiotic resistance genes) due to human interaction; and testing the reliability of methods previously employed to carry out these types of prevalence studies. For prevalence testing, nasal swabs were taken from 113 dogs visiting a local Veterinary Hospital in Durban of which 35% (n = 40) were found to carry Methicillin Susceptible *S. aureus* (MSSA). No cases of Methicillin Resistant *S. aureus* (MRSA) were observed. Prevalence of virulence and antibiotic resistance genes were estimated using conventional PCR. Kirby-Bauer Disc diffusion was used to detect resistance to 9 classes of antibiotics. The most notable findings were 12.5% tetracycline resistance attributable to *tetK* and *tetM*; 15% of samples carried immune evasion clusters (IEC) carried by βC-ϕ's; 7.5% of isolates were linezolid and vancomycin resistant (LR-VRSA) not attributable to resistance genes *cfr* and *vanA* respectively and were not induced by veterinary practices. This is of great concern as LR-VRSA has never been detected before in animals or outside of India and it is clear that some isolates are surviving beyond treatment, hidden in reservoir populations, like dogs. The Reliability of Methods tested if Mannitol Salt Agar (MSA) was sensitive and selectively powerful enough to isolate *S. aureus* from dog nasal swabs as compared to HiCrome™ Aureus Agar Base (AAB) which is suggested to be more suitable for environmental samples by the manufacturer. Fifty-six samples were collected and processed on both MSA and AAB media. Presumptive results were confirmed as *S. aureus* if the species-specific gene *nuc* was detected. Presumptive results from selective media and genomic confirmation of the *nuc* gene were compared to the true states of each sample so to measure the sensitivity, percentage of type I errors due to poor selective power and accuracy of true state prediction in conjunction with *nuc* testing of each media. MSA was 60.9%, 60%, and 84% respectively. AAB was 95.7%, 0% and 98.2% respectively. Odds ratios determined AAB as 14.08 times more likely to detect *S. aureus* from dog nare samples than MSA. This suggests that MSA is not suitable for the context of *S. aureus* isolation from dogs and that previous prevalence rates may be underestimated by as much as 60%. Both investigations demonstrated that dogs are a grossly under-represented reservoir population for *S. aureus*, both in prevalence and potential for disease.

Keywords: *Staphylococcus aureus*; Antimicrobial Resistance; Virulence; Dogs; Selective Medium Comparative Analysis.

PREFACE

The experimental work reported in this thesis was completed by Mary-Anne Frances Brouckaert in the Discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science at the University of KwaZulu-Natal, Westville Campus, under the supervision of Dr Oliver Tendayi Zishiri and Dr Matthew Adekunle Adeleke.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

I certify that the above information is correct

Dr Oliver Tendayi Zishiri	Dr Matthew Adekunle Adeleke
(Supervisor)	(Co-supervisor)
Date: _____	Date: _____

PLAGIARISM DECLARATION

I, Mary-Anne Frances Brouckaert declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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LIST OF ABBREVIATIONS

Symbol	Meaning
°C	Degrees Celcius
µl	Microlitre
µM	Micromolar
AAB	HiCrome™ Aureus Agar Base
AAC	*N*-acetyltransferases
AHP	*O*-phosphotransferases
AIDS	Auto-Immune Deficiency Syndrome
ANT	*O*-nucleotidyltransferases
APC	Antigen Presenting Cell
bp	Base Pairs
BPA	Baird-Parker Agar
CBA	Columbia Blood Agar Base
cm	Centimeter
$cMLS_B$	constitutive Macrolide Lincosamide Streptogramin B
CoNS	Coagulase Negative Staphylococci
CoPS	Coagulase Positive Staphylococci
CP	Capsular Polysaccharide
DNA	Deoxyribonucleic Acid
GI	Gastrointestinal
HIV	Human Immuno-deficiency Virus
hrs	Hours
IEC	Immune Evasion Cluster
$iMLS_B$	inducible Macrolide Lincosamide Streptogramin B
ISS	Insertion Site Sequence
IV	Intravenous
LRE	Linezolid Resistant Enterococci
LRSA	Linezolid Resistant *S. aureus*
LR-VRSA	Linezolid and Vancomycin Resistant *S. aureus*
MHC	Major Histocompatibility Complex
ml	Mililitre

MLS_B	Macrolide Lincosamide Streptogramin B
mRNA	messenger Ribonucleic Acid
MRSA	Methicillin Resistant *S. aureus*
MSA	Mannitol Salt Agar
MSSA	Methicillin Susceptable *S. aureus*
NAM	N-acetylmuramic acid
PCR	Polymerase Chain Reaction
PGFE	Pulse-Field Gel Electrophoresis
pH	Percentage Hydrogen
RAPD	Random Amplification of Polymorphic DNA
rRNA	ribosomal Ribonucleic Acid
SA No.110	Staphylococcus Agar No. 110
SaPI	*S. aureus* Pathogenicity Island
SCC	Staphylococcal Cassette Chromosome
SE	Staphylococcal Enterotoxin
SNP	Single Nucleotide Polymorphism
TcR	T-cell Receptors
tRNA	transfer Ribonucleic Acid
TSB	Tryptone Soy Broth
V	Volts
VJA	Vogel-Johnson Agar
VRE	Vancomycin Resistant Enterococci
VRSA	Vancomycin Resistant *S. aureus*
xg	Times Gravity
βC-ϕ's	B-haemolysin Converting Bacteriophages
χ^2	Chi-Squared value

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Background and Rationale

Staphylococcus aureus is a Gram-positive coccus bacterium with no natural appendages that tends to group cells in grape like clusters (Harris *et al*., 2002). It replicates via binary fission (asexual reproduction) but progeny cells do not always separate completely from each other, causing them to cluster as they do. Colonies are usually a pale yellow to yellow colour (thus *aureus*, meaning gold in Latin) when grown on standard nutrient agar and will change the indicator dye in mannitol salt agar yellow (Ryan & Ray, 2004). It is a catalase positive and β-haemolytic cocci (Matthews *et al.*, 1997). It is a ubiqutous organism that can be both commensal (of no harm to its host) or pathogenic (disease causing) and will present in 80% of all humans throughout their lives (Kluytmans *et al.*, 1997). In its virulent or pathogenic form, it can cause several diseases as a result of infection such as infective endocarditis (infection of the membrane lining the inside of the heart), osteoarticular infections (infection of joints), pleuropulmonary infections (infection of the membrane lining the lungs and thorax), infections of skin and soft tissue and also severe infections involving internalised devices such as catheters, IV drips and other devices (Kluytmans *et al*, 1997; Liu, 2009; Tong *et al*, 2015).

Dogs have long since evolved alongside humans as companion animals for a few millennia (Thalmann *et al.*, 2013). In the UK alone, 31% of households own at least one dog (Murray *et al*., 2010). No consensus data is available on this matter for South Africa, but dog ownership is not uncommon. This would then stand to reason that man and animal have been sharing the same environment and thus, the same pathogens, and they too have been evolving alongside man and dog. Opportunistic bacteria like *Staphylococcus aureus*, being ubiquitous to the types of environments people live in, are also found living on and in companion animals (and humans) as natural and unnatural flora (Jarraud *et al.*, 2002). These types of non-target-species specific bacteria can be transmissible between companion animals and humans (Österlund *et al.*, 1997). As it is understood that *S. aureus* is transmissible through contact (Jacobs, 2014; Koenig *et al*., 2016), more virulent forms of these bacteria can also be transmitted between people and companion animals.

The domestication of these animals means that their population numbers are predominantly controlled by humans, for the most part, as a form of revenue for the breeder. However, with peoples' poor forethought regarding the responsibility of caring for these animals there are

many companion animals that become stray or are born of other strays and do not receive the same medical upkeep as compared to some animals kept as pets would (Bartllett *et al.*, 2005). As a result, this and the generally unhygienic environments (rural farms or unmaintained urban areas) and lack of care, these stray animals are often susceptible to being diseased. A common disease for many furred mammals is a skin condition commonly called mange (Fourie *et al.*, 2007). It is caused by tiny mites of varying genus's that often naturally occur in the fur of these animals, but poor hygiene and lack of care (poor diet, lack of vaccinations, poor grooming practices) will compromise the animal's immune system, causing the mite population to grow. The mites compromise the integrity of the skin and can cause small lesions and hair loss as they irritate the skin on the animal (Paradis, 1999). Commonly associated with mange is secondary infection by pyogenic bacteria, such as *Staphylococcal* species, including *S. aureus* (although not the most common *Staphylococcal* species), and others like *Streptococcus pyogenes*. These bacteria will colonise the compromised skin causing the formation of pus (thus pyogenic) and due to the already subdued immune system of the animal, are very difficult to remove from the animal without the aid of antibiotics (Shipstone, 2000). Unfortunately, antibiotic use, since they were first discovered and synthesised, have not been used responsibly (unnecessary prescription for non-bacterial causing illnesses and patients not completing treatment courses), both in humans and our pets, thus causing the rise in bacterial strains that are no longer susceptible to some antibiotics (Goossens *et al.*, 2005; Lee Ventola, 2015). In the case of *S. aureus*, there is a great concern for methicillin resistant strains which are no longer susceptible to many penicillin-based antibiotics known as β-lactams (Lowy, 2003).

Taking into cognizance the fact that this bacterium is opportunistic and can infect humans, there is always the risk that contact with a companion animal, especially one that is sick or diseased, may lead to an infection with antibiotic resistant forms of these bacteria in the human, especially if the animal causes the human's skin integrity to be compromised through a bite or scratch. While most studies suggest that transmission to humans is rare between owners and pets, human to pet is not uncommon and as dogs can carry *S. aureus* asymptomatically, there is a risk that companion animals may provide an under-reported reservoir for drug resistant (including methicillin, tetracycline and vancomycin, some of the most widely used and otherwise most effective) *S. aureus* (Boost *et al*., 2008; Kottler *et al*., 2010; Han *et al*., 2016). Boost *et al*. (2008), also reported that observed strains of *S. aureus* were healthcare associated, suggesting that dogs act as a reservoir of nosocomial infections outside healthcare facilities.

A number of case studies have arisen where immuno-compromised patients would have recurring infections with the same strains of virulent Methicillin Resistant *S. aureus* (MRSA) upon completion of treatment (Manian 2003; van Duijkeren *et al*., 2005). Investigations had to be undertaken in order to locate the source of these infections as it was clear that they were surviving outside of patients during and after treatment. Upon investigations of the patients homes, it was found that the same strains of MRSA were residing in the family pet, asymptomatically. It was only after the pets were also treated did the reinfection of the patients cease. This highlights the potential pets have as a reservior for *S. aureus*. This is of particular concern due to the high rates of HIV infections in South Africa (Human Sciences Research Council, 2014).

As such, it has become the interest of the University of Kwa-Zulu Natal's Genetics research team to investigate the prevalence of antibiotic resistant strains of *S. aureus* isolated from companion animals, to determine if dog populations are over-looked as potential reservoirs for drug resistant *S. aureus*. This information may help identify possible sources of drug resistant *S. aureus* in dogs in the greater Durban area, expose evidence of poor antibiotic regulation in veterinary and human medicine and provide other information to draw up risk assessments and epidemiologic profiles of this pathogen as a basis of future antibiotic management and management for veterinary and healthcare services with regard to resistant *S. aureus* outbreaks.

S. aureus is not the most prevalent *Staphylococcal* species which inhabits dogs. That title goes to *S. pseudointermedius* which is indistinguishable from *S. aureus* in culture methods and requires genetic testing to differentiate between the two (Hanselman *et al*., 2009). Since virulence in *S. aureus* is not specific to the immune systems of dogs, *S. aureus* is somewhat in a delicate state when it must be isolated from dog samples due to its disadvantage existing in a dog host. This also means that samples are under a lot of competitive stress when they are~~it is~~ cultured. Currently, most prevalence studies for sampling *S. aureus* in dogs (Boost *et al*., 2008; Kottler *et al*., 2010; Han *et al*., 2016) use Mannitol Salt Agar (MSA) as a standard for selecting against competing microbes for *S. aureus*, even though it is not recommended for environmental samples and can accommodate growth of many halotolerant microbes (not only bacteria) (Gostinčar *et al*., 2009). This competition and poor selective power has the potential for *S. aureus* present to be masked and considered absent, thus further under-representing the potential dogs could play as a reservoir for *S. aureus*. Thus, it would be wise to assess the rate of incorrect *S. aureus* detection due to the MSA's ability to select against competing microbes

in comparison to a medium more suited to environmental samples such as HiCrome™ Aureus Agar Base (AAB) (HiMedia, 2015).

1.2. Hypothesis

The first hypothesis is that virulence genes and antibiotic resistance genes detected in *S. aureus* would indicate the pathogenic status of *S. aureus* carried by dogs in the greater Durban Area. The second hypothesis is that there is no significant difference in performance of MSA and AAB in its ability to accurately detect *S. aureus* from the nares of dogs. The final hypothesis is that these two investigations will detect no underestimation of risk associated with dogs as reservoir populations for *S. aureus*. pathogenic

1.3. Aims and Objectives:

- **1.3.1.** To enrich and select for *S. aureus* from samples using peptone water and HiCrome™ Aureus Agar Base and/or Mannitol Salt Agar, respectively.
- **1.3.2.** To isolate DNA from presumptive colonies using a simplified organic extraction method.
- **1.3.3.** To confirm species by detecting the *nuc* gene using conventional PCR.
- **1.3.4.** To establish resistance profiles of positive isolates using the Kerby-Bauer Disc Diffusion method and using PCR to detect common genes that cause such antibiotic resistance.
- **1.3.5.** To detect virulence genes using PCR so to draw pathological information of *S. aureus* in dogs of the greater Durban Area.
- **1.3.6.** To statistically interpret comparative selection data to gauge the sensitivity, selective power and accuracy of *S. aureus* selective media.
- **1.3.7.** To interpret the prevalence rates detected and the selection outcomes as a reflection of the potential~~risk~~ dogs have~~pose~~ as a reservoir population for *S. aureus*.

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CHAPTER 2: LITERATURE REVIEW

This literature review aims to scour previous research for knowledge that is relevant to *S. aureus* infection, transmission and drug resistance, with particular respect to human and companion animal interactions. It also includes the various methodologies available for isolation and selection of this organism. Gaps in knowledge in all these areas are explored as well as the detailed genetic and biochemical mechanisms that allow for *S. aureus* its range of pathogenicity.

2.1. Infection

2.1.1. Pyogenic diseases

Humans carry many different types of bacteria on their skin, some occur naturally as part of a healthy microbial ecosystem and some occur as a result of contact with the surrounding environment. *S. aureus* is an opportunistic bacterium that occurs abundantly in the environment and occasionally in human skin and other system flora but are often associated with pus-forming (pyogenic) diseases (Österlund *et al.*, 1997; Jarraud *et al*., 2002). The most common pyogenic disease caused by this bacterium is Impetigo, a skin infection in the form of skin lesions and sores which may produce pus which is highly contagious through direct contact, especially when skin integrity is compromised as a result of scratching (Kumar *et al.*, 2007). Other conditions associated with pyogenic staphylococcal infections include boils, pimples and abcesses. Burn victims and post-operative patients are also at great risk of contracting severe *S. aureus* infections in their wounds (Howden *et al.*, 2004).

2.1.2. Mange in companion animals

Mange affects most mammals with fur and is characterised as an uncontrolled increase of parasitic mites that populate the animal's skin. Mange appears as severe itching and inflammation of the skin, eventually resulting in hair loss, lesions and nasty scabs that can exist locally and globally on the animal's body (Fourie *et al*., 2007). There are two types of mites that can cause mange like symptoms in an animal: those that burrow into the skin and those that remain on the skin surface causing skin irritation. Of the burrowing category, there are two

species, namely, *Sarcoptes scabei* and *Notoedres cati* which cause sarcoptic and notoedric mange, respectively. Both species have females that burrow into the skin to lay eggs which hatch a few days later. *S. scabei* infects most domestic animals except cats, while *N. cati* is the most frequently identified mite on cats, although both can infect humans for short periods of time (a condition commonly referred to as scabies), (Stone *et al.*, 1972; Chakrabarti, 1986). In the non-burrowing category, there are three species of mite that can cause mange symptoms but are not transmissible to humans. *Demodex canis* causes demodectic mange and is most found on most domestic animals but is rare in cats and horses. This mite situates itself in the pilosebaceous glands of hair follicles causing skin irritation (Nutting, 1976). *Otodectic cyanotis* causes otodectic mange and infects the ear canals of cats and dogs (Sweatman, 1958a). *Psoroptes cuniculi* causes psoroptic mange which is most commonly found on the ears of rabbits. These mites feed on the animal's skin causing the irritation (Sweatman, 1985b). All 5 of these skin conditions will result in secondary infections caused, predominantly, by pyogenic opportunistic pathogens like *S. aureus* and *S. pyogenes* (Paradis, 1999).

2.1.3. **Attachment and basic immune system evasion**

S. aureus typically will start its infection by attaching to epithelial cells, usually in the nasal cavities, on the mucosal membranes. This is allowed for by surface proteins (figure 2.1) that can recognise fibronectin (glycoprotein that aids cell adhesion in the extracelluar matrix between cells), fibrinogen (a fibrin producing protein found in the plasma of blood) and collagen (primary protein for structural stability in conective tissue) (Ryan & Ray, 2004). *S. aurues* can then survive both among and within host cells ~~(Liu G. , 2009)~~ (Liu G., 2009). Once exposed to host tissues, *S. aureus* virulence genes are upregulated, allowing for infection to establish (Novick, Autoinduction and signal transduction in the regulation of staphylococcal virulence, 2003). Among these virulence genes are those that allow the cells to generate a capsule that has, specifically, protein A (Foster, 2005). This protein binds to the Fc subgroup on IgG (immunoglobulin G, a protein active in humoural immunity) preventing it from marking the *S. aureus* for opsonization (destruction via phagocytosis) (Rooijakkers *et al.*, 2005a). *S. aureus* can also produce a coagulase enzyme that can prevent phagocytes from reaching the site of infection effectively (Ryan & Ray, 2004). It has also been theorised that enterotoxins (toxins released in the intestines), toxic shock syndrom toxins and extracellular adhesion proteins, all produced by *S. aureus*, can interrupt the T-cell receptor activation pathway,

preventing long term molecular memory of *S. aureus* infections. This is considered the most probable reason that *S. aureus* infections can recur and persist in individuals without any wane in severity of infection (Llewelyn & Cohen, 2002; Liu G.-, 2009).

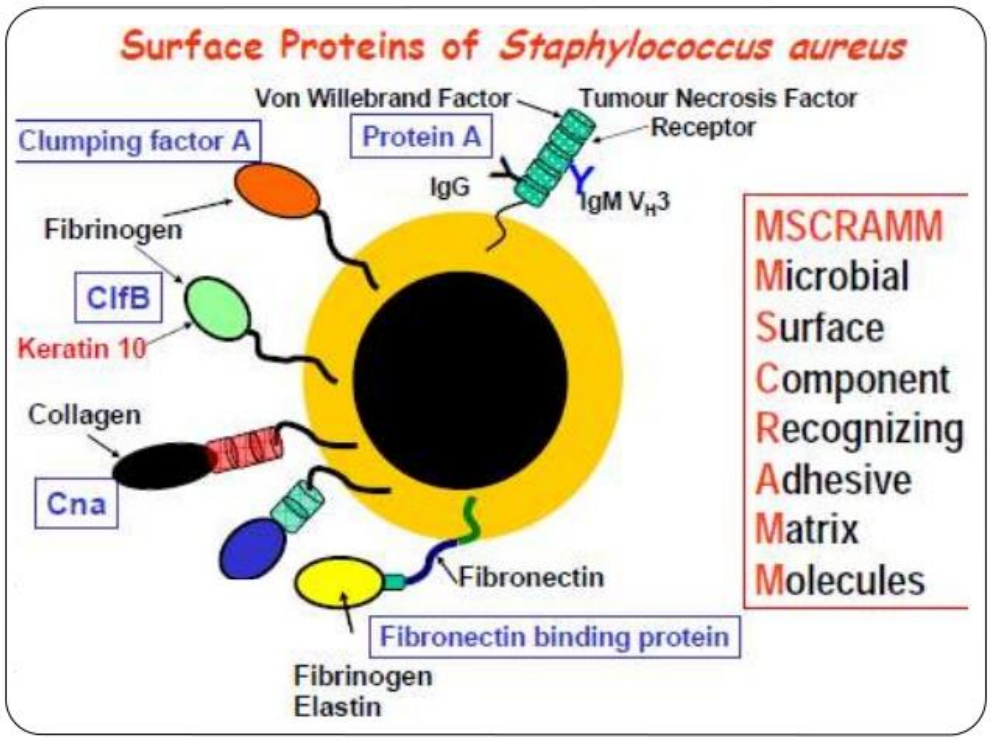

Figure 2.1: Showing the surface proteins of *S. aureus* used for recognising attachment surfaces in host cells.

2.2. Virulence of *S. aureus*

As *S. aureus* is generally considered commensal when inhabiting an animal host but it is not unknown that it has the capacity to become virulent and cause a range of diseases under the right circumstances (presence of appropriate surface proteins for the given host tissue, lowered host immunity due to other diseases such as mange in animals or HIV in humans). These infections generally involve the skin and and soft tissue which can evolve into scepticemia and blood poisoning when toxins produced by the bacteria enter the bloodstream. This is particularly dangerous as it puts patients and high risk (~12% of cases in children) of infective endocarditis, which is associated with a high mortality rate (Corey, 2005) (Valente *et al.*, 2005). Virulence in *S. aureus* is generally attributable to specific molecules (virulence factors), some coded for by specific genetic factors, that increase the potential of the bacteria to cause disease within the host organism. In general, these factors can be classified into 2 major groups (figure

2.2): those factors involved with the cell surface, i.e. factors that affect host cell attachment and host immune evasion (e.g. capsules), and those that are secreted by the cell. The secreted factors can be further split into groups including superantigens (enterotoxins), cytolytic toxins (those that cause tareget cell leakage or lysis, depending on concentration secreted), exoenymes that target specific host molecules for breakdown (e.g. lipases, proteases, etc.), and miscellaneous proteins (other proteins that impact the host's immune system/immune response) (Lin & Peterson, 2010; Costa *et al.*, 2013). Antibiotic resistance is considered a form of virulence, however, unless a strain posseses virulence factors of the above mentioned types, the antibiotic resistance will not affect the severity of infection of the bacteria. That being said, virulent strains which acquire antibiotic resistance prove to be some of the most troublesome pathogens encountered by modern medicine (such as MRSA).

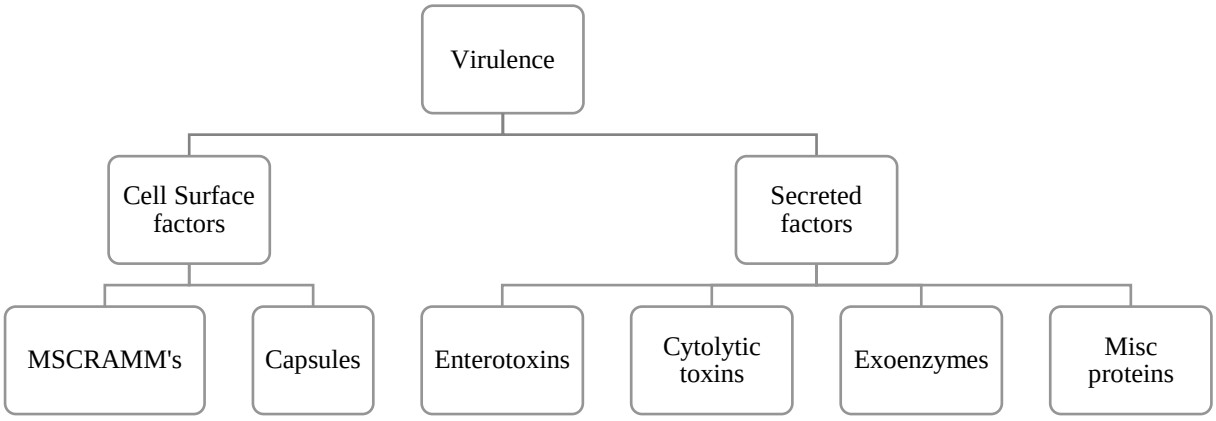

Figure 2.2: General classifications of Virulence factors found in *S. aureus*

As it has been known for the last two decades, *S. aureus* is most famous for its huge variety of different virulence factors, with different mechanisms and functions and so discussing each factor, its metabolic pathways, mechanisms and genetic elements in great detail would be more appropriately done so in a textbook or a series of reviews. So discussed below, in a more general fashion, are some of the more prevalent virulence factors expressed by *S. aureus*, their genetic elements and means of acquisition of such virulence factors.

2.2.1. **Cell surface factors**

Cell surface virulence factors are those that directly involve the interaction between bacterium cell surface and host molecules or surfaces. There are three main groups of cell surface factors, Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM's),

capsule polysaccharides and staphyloxanthin. All these factors are expressed or produced during the initial and log phases of growth in order to establish residence within host tissues.

2.2.1.1. **MSCRAMM's**

MSCRAMM's are directly involved with the first steps of infection via the attachment to host tissues (figure 2.1). The two most prevalent of these, are staphylococcal protein A (SpA) and fibronectin-binding protein A (FnbpA or FnbA). (Bhatty *et al*., 2013; Votintseva *et al*., 2014).

SpA, coded for by *spa* is a chromosomally located gene, and can be found in all naturally occuring strains (Patel *et al*., 1989). It has the important function of binding to the Fc site of immunoglobulin G (the site that triggers phagocytosis, see figure 2.3), thus hiding themselves from being marked for phagocytosis by host macrophages. This defense mechanism works incredibly well with capsule polysaccharides which can be excreted as they too make phagocytosis and opsonization difficult for the host and allow for colonies to persist within mucosal membranes of the host (nasal, oral, vaginal, intestinal).

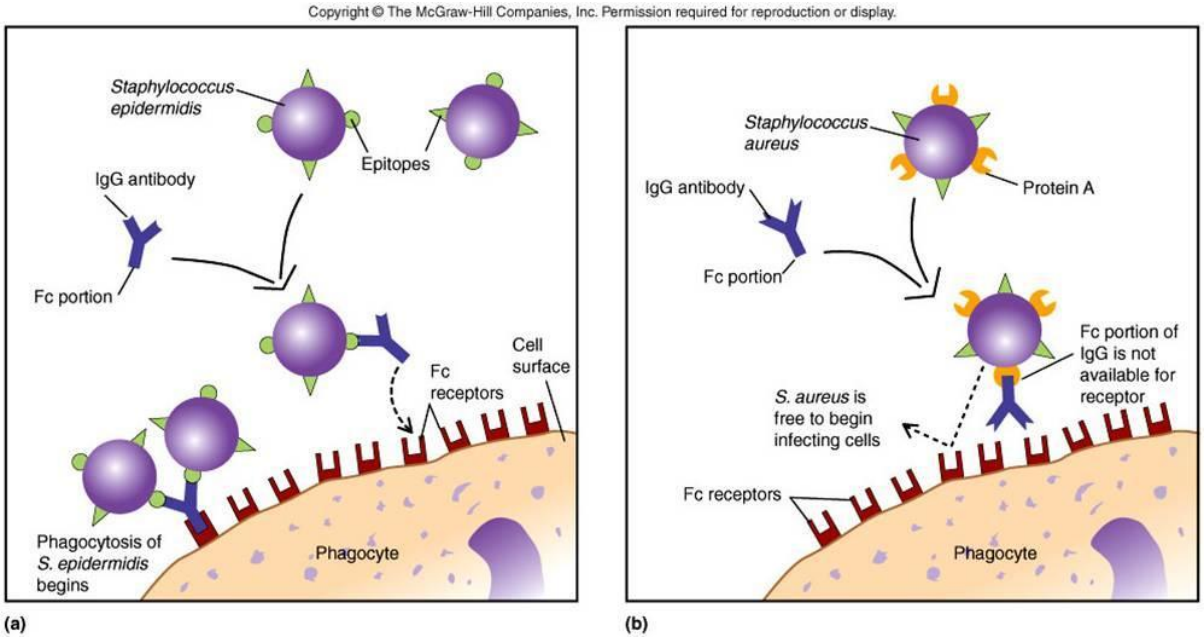

Figure 2.3: Showing the mechanism of action for immunoglobulin G for marking invading bodies for phagocytosis, in the absence (a) and presence (b) of staphylococcal protein A

Despite being present in all strains, there is plenty sequence variation in the *spa* gene. Some variants were recently untypeable until a new typing method was developed in 2014 (Votintseva *et al*., 2014). This study suggested that some previously untyped strains that have

been found commonly in livestock have most likely been underrepresented in human typing studies, suggesting that there may be even more sharing of *S. aureus* strains between animals and humans than previously though. However, it is not known by how much it has been underestimated. This also proves that there as been a great underestimation of the diversity and prevalence of the carriage of mixed strains, but by how much is still unknown.

The *spa* gene is an approximately 2kb gene made up of 3 distinct regions (figure 2.4), the F_C region which codes for the subunits for IgG binding of protein A, the X_c region which codes for the subunits for the binding of protein A to *S. aureus*'s cell surface, and the X_r region which does not have a clear function but does generate peptides that can induce type I interferon (type I IFN) signaling in host cells (Martin *et al*., 2009; Furuya *et al*., 2010). As the type I INF pathway is primarily used for halting various stages of viral infection which inludes inhibiting translation mechanisms, this results in an increased virulence for infecting *S. aureus* cells (Schoggins & Rice, 2011; Markušić *et al*., 2014). The X_r region varies in size as it is a short sequence repeat region (SSR) that has 24bp repeat units that vary in sequence and number, combinations of which are specific to the *S. aureus* strain. Typing of this region involves the amplification of the X_r region using conventional PCR primers and then sequencing of the product (Shopsin *et al*., 1999; Harmsen *et al*., 2003; O'Hara *et al*., 2016). Sequences can then be compared to those in the Rindom SpaServer database (http://www.spaserver.ridom.de/) run by Rindom Bioinformatics and a *spa*-type will be assigned. The known *spa*-repeats can theoretically define as many as 17563 *spa*-types in varying combinations.

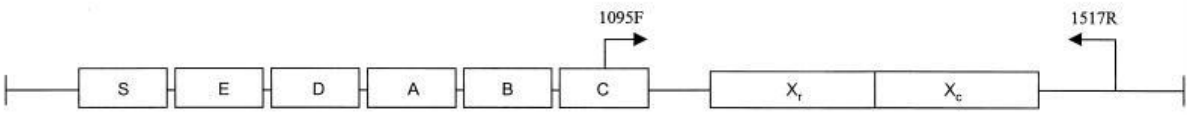

Figure 2.4: Structure of *spa* gene showing F_C subunits S, A-E, and X_r and X_c regions. Arrows indicate the region from which primers are designed for *spa* typing.

Fibronectin-binding protein A (FnbA) is one of the most prevalent virulence factors found in *S. aureus*, with its gene *fnbA* being present in about 83 to 88% of clinical isolates while its relative fibronectin-bindimg protein B (FnbB) with its gene *fnbB* occurs at about half that frequency (Bhatty *et al.*, 2013; Mirzaee *et al.*, 2015). Other studies showed more varied frequencies (up to 98% for *fnbA* and 99% for *fnbB*), whose variation was simply attibuted to sampling biases, source of isolated samples, and the genetic background of said samples. The only consistant variation seen was that MRSA isolated generally had higher instance of FnbP

being present than in MSSA isolates (difference averageing at ~10%) and that isolates from healthy patients (usually nasal carriage) had much lower FnbP gene frequencies (76%) (Rice *et al*., 2001; Arciola, *et al*., 2012).

The genes *fnbA* and *fnbB* are found chromosomally and are not transmitted horizontally between strains but rather are inherited. These proteins allow for *S. aureus* to bind to fibronectin proteins found which are important structural and antimicrobial molecules found in fibrous connective, blood clots at wound sites and in the saliva of vertabrate animals. In saliva, fibronectin can prevent the adhesion of certain bacteria to the pharynx and buccal cells (Hasty & Simpson, 1987; Valenick *et al*., 2010).

As *S. aureus* can use fibronectin binding as a means of anchorage to host tissue, this allows it to readily infect wounds, especially those involving skin and soft tissue. Mutant studies showed that when strains with non-functional FnbA proteins were introduced to blood clot assays, there was no initiation of infection, suggesting that this virulence factor is probably the most important factor for the pathogenesis of *S. aureus*, and even without any other virulence factors present, *S. aureus* can cause illness: even polystyrene beads coated with fibronectin binding protein established infection in cell cultures of human embryonic kidney cells (Sinha *et al.*, 2000). With such information, it is would be of great benefit to develop a drug, or even a topical treatment that inactivates the FnbP's that could be applied to wounds as well as internalised devices such as catheters, IV drip needles, etc.

2.2.1.2. **Capsules**

Capsules can also be produced by *S. aureus*. This is the formation of a polysaccharide matrix that surrounds the cell, exterior to the cell wall, that has a multifaceted function. The most important function is that the matrix protects surface proteins from being recognised by host immune antibiodies and so the bacteria cannot be marked for phagocytosis (O'Riordan & Lee, 2004). It has been understood for some time that the biosynthesis of this extracellular matrix is determined by a gene cluster designated as a CP (capsular polysaccharide) biosynthetic cluster, of which there are multiple serotypes, the most common ones being CP5 (50% of isolates) and CP8 (25% of isolates) which have many genes within of which 75% are almost identical, with *cap5* and *cap8* genes being allelic in the respective clusters. Despite the detailed sequence understanding of these clusters, it has only recently been elucidated, how the some of the enzymes coded for in within, participate in the biosynthesis of these polysaccharides (Li *et al.*,

2014). This has opened up the opportunity to develop drugs that can target the enzymes involved in polysaccharide precursor synthesis so to bypass the pathogenicity of *S. aureus* enabled by the presence of a capsule.

These gene clusters have no affiliation to plasmids and are situated chromosomally. While serotypes 5 and 8 are most common, they are not the most pathogenic. CP1 has proven to cause the most virulent infections, as it allows for greater resistance to phagocytosis and is even lethal to mice (Lee *et al*., 1987). This is due to the presence of the gene *cap1*. Unlike *cap5* and *cap8*, *cap1* is not allelic but rather resides in its own discrete genetic element with flanking regions. What is so interesting about this is that this element was found to resemble that of the Staphylococcal Cassette Chromosome (SCC) so often associated with carrying the methicillin-resistance determining gene, *mecA* (SCC_{mec}) (Luong *et al*., 2002) (see part 2.4 for disccussion of SCC_{mec}).

However, the element is not mobile, thus it cannot be transmitted between strains. Flanking the SCC_{cap1} locus was the gene for a (at the time) novel enterotoxin. It was suggested that the 2 genetic elements were inserted into genome at independent events but are carried together, suggesting that the lethality of CP1 was actually attributable to that toxin and not the capsule as previously thought. This means that if expression of that toxin can be deactivated or the toxicity of the molecule produced is neutralised, the pathogenicity of CP1 type strains of *S. aureus* can be greatly reduced.

2.2.2. **Secreted Factors**

Secreted virulence factors are proteins that are synthesised within the cell but their primary funtions are outside the cell, away from the cell surface, usually interacting with host tissues/molecules. There are four categories of secreted virulence factors including superantigens (also referred to as enterotoxins), cytolytic toxins, exoenzymes and miscellaneous proteins (Costa *et al*., 2013). All of these factors are expressed during the stationary phase of growth, after infection is established. Discussed here are those secreted factors that have been found on mobile genetic elements, (discussing all factors would be time consuming and more detailed information can be easily found in other sources).

2.2.2.1. **Superantigens**

Superantigens include both the enterotoxins and the toxic shock syndrome toxin-1 (TSST-1). These superantigens are enzymes that have a poisonous affect on the host. This does not include enzymes involved in interactions with the host immune system directly. There are 22 enterotoxins (A through V), including staphylococcal-like enterotoxins (SE*l*'s) all of which are coded for by mobile genetic elements. Most famously, these enterotoxins (SE's) are known to cause food poisoning that involves a range of symptoms, sometimes including severe dehydration due to excessive vomiting and diarrhea, commonly reffered to as Staphylococcal Food Poisoning (SFP). This is generally self-limiting as the infection will clear within a few days once the body has flushed it out, and so hospitilisation is rarely neccesary except for cases with extreme dehydration (Argudín *et al*., 2010). Even when *S. aureus* is destroyed by the gut or by high temperatures caused by a fever, the toxins will remain intact and are even resistant to proteolytic enzymes, causing damage to the host until completely passed throught the gastrointestinal tract (Larkin *et al*., 2009). The two major groups of staphylococcal superantigens (SE's and SE*l*'s) are grouped according to their ability to cause an emetic resposnse in a primate model (SE's) or the lack there of (SE*l*'s). The SE's are most famous for the five classic toxins SEA to SEE, most highly associated with SFP. However, according to amino acid sequence, these enterotoxins can be grouped into four or five groups (table 2.1) (four if SEH is put with group one or five if SEH is grouped seperately, depending on the author) (Proft & Fraser, 2003; Thomas *et al*., 2007).

Table 2.1: Grouping of staphylococcal and staphylococcal-like enterotoxins according to amino acid sequence. Note: depending on the author SEH can fall into group 1 or its own group.

Group	*Superantigen*
1	SEA, SED, SEE, SEH*, SE*l*J, SE*l*N, SE*l*O, SE*l*P, SES
2	SEB, SEC, SEG, SER, SE*l*U, SE*l*U2
3	SEI, SE*l*K, SE*l*L, SE*l*M, SE*l*Q, SE*l*V
4	SET
5	SEH*

Superantigens work by interacting with class II MHC and with T-cells. Under normal circumstances antigen-presenting cells (APC's) will engulf and breakdown microbial antigens, only to use them as small peptides bound to class II MHC molecules on its cell surface. This

invites T-cells to recognise this complex with their T-cell receptors (TcR's) in order to trigger an immune response against that specific antigen (0.01 to 0.001% of T-cells are activated) (Lanzavecchia, 1985; Holling *et al*., 2004). Superantigens will complete this process while bypassing the antigen recognition step by directly crosslinking the class II MHC of the APC to the TcR's on T-cells. Because this is non-specific antigen-mediated binding, up to 30% of T-cells can be activated at once, causing a severly over-exaggerated immune response, causing shock (Fraser, 2011) (figure 2.5).

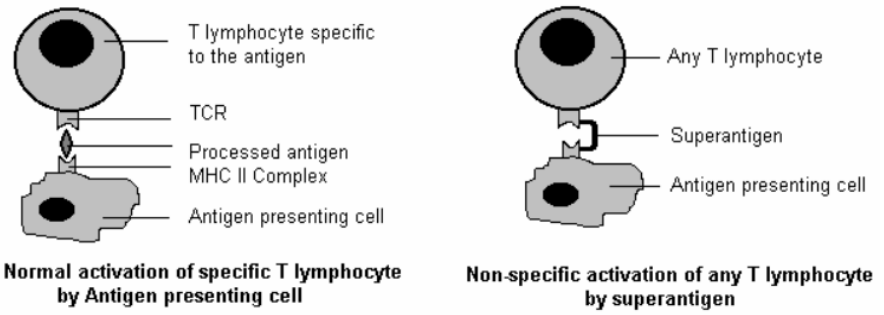

Figure 2.5: Showing the response to foreign antigens under normal circumstances and in the presence of a super antigen

It must be clearly differentiated that this is superantigenic activity which can only occur when superantigens enter the blood stream and can be potentially life threatening. This is not the activity observed when enterotoxins remain in the GI tract. The mechanism as to how these toxins cause GI distress is not well understood but is but is most likely involved with the activation of cytokine release, causing damage to the surrounding tissue due to cell apoptosis (Lin *et al*., 2011; Otto, 2014). Table 2.1 shows that there is no obvious correlation between the related sequence group and whether the superantigen has an emetic effect. Much research has gone into trying to find the sequence related origin of what causes emesis with these superantigens, but with no luck, no difinitive evidence has arisen (Proft & Fraser, 2003). However, if the mechanisms by which enterotoxins maintain their toxicity within the GI tract, it may shed light onto how the sequences contribute to that, which in turn could highlight the sequence responsible for emetic reactions to particular SE's.

2.2.2.1.1. **Mobile Genetic Elements of Superantigens**

Genes which code for SE's and SE*l*'s have been found carried by a plethora of various mobile genetic elements including plasmids, prophages, pathogenicity islands (SaPI's) and genomic islands.

Genomic Islands

Unlike the other elements, genomic islands make up part of the genome and so far no evidence has been found that they may move out of the genome for purposes of horizontal gene transfer. Two major pathogenicity islands found exclusively in *S. aureus* are *v*Saα and *v*Saβ both of which will occasionally carry an enterotoxin gene cluster (*egc*). This cluster has four main variants which include the genes *seg*, *sei*, *selm*, and *selo*, among others including *seln*, *selv* and *selu* which differ per variant (Collery *et al*., 2009). It has been hypothesised that these *egc*'s were originally from on SE gene that through its own duplication and then subsequent variation, gave rise to the *se* genes currently observed in *egc*'s (Jarraud *et al*., 2001). One of the most curious things about these gene clusters is that they can be found within commensal type strains of *S. aureus* without any sepsis occuring in the host.

Unlike other toxins (with special mention of SEA, coded for by *sea*), which can cause severe symptoms in the host, these *egc* genes refrain from causing major damage to the host. van Belkum *et al*. (2006), concluded that this must be as a result of one of the *egc* genes providing the host with some protection against sepsis (this is beneficial for the bacteria as sepsis is self limiting whereas commensalism is preferable for long-term survival of the bacteria). However, this theory is yet to be investigated. They also found that in invasive isolates of *S. aureus*, there was a slightly lower prevalence (54% versus 63%) of the *egc* elements than in isolates from healthy carriers ($P = 0.03$) (van Belkum *et al*., 2006).

Staphylococcal Pathogenicity Islands (SaPI's)

SaPI's, the most common of *se* and *sel* associated mobile genetic elements, are able to move in and out of the genome at specific sites, in a specific orientation. There are many SaPI's, all with similar in structures, with the same insertion site and orientation with the *S. aureus* genome (Argudín *et al*., 2010). Their structure is also quite conserved when compared to most temperate bacteriophages, in fact SaPI's even require particular certain helper phages to induce their replication outside of the *S. aureus* genome, in order for copies to be transmitted between bacteria horizontally. The great number of pathogenicity island variants (as of 2010, at least 20 have been fully sequenced) even allow for some to be transmittable between different *Staphylococcus* species (Tallent *et al*., 2007). These pathogenicity islands usually carry the

toxic shock syndrome toxin (TSST-1) gene, *tst* along with two to three other *se*'s/*sel*'s (Dinges *et al*, 2000; Novick & Subedi, 2007).

TSST-1 differs from the enterotoxins in that it can cross mucosal surfaces and does not specifically have to be ingested or absorbed through the GI tract. This is why so many cases of toxic shock syndrome (TSS) are mentrual related (around 90% of TSS cases are menstrual related (Lin & Peterson, 2010)). However, not all TSST-1 induced cases of TSS are menstural. Some cases are as a result of post-surgery complications, burns or pneumonia, particularly at sites where there is a mucosal surface to travel across. About half of cases of non-menstrual TSS have been reported with enterotoxins SEB and SEC being the primary cause and not TSST-1. According to Foster (2005) 25% of clinical *S. aureus* isolates carry the *tst* gene. However, it has been noted that not all of the alleles are functional. One allele which allows for the substitution of H135A which results in a structurally decent yet functionally inactive version of the TSST-1 protein (a toxoid protein). In a study by Spaulding *et al*, (2012), this version was used as a vaccination in rabbits to successfully prevent superantigenic reactions from the functional versions of TSST-1 that cause serious illness. This is an important step for developing better combative mechanisms against superantigenic activity of *S. aureus* and further toxoid substances should be developed or sought out for toxins produced by other bacteria as well as *S. aureus*.

Plasmids

Plasmids are some of the most well understood mobile genetic elements and are especially important in that they possess the unique ability to perform conjugation, an elegant manner of horizontal gene transfer. *S. aureus* too utilises plasmids for harbouring *se* and *sel* genes. Two types of plasmids that carry enterotoxins have been characterised, pIB485 and pF5 (including pF5-like) which carry *ser* and *selj* genes. pIB485 also carries *sed* and pF5 will occasionally also carry *ses* and *set* (Omoe *et al*., 2003). The gene for SEB, *seb* has also been observed within a plasmid, pZA10, along with *sec1*, both of which are more often seen carried by staphylococcal pathogenicity islands (SaPI's) than pZA10 (Hu & Nakane, 2014).

Prophages

Prophages are bacteriophages that integrate their genetic material into the host's genome. Prophages that are specific to infecting *S. aureus* with additional *se* and *sel* genes most often belong to the *Siphoviridae* family. These phages will infect *S. aureus* with the usual mechanisms of bacteriophage infection but the conversion into a prophage is achieved through a recombination event, directed by an integrase (coded for by *int* on the phage genetic material) that inserts the *attP* site of the phage DNA into the *attB* site in *S. aureus*'s genome (Deghorain & Van Melderen, 2012). The *attB* site in the *S. aureus* genome actually falls within the β-haemolysin gene (*hlb*), another virulence gene. As expected, this does inactivate this naturally occuring virulence gene but this loss is recompensed by the acquisition of new virulence genes, sometimes more than just one including *sea*, *selk*, *selq* and *selp*.

The most toxic of the enterotoxins and the most highly associated with cases food of food poisoning is SEA coded for by *sea*. Some *sea* only phages have been found in troublesome strains of MRSA and VRSA (Argudín *et al*., 2010). It has also been found that most strains of MRSA are toxin carrying strains (Schmitz *et al*., 1997). SEE, coded for by *see* is also a phage-mediated enterotoxin gene and also the second most prevalent enterotoxin associated with SFP (Mashouf *et al*., 2015). To date there are at least 8 characterised phages that carry *sea*, and what with the ubiquitous nature of bacteriophages, there are any number of bacteriophages that could potentially carry SE's (Schelin *et al*., 2011). Naturally, these prophages all have the ability to revert to the lytic cycle if conditions become unfavourable, in order to release progeny prophages, which can then infect other *S. aureus* strains, thus proving their threat through both vertical and horizontal gene transfer.

2.2.2.1.2. **The Immune Evasion Cluster (IEC)**

Apart from enterotoxin genes, many of these prophages have been found carrying other virulence genes such as *chp*, *scn* and *sak* genes which are closely situated on the phage genome, making up part of the innate immune evasion cluster (van Wamel *et al*., 2006). The Immune Evasion Cluster (IEC) is a gene cassette carried most often by β-haemolysin converting bacterophages (βC-ϕ's) and carries a number of immune modifying enzyme genes that enable the infecting bacterium to better bypass the host's innate immune system. The genes most often carried within this cluster include chemotaxis inhibitory protein of *S. aureus* (CHIPS coded for by *chp*), staphylococcal compliment inhibitor (SCIN coded for by *scn*), staphylokinase (SAK

coded for by *sak*), enterotoxin A (*sea*) or enterotoxin-like P (*sep*). All seven of the characterised variants of IEC carry the gene for *scn* and some combination of the other three genes (Verkaika *et al*., 2011; van Wamel *et al*., 2006)(see figure 2.6).

The vehicle for IEC's are aptly named βC-ϕ's as the attachment site for the prophage is found within the β-haemolysin gene, *hbl*. As mentioned before, this will result in the inactivation of *hbl* but this form of virulence is recompensed by the addition of multiple new genes that allow for better protection against the host's innate immune system. All genes products included in the IEC (with the exception of *sea*) show strict specificity to the human immune system and would otherwise not affect dogs, however, if isolates are carrying genes from the IEC, if transmitted to humans, would still be effective against human hosts (Rooijakkers *et al*., 2006).

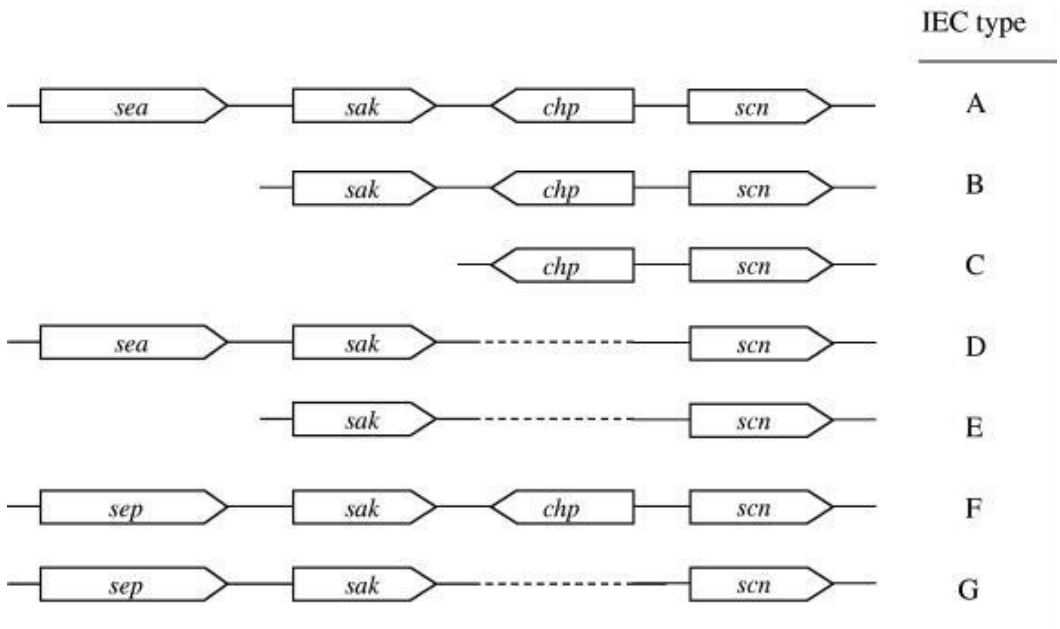

Figure 2.6: Variations of Immune Evasion Clusters that can be found in β-haemolysin converting prophages (van Wamel *et al*., 2006)

Chemotaxis Inhibitory Proteins (CHIPS)

Anaphylatoxins are specialised proteins produced as part of the human immune compliment system. These proteins (namely C3a, C4a and C5a) are part of a biochemical process that causes an inflammatory response to an antigen, in context of this study, an *S. aureus* infection (Fritzinger *et al*., 1992). Another type of protein that also allows for chemotaxis as well as the activation of phagocytes is the chemotactic factor, also referred to as fMLP (N-Formylmethionyl-leucyl-phenylalanine) (Panaro & Mitolo, 1999). These proteins specifically

allow for chemotaxis, which is the process whereby neutrophils and monocytes (phagocytic white blood cells) move out of the blood stream (diapedesis) and toward the site of infection by following an increasing concentration of these anaphylatoxins. Once at the site of infection the neutrophils and monocytes will attempt to destroy invading pathogens via phagocytosis (Rooijakkers *et al*., 2006; Postma *et al*., 2004). CHIPs (chemotaxis inhibitory proteins) coded for by *chp*, bind to C5a and fMLP proteins, thus preventing chemotaxis and stalling the early detection systems of the host immune system. While *chp* on its own does not allow for more serious morbidity, in conjunction with other virulence factors, with specific reference to the enterotoxins, the presence of *chp* can allow for a longer window of opportunity to cause as much toxicity as possible before the host's immue system can successfully target the invading pathogen. This is especially effective as *chp* is expressed during the exponential phase of growth (Rooijakkers *et al*., 2006).

Staphylococcal Compliment Inhibitor (SCN)

Also expressed during exponential growth is *scn* which codes for the protein SCIN, Staphylococcal complement inhibitor. According to the conditions used in the study by Rooijakkers *et al*., (2006), the promotor region for this gene is five times more active than the other genes making up the IEC. SCIN, like CHIPS, prevents opsonisation and phagocytosis by interacting with the compliment pathway. Unlike CHIPS, SCIN binds specifically with C3 convertases that are already attached to the microbe, thus preventing the formation of downstream molecules that allow for phagocytosis and opsonisation (Rooijakkers *et al*., 2005; Rooijakkers *et al*., 2007). Naturally, it would be more economical that *scn* is expressed more than *chp* as it targets molecules upstream of those targeted by *chp*, however, this does not necessarily apply to conditions not specified by that study (Rooijakkers *et al*., 2006), and more research should be conducted *in vivo* to determine if this is the case in actual infection conditions.

Staphylokinase (SAK)

Staphylokinase or SAK, expressed product of *sak*, is produced during the late exponential phase an and employs two immune evasion tactics. It is able to bind to both plasminogins and α-defensins. *S. aureus* is able to capture host plasminogens (the inactive precursors to plasmin)

on its cell surface it can allow for SAK-mediated activation of the plasminogen into plasmin (Molkanen *et al*., 2002). Once activacted, plasmin, a broad spectrum proteolytic enzyme which is able to break down the fibrin network formed to clot blood (Law *et al*., 2012). This prevention of blood clotting may help prevent lacolising the site of infection. SAK also binds with defensins.

Defensins are host produced proteins that are rich with positively charged amino acid functional groups. This draws the defensins to the negatively charged phospholipids of the bacterial cell membrane. Defensins are also folded in a polar manor such that opposite to the positive end are hydrophobic amino acid groups. Once in contact with the bacterial cell wall, the hydrophobic end of the defensin will bury itself into the cell membrane with phospholipid heads clustered all the way around the molecule, forming a hole in the cell membrane (Hazlett & Wu, 2010). Naturally, enough holes will cause the invading pathogen to lose osmotic pressure and die. SAK can bind to and inactivate these defensins preventing this from occuring (Bokarewa *et al*., 2006) . However, the role SAK plays in the infection processes is not fully understood as Jin *et al*. (2003) observed that patients with *S. aureus* isolates that did not carry the *sak* gene were 3 to 4 times more likely to be lethal than those isolates that did carry it (Kwieciński *et al*., 2010) and it has also been noted that the *sak* gene is expressed far more in mucosal and skin isolates than isolates invading internal organs.

2.3. Transmission

While *S. aureus* is opportunistic and is transferred between humans primarily through contact (Koenig *et al*., 2016) (Jacobs, 2014), and is commonly understood to be a zoonotic microbe (Public Health Agency of Canada, 2011), there is some uncertainty about the rates of transfer from dogs to humans and humans to dogs. A cross-sectional study done by Boost *et al*., (2008) showed that of 17 owners and their dogs (not limited to one dog per owner) that tested positive for *S. aureus*, 6 sets had isolates with indistinguishable Pulse Field Gel Eelectrophoresis (PFGE) profile. This suggested that there was transfer between owner and dog. However, according to the questionaires completed by the owners, transfer was less associated with the manner of contact with the pets (petting, licking the owner's face etc.) but rather with the profession of the owner. More specifically, healthcare workers showed the highest association with identical PFGE profiles between owner and dog than any other profession. This suggests that transfer from owner-to-dog is more likely than dog-to-owner. The authors did however

suggest that a larger sample size with bigger control groups should be conducted to prove or disprove that hypothesis.

Earlier studies have confirmed that MRSA carriage in companion animals can still pose a threat to their human owners. A surveillance study by Middleton *et al*. (2005) at Veterinary teaching hospitals across the United States showed that 65 animals (mostly dogs and horses but also including birds, cats and cattle) carried 70 isolates of *S. aureus*, 58 with unique typing profiles and all MRSA strains (14% of animals carried MRSA) were distinguishable by PFGE. What is interesting to note is that, Middleton *et al*. (2005) reported that the infections appeared to be community-acquired as opposed to hospital-acquired, and that acquisition from the community occurs at rates similar to hospital-acquired *S. aureus* infections (Duquette & Nuttal, 2004). There is currently no data for South African domestic animals as focus has mainly been on livestock (Schaumburg *et al*., 2014). While it is understood that transfer to healthy humans is rare, there is still great risk for those individuals who are immunocopmromised.

A case study showed the chronic MRSA reinfenction of the leg stump of man with diabetes mellitis and his wife, a transplant patient also with diabetes, symptomatic of cellulitis (Manian, 2003). They jointly owned an asymptomatic dog that shared very close contact with them, such as kisses, licking of faces and sleeping on the bed with its owners. It was found that the dog carried a strain of MRSA indistinguishable by PFGE to that which kept recolonising the two patients. Only after decolonisation of the MRSA from the dog and the limited intimate contact with the dog were decolonising therapies of the couple successful. The dog was healthy and had no prior exposure to antibiotics and so it is assumed that its MRSA was originally acquired from its owners, who most probably acquired it nosocomially. In turn, the dog acted as a reservior of MRSA which constantly reinfected its owners.

Another study observed a woman admitted to hospital for diabetic foot that had ulcerated with positive cultures for fusidic acid and tetracycline resistant MRSA (van Duijkeren *et al*., 2005). The patient was successfully treated for the infection but it recurred as a urinary tract infection months later with resistance patterns identical to the previously isolated strain. After the second round of treatment, the patient followed a 6 month screening regime to monitor if she would reacquire that strain of MRSA as a carrier. On the final screening she was tested positive for the same strain of MRSA. Subsequent screening of her husband, son and dog showed they too were carriers of the same strain (indistinguishable by PFGE). All were treated with successful eradication of that MRSA strain for at least the 6 month screening process subsequent to

antibiotic treatment. It was assumed that the woman initially transmitted the MRSA to the other members of her household who subsequently reinfected her, although there was no medical history given for the dog. Without that information, it cannot be determined whether the dog acquired it communally or through its own exposure to antibiotics.

2.4. Antibiotic Resistance

Penicillin was the greatest combatant against *S. aureus* in the early to mid-20th century and by the 40's, penicillin resistant strains arose, which led to the increased use of methicillin as an alternative to penicillin, in turn selecting for methicillin-resistant strains which arose in the early 60's: the infamous MRSA (methicillin resistant *S. aureus*) (Lowy, 2003). This strain had developed a broad resistance to penicillin-derived antibiotics known as β-lactams including oxacillin and cefoxitin (Fernandes *et al.*, 2005). It did so due to an acquired plasmid carrying the code for an enzyme called penicillin binding protein 2a (PBP2a) that can hydrolyse the β-lactam ring which confers antibiotic capabilities to β-lactam antibiotics like penicillin and its derivatives (Chambers & DeLeo, 2009). In 2010, a meta-analysis was carried out by Logman *et al*., to investigate which non-β-lactam antibiotics are effective against MRSA and it was found that linezolid and some glycopeptide antibiotics were effective against skin and soft tissue infections of MRSA (Logman *et al*., 2010). However there have been increasing incidents of resistance to most types of antibiotics, not only the above mentioned, worldwide, making the need to study and understand the mechanisms of resistance and resistance conference all the more important.

According to the data collated by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the following antibiotics in Table 2.2 (from different antibiotic classes) are the more prevalent antibiotics used against *S. aureus* all of which have documented cases of resistance (EUCAST, 2016). There are other antibiotics that have prevalent use agains *S. aureus* but have been ommited as more prevalent representatives of that class have been included below.

Table 2.2: Names and classes of antibiotics discussed in this review.

Antibiotic Name	***Antibiotic Class***
Cefoxitin (methicillin alternative)	Cephamycin
Ceftoroline	Cephalosporin
Ciprofloxacin	Fluoroquinolone
Clindamycin	Lincosamide
Erythromycin	Macrolide
Gentamycin	Aminoglycoside
Linezolid	Oxazolidinone
Teicoplanin	Glycopeptide
Tetracycline	Tetracycline
Vancomycin	Glycopeptide

2.4.1. **Cefoxitin**

Cefoxitin is a 2nd-generation cephamycin (often grouped with cephalosporins), a sub-class of antibiotics grouped with penicillins to form the class of β-lactams, as both groups contain β-lactam rings. It is used against a broad spectrum of both Gram-positive and Gram-negative bacterial infections (Onishi *et al.*, 1974; Guignard *et al*., 2004). Cefoxitin was originally used against extended spectrum β-lactamase producing organsims such as MRSA (Jarlier *et al.*, 1988; Fuda *et al*., 2005). It works in a very similar way to penicillin in that it binds to the reaction site of transpeptidase enzymes originally purposed for catalysing crosslinkage between peptidoglycan chains, thus reinforcing the structure of the cell wall (Stapleton & Taylor, 2002) (figure 2.7). These enzymes are also reffered to as penicillin binding proteins (PBPs). Before the development of 2nd generation penicillins (methicillin and oxacillin) and cephamycins (cefoxitin), penicillins could be broken down if the target cells carried β-lactamase which could hydrolyse the β-lactam ring in penicillins and cephalosporins before they took effect. These 2nd generation β-lactams were designed to have functional groups that sterically hinder the enzymes from recognising them as substrates (Chambers, 1997). However, resistance to these too emerged in the form of a modified transpeptidase enzyme that could carry out its function as a transpeptidase but will not bind to these 2nd generation β-lactams (figure 2.7).

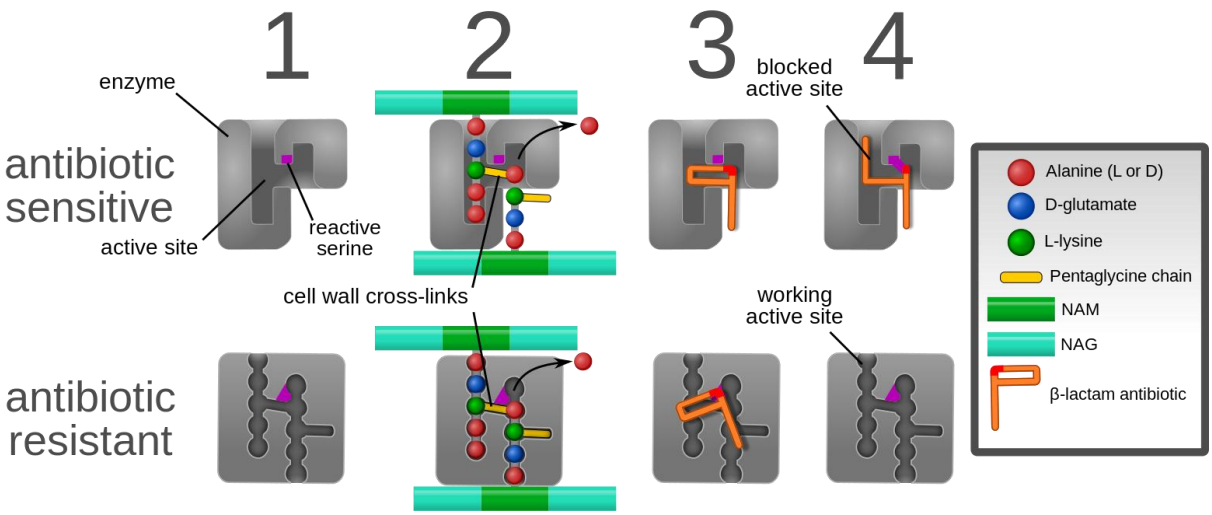

Figure 2.7: The mechanism of action of β-lactam antibiotics and the mechanism of resistance to β-lactam antibiotics with modified transpeptidase enzyme.

Strains resistant to 2nd generation β-lactams have PBP2a which is coded for by the *mec* gene set which includes *mecA* which codes for the PBP2a protein and *mecR1* and *mecI* which code for transcription regulation elements. It is suggested that the most probable source of *mec* was through the acquisition of some cromosomal DNA via mobile genetic elements from *Staphylococcus fleurettii* (Tsubakishita *et al*., 2010). Research by Tsubakishita *et al*. (2010) also suggests that this segment of DNA with *mec* is most likely what evolved to be the Staphylococcal Cassette Chromosome (SCC*mec*) which is so broadly studied today.

Staphyococcal Cassette Chromosome (SCC*mec*) can be found integrated with *S. aureus*'s chromosome or may remain as a mobile genetic element. Within it are four definitve elements (Figure 2.8), two of which are also used to classify variants of SCC*mec* into allotypes, the *mec* region and *ccr* region (table 2.3). The *mec* element is composed of the *mec* gene set (*mecA*, *mecR1* and *mecI*) coding for resistance to methicillin and some other β-lactams. The *ccr* element is composed of the *ccr* gene set and open reading frames (ORF's) surrounding the *ccr* gene set (Chongtrakool *et al*., 2006).

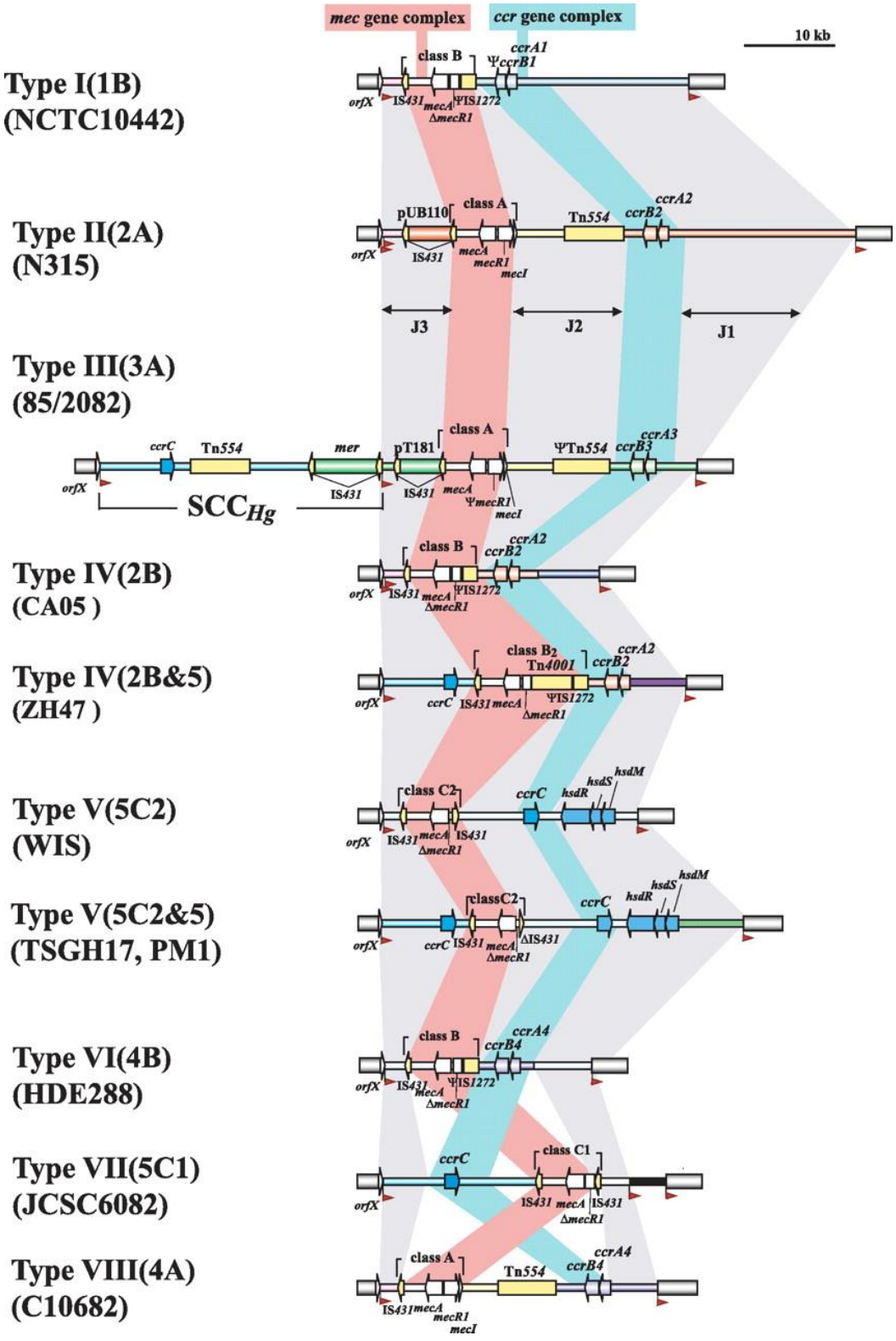

Figure 2.8: Showing anatomy and homology of the SCC*mec* types

The *ccr* gene set carries the sequences of unique site specific cassette chromosome recombinases which direct site and orientation specific excision and integration of SCC*mec* in and out of the host's chromosome. The surrounding ORFs are of unknown function. There are

three main *ccr* types (A, B and C), each with less than 50% sequence identity. Types A and B each have four (A1-4 and B1-4) subtypes and will often occur together (see table 2.3). The other two elements are the insertion site sequence (ISS), found upstream or downstream of the *mec* complex, and the direct repeats which flank the ISS. The ISS is the target of the *ccr* recombinases, allowing the integration of the SCC*mec* at a specific site in the staphylococcal chromosome. The different insertion sequences that associate with the *mecA* gene are what is used to distinguish the classes of the *mec* complex. (IWG-SCC, 2009). A fifth element, not essential to the functioning of the cassette are the J-regions. They are regions of DNA that "join" the *mec* and *ccr* regions (between and on either side). These regions are acknowledged as they may carry resistance genes to other antibiotics (Valsesia *et al.*, 2010).

Table 2.3: Showing *mec* complex classes and *ccr* complex types that define the 8 major allotypes of the SCC*mec*

SCCmec Type	*mec complex Class*	*ccr complex Type (included subtypes)*
I	B	1 (A1B1)
II	A	2 (A2B2)
III	A	3 (A3B3)
IV	B	2 (A2B2)
V	C2	5 (C)
VI	B	4 (A4B4)
VII	C1	5 (C)
VIII	A	4 (A4B4)

2.4.2. **Ceftaroline**

Ceftaroline is a 5th-generation cephalosporin antibiotic used to treat skin and soft tissue infections and has proven effective against a broad spectrum of Gram-positive bacteria including MRSA and multidrug resistant MRSA, including vancomycin resistant strains (Kollef, 2009) (Duplessis & Crum-Cianflone, 2011). It is administered as a prodrug in acetate form (ceftaroline fosamil) which breaks down into the active ceftaroline drug and the inactive ceftaroline-M1 metabolite (Yukihiro & Junko, 2008). It is made up of a β-lactam ring and a cepham ring like most cephalosporins but its most usful structure is a thiazole ring which has high binding affinities with PBP2a expressed by MRSA, rendering the bacteria susceptable to

the β-lactam and cepham ring structures of a second ceftaroline molecule which block the active site of PBP2a from carrying out its function as a crosslinking enzyme in the peptidoglycan layer of the cell wall (Ishikawa & Nobuyuki, 2003; Harrison *et al.*, 2015). In a recent study by Long *et al.*, (2014), ceftaroline-resistant strains of MRSA were isolated from the blood of a cystic fibrosis patient in addition to the 5 other strains of MRSA isolated from the patient's airway. This resistance was not community acquired as it was a direct result of antibiotic exposure. It was found that the resistance to ceftaroline was confered by 2 non-synonymous SNP mutations in the *mecA* gene (which codes for PBP2a), causing amino acid changes Y446N and E447K that prevented ceftaroline from binding to and inactivating the PBP2a protein (Long *et al.*, 2014). These mutations have also been related to resistance of MRSA against cetobiprole, another cephalosporins closly related to ceftaroline (Banerjee *et al.*, 2008).

2.4.3. **Ciprofloxacin**

Ciprofloxacin is a 2nd-generation fluoroquinolone that is used to treat internal infections (gastrointestinal, respiratory, urinary-tract, joint) as well as some skin infections, and can be used against a broad spectrum of bacteria, including both Gram-negative and Gram-positive bacteria (Committee on Antimicrobial Agents, 1994). It works by inhibiting the functions of DNA gyrase and topoisomerase IV, both are type 2 topoisomerases, which control the seperation of DNA strands for replication, thus preventing effective cell replication (Drlica & Zhao, 1997) (Piercy *et al.*, 1989). It does this by ciprofloxacin binding to subunits GyrA and GyrB of DNA gyrase or to the respectively homologous subunits of topoisomerase IV, ParC and ParE. As these enzymes form a complex with DNA during replication in order to unwind it trough use of double stranded breaks, when the fluoroquinolone binds to it, the double stranded break cannot successfully be resealed (Hooper, 2001).

Resistance to ciprofloxacin treatment by clinical *S. aureus* samples was first published in 1986 and by 1994 it had been established that resistance was due to SNPs in *grlA* and *glrB* in topoisomerase IV, which code for subunits ParC and ParE, respectively (ciprofloxacin's primary target in *S. aureus*) and in SNPs in *gyrA* and *gyrB* in DNA gyrase, which code for subunits GyrA and GyrB respectively (ciprofloxacin's secondary target in *S. aureus*) (Campion *et al.*, 2004) (Ferrero *et al.*, 1994) (Kelley *et al.*, 1986) (Raviglione, et al., 1990). There are many SNPs associated with resistance, some causing amino acid changes in the enzyme

subunits, some not (Schmitz *et al*., 1998). Either way, these SNP's allow for the prevention of ciprofloxacin binding to the topoisomerases. It would interesting to see how these synonymous mutations confer resistance and should be investigated further, with particular focus on the binding mechanism between the fluoroquinolones and the drug targets as it is still not clearly understood.

Another mode of resistance to ciprofloxacin employed by *S. aureus*, is the utilisation of the efflux enzyme NorA (coded for by the gene *norA*), which can transport fluoroquinolones out of the cell (Campion *et al.*, 2004). NorA is multidrug efflux pump and is coded for in the host's chromosome but is only effective against ciprofloxacin when overexpressed. This can be achieved through mutations in the promotor region of *norA* which prevent repressor binding (Fàbrega *et al.*, 2009). Multiple fluoroquinolone resistance mechanisms can be present in one organism and can be found in both methicilin resistant (MRSA) and methicilin susceptable *S. aureus* (MSSA) (Kwaatz & Seo, 1997). These mutations are not trasmitted horizontally but rather are the direct result of expsosure to the drug.

2.4.4. **Clindamycin**

Clindamycin is a lincosamide that can be used to treat some aerobic Gram-positive and some anaerobic Gram-negative bacteria (Smieja, 1998). Like most macrolides and streptogramin B class antibiotics, they bind to the 23S-rRNA receptor site of the 50S subunit of the ribosome, preventing protein synthesis, imparing the bacterium's functionality (Bottega *et al*., 2014) (Leclercq, 2002). The most common form of resistance is most often associated with the presence of the genes *ermA* or *ermC* (Leclercq, 2002)(Yoon *et al.*, 2008). It has also been noted that *ermB* can also be found in *S. aureus* but at a much lower frequency (Weisblum, 1995a). One study by Nicola *et al.*, (1998) noted that one sample carrying the *ermB* gene showed strong constitutive resistance to erythromycin and clindamycin, however no other reports have been made except in other species including *Streptococcus pneumoniae* and *Clostridium difficile* (Waites *et al*., 2000; Pituch *et al*., 2003).

These *erm* genes code for a specific methylation pattern, whereby an adenine (A2058) in a conserved region of domain V of the 23S rRNA receptor site is methylated, preventing binding of macrolides, lincosamides and streptogramin B antibiotics, causing the MLS_B phenotype (Macrolide Lincosamide Streptogramin B resistance (Weisblum, 1995a; Levin *et al.*, 2005).

This form of resistance prevents binding of the antibiotic. This is can result in constitutive resistance to clindamycin ($cMLS_B$) or inducible resistance ($iMLS_B$) which is induced when erythromycin resistant *S. aureus* (also coded for by *erm* genes) is in the presence of erythromycin (Siberry *et al.*, 2003; Levin *et al.*, 2005; Baragundi *et al.*, 2013; Bottega *et al.*, 2014; Banik *et al.*, 2015). Induced resistance will still be founded on the presence of an *erm* gene, but the expressed mRNA's are inactive and cannot translate into protein, requiring a macrolide antibiotic to be present in order to induce the activation of said mRNA's (Weisblum, 1995b; Leclercq, 2002).

This form of induced resistance is difficult to measure using broth dilutions or MIC's as a two dimensional analysis would have to be carried out (increasing concentrations of clindamycin against increasing concentrations of erythromycin). A substantially easier way to test inducible resistance is to utilise the disc diffusion method by placing erythromycin and clindamycin discs adjacently. If the bacteria have the $iMLS_B$ phenotype then there will be no zone of clearing around erythromycin (erythromycin resistant) and a 'D'-shaped zone of clearing around clindamycin (figure 2.9), thus indicating that the presence of erythromycin induces resistance to clindamycin. (Yoon *et al.*, 2008) (Sasirekha *et al.*, 2014). It has also been found that there are higher instances of clindamycin resistance (both constitutive and inducible) in MRSA isolates (68%-92% of MRSA isolates) than in MSSA (19%-30% of MSSA isolates) (Schreckenberger *et al.*, 2004) (Yilmaz *et al.*, 2007).

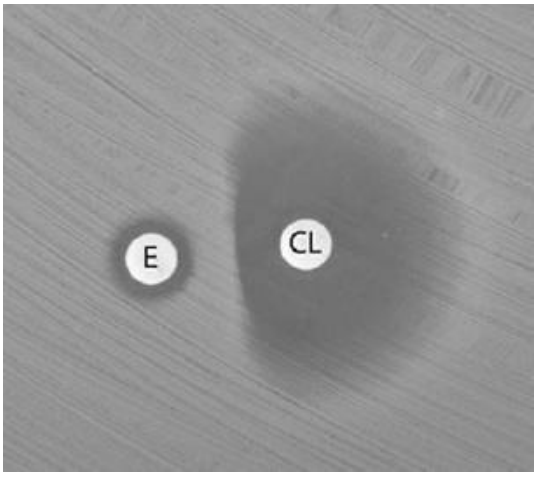

Figure 2.9: Positive D-test result indicating an $iMLS_B$ phenotype (E – Erythromycin; CL – Clindamycin) (Levin *et al.*, 2005)

2.4.5. **Erythromycin**

Erythromycin is a macroide antibiotic used mostly for surface and soft tissue infections as well as other infections by a range of both Gram-negative and Gram-positive bacteria (Weisblum, 1995a) it functions very similarly to clindamycin in that it targets the 23S rRNA receptor site on the 50S subunit of the ribosome in order to prevent protein synthesis (Leclercq, 2002). As previously mentioned, the most common forms of resistance to erythromycin comes from the host harbouring *erm* genes. *S. aureus* most commonly carries *ermA* and *ermC* genes which code for the enzymes that will methylate the sites on ribosomes where the antibitics bind in order to deactivate protein synthesis in the cell. Another previously mentioned gene, *ermB*, also confers erythromycin resistance but is not found as commonly as *ermA* and *ermC* (Nicola *et al.*, 1998; Leclercq, 2002). There are other mechanisms of erythromycin resistance, a common one being the efflux of the antibiotics out of the cell before they are able to deactivate protein synthesis in the cell. This can be controlled by the *msrA* which codes for an efflux pump that resembles that of an ABC transporter, requiring 2 ATP-binding domains (Ross *et al*., 1990) (Leclercq, 2002). However, this mechanism only allows resistance to macrolides and streptogramin B antibiotics and not lincosamides like clindamycin (Baragundi *et al*., 2013).

2.4.6. **Gentamicin**

Gentamicin is effective as a broad spectrum antibiotic, and is often used as a stopgap while cultures determine the infecting pathogen (Mingeot-Leclercq *et al.*, 1999). It belongs to the antibiotic class of aminoglycosides along with streptomycin, kanamycin A, amikacin, tobramycin and some others which are made up of a collection of various aminated sugars linked together, with glycosidic linkages to a cyclitol (Dowding, 1977; Mingeot-Leclercq *et al.*, 1999; Xie *et al*., 2011a). These antibiotics are mostly used against Gram-negative bacteria but have broad spectrum capabilities. They work by binding to the 30S subunit of the ribosome, primarily, preventing the elongation of polypeptides as well as disabling the proofreading mechanism, thus interupting and interfering with protein synthesis (Melancon *et al.*, 1992; Mingeot-Leclercq *et al.*, 1999). *S. aureus*'s resistance to this gentamicin usually extends to most aminoglycosides as the mechanisms entail the use of modifying enzymes that occur naturally in the cell, such as *N*-acetyltransferases (AAC) which disrupt amino functions and *O*-nucleotidyltransferases (ANT) and *O*-phosphotransferases (AHP) which disrupt hydroxyl

functions (Figure 2.10) (Shaw *et al.*, 1993). These all result in the inability of the antibiotic to bind to the ribosome.

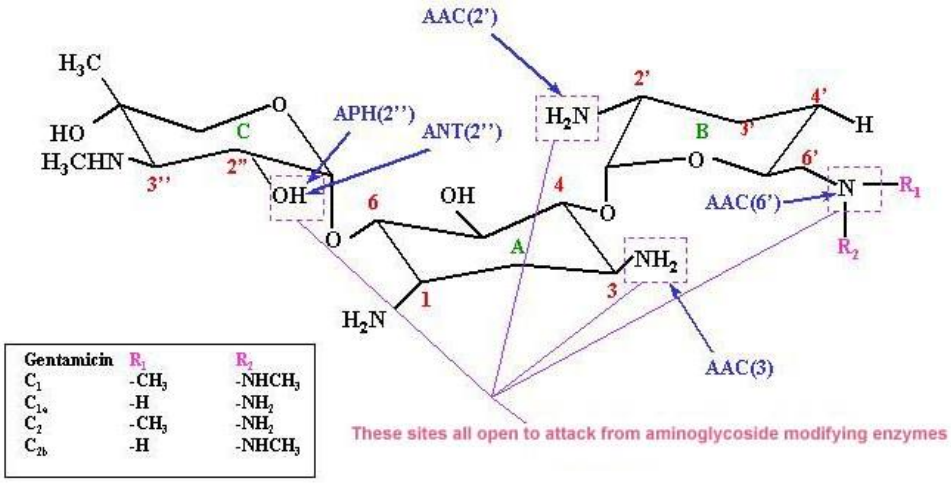

Figure 2.10: Sites of enzyme modification on gentimicin (adapted from Mingeot-Leclercq et al., 1999)

The enzymes are named according to the site to which they target (Figure 2.6). In general, the genes coding for these enzymes are found on transposable elements such as plasmids, gene cassettes, integrons, genomic islands etc. and are transmissable within and between species. One in particular to *S. aureus* called *aac(6')-aph(2'')* codes for bifunctional enzyme that targets 2 sites with 2 different enzyme activities (Ramirez & Tolmasky, 2010). This gene is commonly found on transposons much like Tn*4001* often found on plamids found in *S. aureus* (Mahairas *et al.*, 1989). However, a study by Storrs *et al.*, 1988 found that that gene could be found chromosomally. In this study, it was also determined that *S. aureus* isolates with high resistance to gentamicin only carried one copy of the gene, suggesting gene dosage is not a factor in how resistant an isolate is, therefore, considering the easy mode of transmission between bacteria and the strength of resistance from this common gene suggest a large risk associated with the use of gentamicin on *S. aurues* infections. While much information is available for these genes found in other species (particularly Gram-negative species) there is not much sequence information regarding those aminoglycoside-modifying enzymes found to be active in *S. aureus* specifically.

2.4.7. **Linezolid**

Linezolid is the first oxazolidinone to be used as an antibiotic (Stevens *et al.*, 2004). It is used to treat Gram-positive infections particularly skin and soft tissue infections by *S. aureus*, in particular, it is effective against vancomycin resistant strains of *S. aureus* (VRSA) (Endimiani *et al*., 2011). It has bacteristatic capabilities in that (like many other antibiotic classes) it inhibits protein synthesis and in doing so, prevents the cells from multiplying. However, this class of antibiotics is unique in that instead of preventing the elongation steps in protein synthesis, it prevents the initiation step of protein translation due to the molecule binding to the 23S rRNA subunit of the 50S unit of the ribosome, specifically, the catalytic site of the peptidyl-transferase centre (see below) (Leach *et al.*, 2011). Crosslinking experiments have shown that the antibiotic will bind in the peptidyl-transferase centre where the amino acid of the tRNA in the A position is situated (marked in blue in figure 2.11) (Leach *et al.*, 2007).

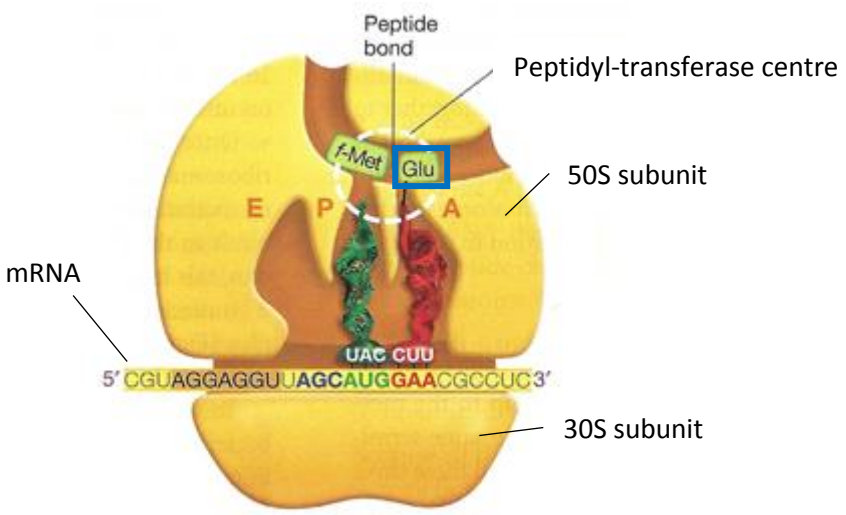

Figure 2.7: The first elongation step in protein synthesis. Oxazolidinones would bind at the peptidyl-transferase centre (white dotted circle) in the A position (blue square), preventing tRNAs from releasing their amino acids and preventing the formation of peptide bonds between amino acids (http://www.discoveryandinnovation.com/BIOL202/notes/lecture13.html edited and retrieved on 11 July 2016).

Resistance to this antibiotic has been seen very rarely with more than 99.9% of *S. aureus* strains (MSSA and MRSA) are still susceptable to linezolid in the US alone and 99.8% are still susceptable globally (Jones *et al.*, 2007a), (Jones *et al.*, 2007b), (Mendes *et al.*, 2008). However, in many cystic fibrosis patients, there have been an increase of instances of *S. aureus* acquiring resistance to linezolid after prolonged use in the patient (Endimiani *et al.*, 2011). The

most recognised mechanism of linezolid resistance by *S. aureus* is a point mutation in domain V of the 23S rRNA genes.

There are three distinct SNPs vis. G2576T, G2447T and T2500A. These mutations cause the 23S rRNA to no longer be compatible with linezolid binding in the peptidyl-transferase centre. This domain V falls within the *rrn* rRNA operon of which *S. aureus* has five or six copies throughout the genome and it has been found that the more copies of these SNPs, the greater the resistance to linezolid (Besier *et al.*, 2008). More SNPs in domain V have been documented more recently (Endimiani *et al*., 2011) suggesting that resistance to linezolid has been as a result of de novo mutations as a result of exposure and not horizontal transmittance. However, in 2010, it was documented that a plasmid mediated gene called *cfr* allowed *S. aureus* resistance to linezolid that is transmissable horizontally (Morales *et al*., 2010). This gene codes for a methyltransferase that methylates an adenine (A2503) situated in the 23S rRNA gene, which confers resistance to linezolid as well as other antimicrobials such ans chloramphenicols and clindamicin (Mendes *et al*., 2008).

Now that linezolid resistance is transmissable, there is much pressure to find new effective therapies for cystic fibrosis pateints, and other patients with vancomycin resistant strains of *S. aureus*, as instances of resistance are likely to be more common. It is important that new methods of counter attack on linezolid resistant (particularly *cfr* carrying) *S. aureus* that prevent resistance (e.g. chemical binding to *cfr* promotor to prevent its expression) be established soon as it is unwise to seek out other antibiotics that will eventually become redundant due to resistance as a result of overexposure.

2.4.8. **Tetracycline**

Tetracycline (the antibiotic) is grouped with doxycycline and minocycline in the class tetracyclines. These are broad spectrum antibiotics which reversably bind to the 30S subunit of the ribosome (unlike some previously mentioned protein synthesis inhibitors which bind on the 50S subunit), sterically hindering incoming aminoacyl-tRNA's at the A site, preventing the continuation of protein synthesis (figure 2.12) (Griffin *et al.*, 2010).

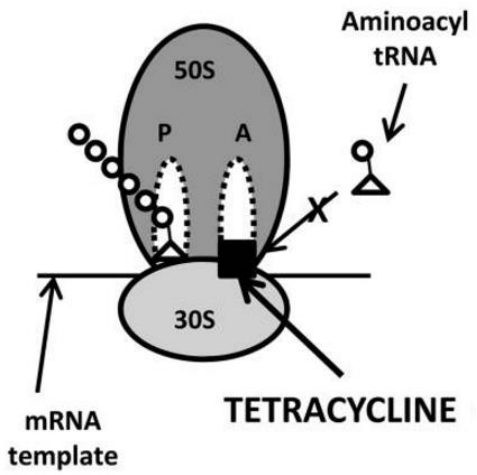

Figure 2.12: Mechanism of action for tetracycline, preventing aminoacyl-tRNA's from reaching the A site on the ribosome (image credit to Griffin *et al.*, 2010)

At the turn of the centuary tetracyclines were the second most prescribed broad spectrum antbiotic after penicillins and derivatives (Trzcinski *et al.*, 2000), and as expected since then many cases of resistant *S. aureus* have arisen since then. Current knowledge suggests that there are 3 mechanisms of resistance to tetracyclines: expulsion of the antibiotic by use of efflux proteins, production of proteins that block tetracyclines from binding to the ribosome and the production of enzymes that metabolically inactivate tetracyclines (Ullah *et al*., 2012). The third has not yet been detected in *Staphylococcal* species (McCallum *et al.*, 2010).

Two common genes that code for efflux proteins, *tetK* and *tetL* are generally located on and acquired from plasmids, most commonly on the pT181 plasmid shown below (figure 2.13) (Noirot *et al*., 1990). These proteins will situate themselves within the cell membrane and actively (in an energy dependant manner) expel tetracycline molecules out of the cell so to prevent a toxic build-up within. *tetK* is more commonly seen in *S. aureus* than *tetL*, with *tetL* only being found in isolates already positive for *tetM* (Bismuth *et al.*, 1990).

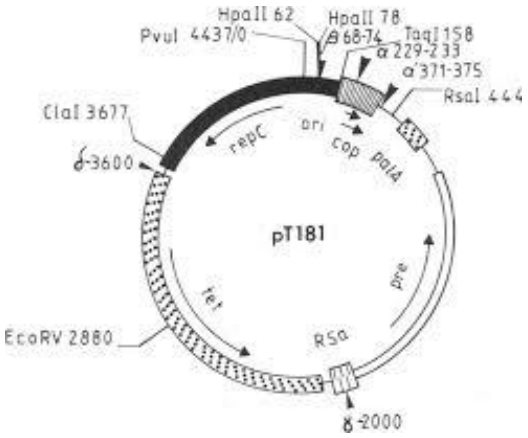

Figure 2.13: pT181 plasmid carrying tetracycline resistance gene *tetK* or *tetL* often acquired by *S. aureus*. Arrows within the diagram represent transcription direction. (Noirot *et al*., 1990).

The most common gene coding for protein mediated ribosomal protection found in *S. aureus* is the *tetM* (*tetO*, another related gene is not commonly found in *S. aureus*). This gene is most commonly carried on transposons like Tn5801 and Tn916 (figure 2.14). These transposons also carry genes which allow for and regulate conjugation, meaning that the horizontal transfer of these elements is not limited by the capabilities of the hosting cell. Therefore, these transposons can indiscriminately move between different species of bacteria, making them powerful transport tools of antibiotic resistance (de Vries *et al.*, 2009). Many MRSA isolates have been found with these genetic elements coding for various forms of tetracycline resistance, often carrying more that one *tet* gene.

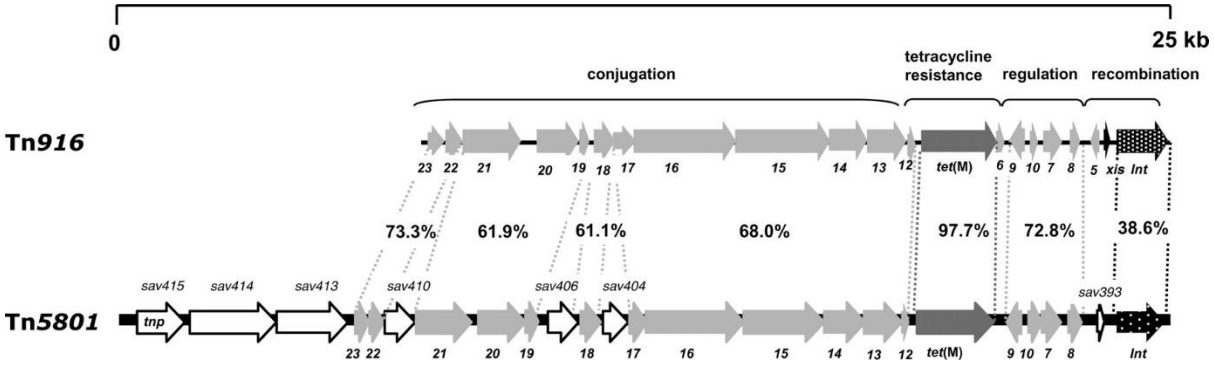

Figure 2.14: *tetM* carrying transposons Tn916 and Tn5801 with genes enconding conjugation. (de Vries *et al.*, 2009).

A recent study in Malaysia showed that MRSA isolates would often be found carrying such mobile genetic elements allowing for multiple drug resistances (Lim *et al.*, 2012). Many *tetM* positive *S. aureus* isolates will be found carrying the *tetK* gene (the *tetKM* genotype) which as

additive effect to the tetracycline resistance of *S. aureus* (Trzcinski *et al.*, 2000). What is not greatly understood is what affinities these elements have to each other and why it is common to find them in tandem. Understanding this may shed light on ways of preventing the spread and birth of more multidrug resistant strains of bacteria.

2.4.9. **Vancomycin and Teicoplanin**

These two glycopeptides have almost identical mechanisms of action and resistance but have been treated as separate antibiotics. Both have been used as effective antibiotics against MRSA, with vancomycin being the gold standard across the board for many years (Liu *et al*., 2011). Both have a glycopeptide core and work by inhibiting the production of crosslinkages in the cell wall, causing the osmotic pressure of the inside of the cell to be too high for what the cell wall with weakened integrity can hold, resulting in the lysis of the cell (Hiramatsu, 2001) (Somma *et al.*, 1984). The glycopeptides do this by hydrogen bonding to the D-alanyl-D-alanine peptide group on the end of N-acetylmuramic acid (NAM) moeities, preventing crosslinking enzymes from securing the pentaglycine chain on the NAM moeity to the D-alanine on other NAM molecules (figure 2.15). Because these glycopeptides target such a vital molecule (peptidoglycan) in the cell and not the enzymes that build them, there has (until recently) been very little wiggle room for the bacteria to change the substrate to which the glycopeptides bind, meaning that binding affinities between the antibiotic and the substrate do not dictate the antimicrobial activity of the antibiotic in susceptable bacteria (Courvalin, 2006).

In understanding that vancomycin and teicoplanin have the same mechanisms of action, it stands to reason that resistance mechanisms would affect them both. Resistance to vancomycin and teicoplanin is mediated by the same group of genes. These *van* genes are suspected to have originated from *Enterococcus sp.* and moved to *S. aureus* strains (Moellering, 2006). Initially only *vanA* clusters had been identified in *S. aureus* (Courvalin, 2006), but a more recent study in Iran showed that *vanB* clusters were identified in 37% of screened isolates (Saadat *et al.*, 2014). There are other *van* gene clusters but are only found in enterococci and other Gram-positive or intrinsically resistant species. *vanA* is found on Tn1546-like transposons, originally non-cojugative elements, but also on some plasmids and occasionally, chromosomally (Périchon & Courvalin, 2009). *vanB* gene clusters are found on transposable elements that can conjugatively move between chromosomes, as well as on Tn916-like transposons. Both gene

clusters produce enzymes that work toward replacing the terminal D-alanine of NAM with a D-lactate, derived from reduced pyruvate, thus preventing glycopeptides with hydrogen bonding and interupting cell wall synthesis (Figure 2.15). *vanA* permits high resistances to vancomycin and teicoplanin while *vanB* has a less clearly described intensity of resistance to either antibiotic when in expressed in *S. aureus*. However, in enterococci it has been noted that *vanB* resistance is inducible only in the presence of vancomycin and so has been noted to confer "variable" resistance (Courvalin, 2006). More research should be conducted to clearly define the resitance profiles *vanB* allows for in *S. aureus*. Good news came in 2011 when a redesigned form of vancomycin was developed that could bind to D-alanyl-D-lactate and D-alanyl-D-alanine subtrates, allowing it to be effective against isolates previously identified as glycopeptide resistant as well as those that are currently identified as susceptible (Xie *et al.*, 2011b).

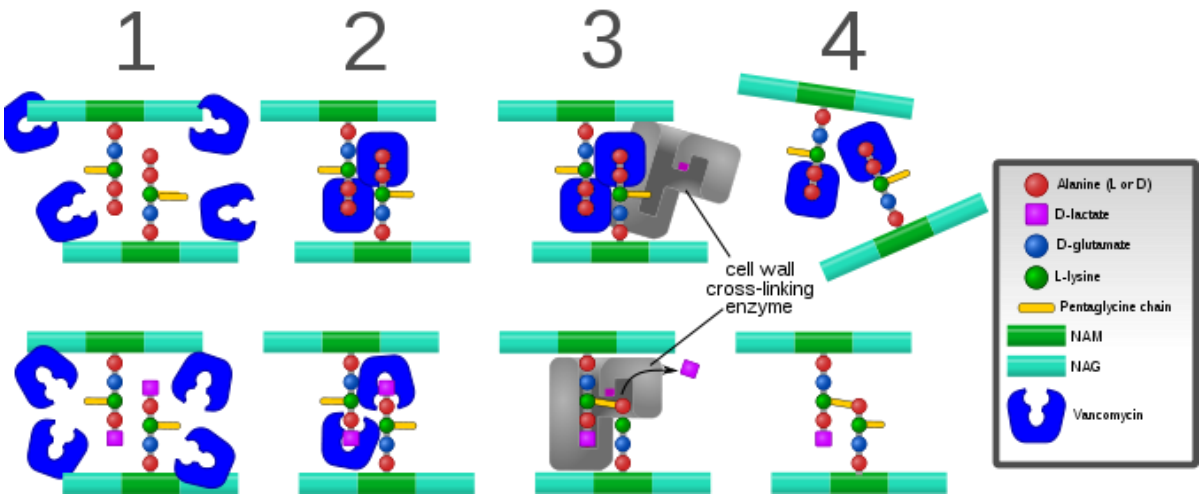

Figure 2.15: Sequence of events when cell wall synthesis is interrupted by glycopeptide antibiotics (vancomycin is shown but mechanism applies to teicoplanin as well). Top line illustrates events when no resistance mechanisms present and the bottom line illustrates events when resistance mechanisms are present

There are other types of resistance not mediated by *van* gene clusters has been described, usually in *S. aureus* isolates with profiles of intermediate resistance and an unknown mechanism of resistance (strains like Mu50). One strain has even been isolated that produces colonies with varying levels of resistance when subcultured, known as heterogenous resistance (strain Mu3) (Sujatha & Praharaj, 2012). More and more MRSA isolates have been acquiring glycopeptide resistance, predominantly through the acquisition of the *vanA* gene cluster most probably from vancomycin resistant enterococci (VRE) co-infecting a wound, but some

isolates are showing intermediate resistance without *vanA*. Most literature has found that in VR-MRSA, disc diffusion does not accurately detect the resistance profile and that minimum inhibitory concentration tests should be utilised instead (Loomba *et al.*, 2010; Thati *et al.*, 2011). More research needs to be conducted on the relationships between haboured genes and resistance profiles, both for vancomycin and teicoplanin and the mechanims by which MRSA and MSSA with intermediate and heterogenous resistance act. Also, investigating ways of preventing conjugation between VRE and *S. aureus* may prove useful in finding ways reducing the rate of *van* gene acquisition.

2.5. Isolation Media for *S. aureus*

It is not an unknown fact that genetic characterisation is the most reliable method for accurately determining the species of an organism (Brakstad *et al*., 1992; DeAngelis *et al*., 2011; Shalaby *et al*., 2011). It even has the power to differentiate individuals within a species that would phenotypically have been characterised as the same (sub-species within a species). In bacteriology, there is much need for genetic species confirmation in both research and clinical or diagnostic settings due to the ubiquitous nature of bacteria, our inability to distinguish them by visual characteristics, regardless of the magnification used to view them and the fact that genetic methods are far more rapid which is especially important for clinical diagnostic purposes. Unfortunately, most DNA related methods, such as conventional PCR, PFGE, sequencing and RAPD techniques, require expensive equipment as well as a high reagent expense per sample (Spiegelman *et al*., 2005). As a result, many biotechnology companies have attempted to develop growth media which select against particular groups of bacteria (based on biochemical charateristics) in order to allow unhindered growth of another group of bacteria (based on other biochemical characteristics), so to reduce the number of samples required to undergo genetic testing by presumptively eliminating samples that do not have the microbe of interest.

This is not just important in order to reduce costs needed in clinical diagnostics or research, but also to ensure accurate detection of the microbe of interest. In the environment, there are a large range of microbes which exist together, including bacteria, fungi, viruses, archaea and other single cell eukaryotes. Like most natural habitats, there is much competition between species for space and food meaning that, unlike in a laboratory setting, there is a lot of pressure against

the survival of most individual species (Hibbing *et al*., 2010; Foster & Bell, 2012). Selective media offer to eliminate some, if not all, competition against the microbe of interest in order to allow it to grow in such a manner appropriate for its detection and downstream processing (Ehrmann *et al*., 2013).

It can often happen that in an environmental sample, even when subjected to selective media, the microbe of interest may still have its growth masked or inhibited by other microbes present that survive in similar selective conditions (Gostinčar *et al*., 2009). This makes it important to choose the appropriate medium for the purposes of ones study. A number of studies have investigated the benefits of using more specifically selective media (examples discussed below) when working with environmental samples specifically, finding that media tailored to inhibit wider ranges of microbes produce more sensitive and accurate results but DNA methods are required to confirm presumptive results.

Discussed below are some examples of different media used for the isolation of *S. aureus*, their composition, how they eliminate competition and the characteristics they confer onto positive *S. aureus* samples.

2.5.1. **Enrichment Media**

2.5.1.1. **Buffered Peptone Water**

Peptone water is a very simple enrichment medium made of 2 parts peptic digest of animal tissues (provides essential nutrients) and 1 part sodium chloride (maintains osmotic balance) (HiMedia, 2011). Buffered peptone water has added anhydrous disodium phosphate and monopotassium phosphate to allow it buffering capabilites (HiMedia, 2017). Both allow for the enrichment of sampled microbes for appropriate analysis in selective media but buffered peptone water offers recovery sub-lethally damaged cells which may be sensitive to fluctuations in pH. This makes it possible also to recover live cells from older laboratory samples (as many as 10 weeks old) that were left in normal sterile storage conditions (4°C refrigerators), that would appear unviable if directly subcultured onto selective media. As environmental samples can carry whole niches of micro-organisms, it is not unusual to have some difficulty detecting your microbe of interest due to all the competitive stressors it has had to endure (Hibbing *et al*., 2010; Edel & Kampelmacher, 1973). This makes normal and buffered peptone water, despite their simplicity, important for pre-enrichment, prior to selection. With

regard to this study, this is a very important step as *S. aureus* is not often found as the majority of a microbial population within the airways of dogs, which if not properly pre-enriched, may be overcome by the competition of other microbes, making it too difficult for it to grow on selective media, resulting in it remaining undetected and the sample exhibiting a false-negative result. The peptone waters, in their simplicity, can have agents added to them which can be selective or differential such as phenol red (pH indicator) for detecting carbohydrate fermentation or EC O157: H7 Selective Supplement for the selection of *Escherichia coli* O157. This is most appropriate for tests on already isolated microbes that are in need of biochemical testing.

2.5.1.2. **Tryptone Soy Broth (TSB)**

TSB contains pancreatic digest of casein and papaic digest of soyabean meal to provide amino acids and other longer chained peptides required for microbial growth. The carbohydrate source is dextrose sugar and dibasic potassium phosphate acts as the buffer for the medium. NaCl maintains the osmotic balance (HiMedia, 2011). This is used as a non-specific general growth medium for most aerobic bacteria and fungi and as such can be used for sterility testing of other media. As such, this medium is not recommended for use with environmental samples when isolation of a specific species is the intended goal. However, once your species of interest is successfully isolated, it may be re-cultured successfully for downstream microbial techniques such as antimicrobial susceptibility testing.

2.5.1.3. **Columbia Blood Agar (CBA)**

CBA is a particularly versatile base medium that can be used for both selective and differential purposes. It is composed mainly of special peptone – a nutrious substance promoting rapid growth of most types of bacteria. It also uses corn starch is primarily a carbohydrate source which doubles as a natural neutraliser of toxic metabolites (HiMedia, 2011a). As it is a base medium, a range of additives can be used to differentiate different species of bacteria and some to inhibit the growth of others. For the isolation of *S. aureus*, one selective suppliment that can be added to the medium is a combination of two antibiotics that have primary action against Gram-negative bacteria, usually nalidixic acid and colistin sulphate (HiMedia, 2012). This

gives the Gram-positive bacteria such as those in the *Staphyloccocus* species less competition when growing on the medium, allowing *S. aureus* species to be detected more easily. The addition of sheeps' blood can allow the researcher differentiation between species which are capable of α-, β- and γ-haemolysis and those incapable (Ellner *et al*., 1966). *S. aureus* has α- and β-haemolytic capabilities and such can be distiguished from other species, if it is already known to be in the sample. However, as it has been discussed above, *S. aureus*'s β-haemolysin gene *hlb* can be interupted and rendered inexpressive by the insertion of phage DNA within the gene. Such samples would appear as only α-haemolytic and since the medium does not offer other differentiating factors, the sample would be overlooked as a negative result.

2.5.2. **High Salt Media**

2.5.2.1. **Mannitol Salt Agar (MSA)**

MSA is currently the go-to medium for growth of *S. aureus* and is the recommended medium by the Korean Food and Drug Administration (KFDA) (Kim & Oh, 2010) and is widely understood to especially suit the growth conditions required by *Staphylococcus* sp. and other cocci. This medium is both selective and differential. For selection, MSA contains high salt concentrations which usually only Staphylococci can survive in. . For differentiation, MSA also offers D-mannitol as a carbohydrate source. D-mannitol if fermented creates excess acid. Phenol red in the medium differentiates between fermenters and non-fermenters by changing to yellow in the presence of an acidic environment (HiMedia, 2015). However, there are a few species of bacteria such as *S. xylosus, S. cohnii, S. sciuri, S. simulans* and *Listeria* spp, among many others, that are able to ferment D-mannitol and produce a positive phenol reaction, if they can survive the high salt concentrations. As a result, it is recommended that a secondary coagulase test ought to be carried out in order to confirm that presumptive colonies are indeed *S. aureus* (Gramoli & Wilkinson, 1978). Many fungi are also able to survive in moderately saline environments such as *Cladosporium sphaerospermum*, its spores very commonly found in indoor and outdoor air (Gostinčar *et al*., 2009), and so, likely to be found in the airways of most animals. This means that MSA can will only be accurate in presumtively identifying *S. aureus* depending on where or what is sampled.

Kim and Oh, (2010) demonstrated that MSA was 96.5% sensitive (*S. aureus*-positive samples were correctly identified) and 66.6% specific (*S. aureus*-negative samples were correctly

identified) when isolating from innoculated commercial food. However, Niskanen and Aalto (1978), originally demonstrated that MSA was 88% sensitive and 75.4% specific at detecting *S. aureus* from innoculated minced meat. Both of these studies used set innoculants which do not accurately represent the biological balances in an environmental sample. This is not to say that MSA has not been used for direct direct selection from environmental samples. In 2006, a study by Sexton *et al*. involved sampling surfaces and air in MRSA isolation wards in a hospital directly with MSA. While many studies have discussed the imperfect results produced by MSA when many different species are involved in a sample, that factor may have been reduced be the fact that surfaces in these isolation rooms had vigourous sterilisation routines that should eliminate much of the unwanted competition that would be seen under normal circumstances. Thus, careful consideration should be given when deciding on a medium for use in a study, if environmental samples are to be used. More studies measuring the accuracy (sensitivity and specifity) of MSA (and other media) for different types of environmental samples should be conducted to standardise the appropriateness of media used, depending on the context of the study.

2.5.2.2. **Staphylococcus Agar No. 110 (SA No.110)**

Also known as Stone Gelatine Agar, SA No.110 is a differential medium for staphylococcal species that also uses D-mannitol reduction as a manner of intial differentiation. Like MSA, it selects against other species by use of high salt conentration. It uses casein enzymic hydrolysate and yeast extract as sources of carbon, nitrogen and other essential nutrients and growth factors but it also includes D-mannitol and lactose as corbohydrate sources as well. Unlike MSA, SA No.110 does not use phenol red as an indicator for mannitol reduction. It also includes gelatin which *S. aureus* is able to digest. These two factors and the colony pigmentation are the differential factors in this medium. Therefore, if an isolate survives on the medium, reduces the mannitol, digests the gelatin, and has yellow to orange coloured colonies, it may be presumptively be considered, *S. aureus* (HiMedia, 2015). However, the limitations of this medium are the same as MSA: while SA No.110 has greater differential power (more differentiating factors are included), it has the same selective power as MSA – any halotolerant species can survive on this medium. This potentially allows for the masking of true *S. aureus* isolates by other microbes, depending on the competitive dynamics in the sample.

2.5.3. **Potassium Tellurite Media**

2.5.3.1. **Vogel-Johnson Agar (VJA)**

VJA is a slightly involved medium that uses the addition of a number of selective agents in the agar, including potassium tellurite as its main selective agent as opposed to the high salt concentrations used in MSA and SA No.110. VJA is both a selective and differential medium. Apart from the basic nutritional components, it includes glycine, lithium chloride and potassium tellurite (1%) as selective agents. Glycine, in high enough concentrations, inhibits the growth of most bacteria due to interference with cell wall synthesis enzymes (Cowles, 1947; Minami *et al*., 2004), but due to their reinforced cell walls, Gram-positive bacteria can survive the higher concentrations. Lithium chloride also acts as an inhibitor towards non-Gram-positive microflora as coagulase-positive *Staphylococcus* sp. (CoPS) have specially adapted cell walls that prevent the intake of lithium chloride. Potassium tellurite when reduced enzymatically produces a superoxidative free-radical that has toxic effects on most microflora, however, CoPS can survive the superoxidatitive effects and continue to reduction pathway to finally form metallic tellurium (Pérez *et al*., 2007). Due to all these inhibiting agents, D-mannitol, is added which acts to promote *S. aureus* growth. Differentiating factors are phenol red and potassium tellurite, therefore mannitol fermenters turn the agar yellow, and potassium tellurite reducing colonies will be black due to tellurium deposits (HiMedia, 2016). The high selectivity of this media allows it to be used for the detection of CoPS in highly contaminated food and clinical samples. It is also approved by the American FDA (Kim & Oh, 2010). This medium scored 88% sensitive and 31% specific in Niskanen and Aalto's innoculated minced meat in 1978, but 100% specificitiy and sensitivity in Hyun-Jung and Se-Wook's innoculated rice and fish in 2010. This little more than prove that this medium's ability to accurately detect *S. aureus* depends more on the type of sample used than the medium itself, and that neither of these results indicate its reliability for environmental samples.

2.5.3.2. **Baird-Parker Agar (BPA)**

BPA is very similar to VJA except that it does not use D-mannitol and it also uses a higher concentration of potassium tellurite (3.5%) (HiMedia, 2015). It also uses stabilised egg-yolk to detect lipase activity exibited by some CoPS species such as *S. aureus* and *S. intermedius* (Hájek, 1976). D-mannitol is not as selective as the potassium tellurite and as such, is not

necessary. The increased concentration of potassium tellurite selects against most other microbes completely except CoPS. With the increased stringency in selection there are fewer and fewer instances where *S. aureus* might be overlooked or masked by contaminating species and as a result it has become the most recommended medium for isolation of *S. aureus* from food, clinical and industrial samples by the KFDA, American FDA, Association of Official Agricultural Chemists (AOAC) and Association Francaise de Normalisation Standards Organization of France (AFNOR) (Kim & Oh, 2010). In both the Kim and Oh (2010) and the Niskanen and Aalto (1978) studies, it was the most accurate medium that could sensitively detect *S. aureus* at all concentrations.

2.5.3.3. **HiCrome Aureus Agar Base (AAB)**

AAB is very similar to BPA except that it is chromogenic and the nutrient sources are slightly different (does not affect nutrient composition). AAB also lacks glycine. With high potassium tellurite concentrations like BPA (3.5%), glycine is likely an unnecessary selective agent (HiMedia, 2015). Same colony morphologies are expected on AAB as are on BPA except that *Listeria monocytogenes* will appear a deep blue, differentiating it from the CoPS. This medium is recommended for the use for environmental samples by the product's company but there is little literature that demonstrates this as such.

2.5.4. **Other Chromogenic Media**

2.5.4.1. **CHROMagar™ staph aureus**

This medium uses similar components to BPA when supplimented with rabbit plasma fibrogenin but also includes a mixture of chromegenic components that allow differentiation between a number of different species (AOAC Research Institute, 2016). This medium does not offer a greater selective power than other media, only the convenience of easier differentiation between colonies.

2.6. Conclusion

Staphylococcus aureus is of great scientific interest due to its potential for disease governed by its wide range of virulence mechanisms, antibiotic resistance mechanisms (summerised in Table 2.3) and its ability to survive in a range of environments. Both its commensal and pathogenic nature are what make it so dangerous, allowing it to be widespread throughout human populations while remaining asymptomatic, waiting to take an opportunity to cause sometimes life-threatening disease (Corey, 2005). While decades of research has been done on this organism, and there are abundnant sources of information on it, its pathogenicity and its genome, there are still some gaps in knowledge, both of the organism's mechanisms of action in particular stages of infection and of effective treatments that limit the pathogen's virulence (as opposed to poisoning it with antibiotics which can be dangerous to the patient involved as well). As such we still find ourselves encountering untreatable epidemics of multidrug resistant *S. aureus*, suggesting that implementation of ourknowledge of this microbe is not being utilised to its full potential.

Beyond this, it is important to recognise that while new treatments take a while to develop and test, it is extremely important to manage and contain epidemics that happen in the mean time and that must be done through careful monitoring of carrier populations where *S. aureus* can thrive asymptomatically until disease is caused. Literature has highlighted that dogs have the potential to be carriers and observation of their domestic populations could prove useful in preventing infection in humans. As *S. aureus* virulence is not tailored to canine immune systems (Boost *et al*., 2008), appropriate and sensitive methods should be utilised to generate the most accurate population representative data. Comparitive studies of isolation and detection methods may prove useful for future survellence studies.

Table 2.3: Antibiotic resistance observed in *S. aureus* and the mechanisms of resistance acquisition.

Antibiotic	*Class*	*Gene*	*Resistance acquisition*	*Reference*
Cefoxatin	Cephamycin	*mecA*	SCC_{mec}	Tsubakishita *et al*., 2010
Ceftoroline	Cephalosporin	Y446N and E447K in *mecA*	Exposure	Long *et al.*, 2014
Ciprofloxacin	Fluoroquinolone	SNPs in *grlA* and *glrB*	Exposure	Campion *et al.*, 2004
		Overexpression of *norA*	Exposure	Fàbrega *et al.*, 2009
Clindamycin	Lincosamide	*ermA*	*Tn554*	Murphy *et al*., 1985)
		ermC	Plasmid	Westh *et al*., 1995
Erythromycin	Macrolide	*ermA*	*Tn554*	Murphy *et al*., 1985
		ermB	*Tn1545, Tn917*	Okitsu *et al*., 2005
			Plasmid	Li *et al.*, 2016
		ermC	Plasmid	Westh *et al*., 1995
		msrA	Plasmid	Matsuoka *et al*., 1998
Gentamicin	Aminoglycoside	*aac(6')-aph(2")*	*Tn4001*	Mahairas *et al.*, 1989
Linezolid	Oxazolidinone	SNPs in 23S rRNA genes	Exposure	Endimiani *et al*., 2011
		cfr	Plasmid	Morales *et al*., 2010
Teicoplanin & *Vancomycin*	Glycopeptide	*vanA*	*Tn1546*-like	Périchon & Courvalin, 2009
		vanB	*Tn916*-like	Courvalin, 2006
Tetracycline	Tetracycline	*tetK*	*pT181*	Noirot *et al*., 1990
		tetM	*Tn5801, Tn916*	de Vries *et al.*, 2009

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CHAPTER 3

COMPARISON OF MANNITOL SALT AGAR AND HICROME™ AUREUS AGAR BASE FOR THEIR POWER IN SELECTING FOR *S. aureus* FROM ENVIRONMENTAL SAMPLES (DOG NARES)

3.1. Abstract

Mannitol Salt Agar (MSA) is the standard selective medium used for *S. aureus*. However, it allows growth for many halotolerant microbes. *S. aureus* is not the most prevalent *Staphylococcal* species in dogs and is under much competitive pressure in order to survive, making it a delicate organism to isolate in this context. This investigation aimed to test if MSA was sensitive and selectively powerful enough to isolate *S. aureus* from dog nasal swabs as compared to HiCrome™ Aureus Agar Base (AAB) which is assumed to be more suitable for environmental samples. 56 samples were collected and placed in peptone water and their true state of *S. aureus* presence was determined by directly testing for *S. aureus* specific gene *nuc*. Each sample was plated onto both MSA and AAB and presumptive results were confirmed with detection of *nuc*. Presumptive and *nuc* results were compared to the true states of each sample so to measure the sensitivity, % of type I errors due to poor selective power and accuracy of true state prediction in conjunction with *nuc* testing of each media. MSA was 60.9% sensitive, 60% of type I errors due to poor selective power and 84% accurate in true state prediction. AAB was 95.7%, 0% and 98.2% respectively. Odds ratios determined AAB as 14.08 times more likely to detect *S. aureus* from dog nare samples than MSA. This suggests that MSA is not suitable for the context of *S. aureus* isolation from dogs and that previous prevalence rates may be underestimated by as much as 60%.

KEYWORDS: *Staphylococcus aureus*, Selective Media Comparitive Analysis, Mannitol Salt Agar, Hicrome™ Aureus Agar Base, Environmental Samples, Dog

3.2. Introduction

Staphylococcus aureus is both a commensal and pathogenic organism that can survive within a range of hosts and habitats (Kluytmans *et al*., 1997; Public Health Agency of Canada, 2011). It is of special interest to many researchers involved with healthcare and disease due to the

microbes' unique abilities to evade the human immune system to cause disease in conjunction with its rising prevalence of resistance to all classes of antibiotics used against it (Leclercq, 2002; Campion *et al.*, 2004; Courvalin, 2006; Yoon *et al.*, 2008; Ramirez & Tolmasky, 2010; Tsubakishita *et al*., 2010; Endimiani *et al*., 2011; Liu *et al*., 2011; Lim *et al.*, 2012; Long *et al.*, 2014; Banik *et al.*, 2015).

Dogs, a proven carrier of *S. aureus*, are not subject to the same virulence demonstrated by *S. aureus* in humans as *S. aureus*'s virulence is tailored specifically to the human immune system (van Duijkeren *et al*., 2011; Garbacz *et al*., 2013). Their risk as asymptomatic carriers of virulent *S. aureus* that can reinfect humans has also been observed (Manian, 2003; van Duijkeren *et al*., 2005). This demonstrates the importance of observing *S. aureus*, its prevalence and the prevalence of carried antibiotic resistance and virulence genes in reservior populations like dogs very important. In studies by Boost *et al*. (2008) and others, dogs are sampled from the the nares which are home to many different competing microbes, as like most environmental samples (Hibbing *et al*., 2010; Foster & Bell, 2012). However, since *S. aureus* is not as virulent in dogs, it is already at a competitive disadvantage, meaning their cell count will be much lower than that in a human sample. This can be problematic for laboratory methods that may not be sensitive enough to detect *S. aureus* in such a competitive environment and as such, the most appropriate methods must be carefully chosen in order to produce the most accurate results.

Mannitol Salt Agar (MSA), the most widely used laboratory medium for *S. aureus* and is the recommended medium by the Korean Food and Drug Administration (KFDA) (Kim & Oh, 2010). This medium is both selective in that it restricts growth to only halotolerant microbes (can survive in high salt conditions but are not essential for growth) and differential in that mannitol fermentation causes the medium to change colour from red to yellow (HiMedia, 2015). These criteria are not specific, allowing the likes of *S. xylosus*, *S. cohnii*, *S. sciuri*, *S. simulans*, *Listeria* spp and even very common airbourne fungi like *Cladosporium sphaerospermum*, among many others, to grow and produce a positive colour change, mimicking the growth of *S. aureus* (Gramoli & Wilkinson, 1978; Gostinčar *et al*., 2009). Studies by Nisakanen and Aalto (1978) and Kim and Oh (2010) demonstrated this lack of specificity (75.4% and 66.6%, repectively) from controlled, innoculated samples. With MSA's lack of specificity and samples taken from environments with a lot of competition against *S. aureus*, MSA may not be the appropriate medium for the isolation of *S. aureus*, specifically

from dog nares, i.e. it is hypothesised that the sensitivity of a medium will change depending on the type of samples innoculated on to it.

An alternative to mannitol fermenting media are the potassium tellurite reducing media like HiCrome™ Aureus Agar Base (AAB) and Baird-Parker Agar (BPA). AAB differs from BPA in that it is chromogenic (can differentiate *Listeria monocytogenes* from coagulase positive *Staphylococci* (CoPS) when grown on AAB) and the nutrient sources are slightly different (does not affect nutrient composition) (HiMedia, 2015). These types of media theoretically have a much higher selective power than mannitol fermenting media as their components select against a much broader rage of environmental microbes including non-Gram positive microbes (Gram-negative bacteria, fungi and other microbes) and other coagulase-negative *Staphylococci* (CoNS) by using potassium tellurite and lithium chloride (Pérez *et al*., 2007). The only limitation this medium would have in the context of sampling from dog nares is that *S. pseudointermedius*, the most abundant Gram-positive microbe in dog airways, can present similarly to *S. aureus* as it has many of the features of *S. aureus* that this medium was designed around (i.e. survives under superoxidative stress and cell wall refrains from taking up lithium chloride) (Hájek, 1976; Hoekstra & Paulton, 2002; Hanselman *et al*., 2009). This medium is recommended for the use for environmental samples by the product's company but there is little literature that demonstrates this as such.

The aim of this study~~project~~ was to detect the true state (difinitive presence or absence of *S. aureus*) of an environmental sample (dog nare swab) by use of genetic methods and then comparing presumptive results from MSA and AAB to them, so to demonstrate the sensitivity and specificity of Mannitol Salt Agar and HiCrome Aureus Agar Base in specific context of dog nare samples, without the biases of designing an experiment with innoculated samples.

3.3. Materials and Methods

Sample collection

Between January and June 2017, samples were taken from 56 dogs housed at a veterinary hospital located in Westville, Durban, (50 pets in for various degrees of veterinary care, 6 were strays left with the clinic to undergo primary care in order to bring the animals to adoption appropriate health) from the nares. They were collected using sterile cotton swabs dipped in autoclaved deionised water.

Enrichment

Each swab was placed into 5ml of autoclaved peptone water and incubated at 37°C for 18-24hrs before selection. This step is used to help recover and condition cells, damaged from environmental and competitive stress to avoid false negatives (Hibbing *et al*., 2010; HiMedia 2017).

Selection

Sigma-Aldrich's HiCrome^{TM} Aureus Agar Base (AAB) supplemented with the equivalent of 3.5% potassium tellurite but without egg yolk emulsion. This was because the egg yolk emulsion (EYE) would not be able to differentiate between *S. aureus* and *S. intermedius*, which is more common in dogs. Since it is very expensive it was decided that its omission would be of little consequence to the project. MSA bought from Oxoid and was made according to product instructions. Onto 60mm plates of AAB and MSA each, 100µl of peptone water culture was spread plated and then incubated at 37°C for 18-24hrs. If plates were overgrown, a second spread plate was performed using a 10^{-1} or 10^{-2} dilution of peptone water, as necessary. For MSA, pale yellow-gold colonies were picked up and isolated on a second 60mm MSA plate using the 4-way streak method and then incubated at 37°C 24hrs (Figure 6.3.1, see Appendix). For AAB, very shiny black colonies were picked up and isolated on a second 60mm AAB plate using the 4-way streak method and then incubated at 37°C for 36-48hrs (Figure 6.3.2, see Appendix). Any plates which had fungal growth with otherwise no presumptive bacterial characteristics were considered a negative presumptive result.

DNA isolation

True State testing: On the same day peptone water culture was inoculated onto each medium, a total of 2ml of said culture was spun down at 15 000xg. Supernatant was removed leaving cells for lysis. 500µl sterile water at 4°C was added to the cells and then vigorously vortexed for 2 minutes, to dislodge the pellet and break the cells. 500µl of 24:1 chloroform and isoamyl alcohol was added at 4°C and vortexed again until the mixture appeared somewhat homogenous. Tubes were then spun down at 12 000xg for 5 minutes. The top aqueous layer was removed into a clean tube. The DNA concentration was measured using NanoDrop technology and diluted as necessary for concentrations appropriate for PCR (approximately 5µM) and then stored at -20°C until PCR.

Media testing: Individual colonies from the presumptively positive 4-way streak plates were picked up using an inoculation loop and broken up into 500µl sterile water at 4°C. Then added was 500µl of 24:1 chloroform and isoamyl alcohol at 4°C. Tubes were vortexed and centrifuged at 10 000xg for 5 min. The top aqueous layer was used directly in PCR (Ruiz-Barba *et al*., 2005).

PCR detection of *nuc*

In 12.5µl of ThermoFisher's DreamTaq® Green PCR master mix (2x), was used with 4µl of the extracted DNA supernatant. 4 µM of each primer synthesised by Inqaba Biotech were used (Forward: 5'-GCGATTGATGGTGATACGGTT-3'; Reverse: 5'-AGCCAAGCCTTGACGAACTAAAGC-3', respectively (Brakstad *et al.*, 1992)) and the reactions were made up to 25µl using sterile nuclease free water. Cycling conditions were initial denaturation at 94°C for 4 minutes then 40 cycles of 94°C for 1 minute, 56.5°C for 30 seconds and 72°C for 1 minute and 30 seconds and then final extension of 72°C for 3 minutes and 30 seconds. To maintain objectivity, *nuc* detection in DNA from the peptone cultures was not carried out until after DNA isolated from selected cells from each medium was tested. True State was determined by the presence or absence of *nuc* from the peptone cultures.

Gel electrophoresis

All products were run through 1.5% agarose gels stained with ethidium bromide at 80V until the loading dye provided by the master mix was ~1cm from the bottom. A representative image is provided below (Figure 3.1).

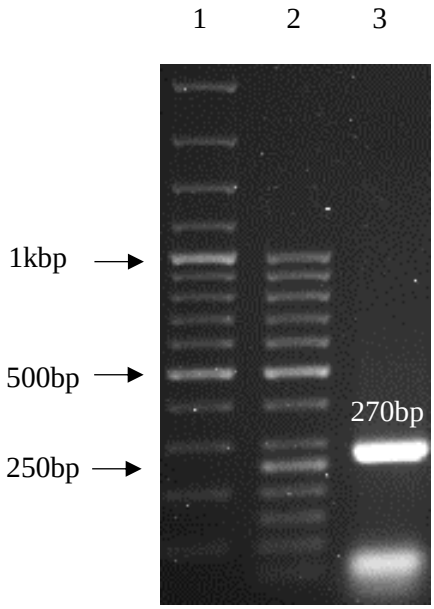

Figure 3.1: Expected product for a positive *nuc* result (Lanes: 1 – 100bp ladder; 2 – 50bp ladder; 3 – *nuc*)

Statistical Analyses

All samples had to be classified according to how their presumptive results related to the true state in order to appropriately draw context to how each media performed in its selection.

True State: *S. aureus* predetermined as **Present**

- True positive – A positive presumptive and *nuc*
- False negative (type II error) – A negative presumptive and *nuc*. This included plates with no growth.
- False false positive (type I error) – A positive presumptive and negative *nuc* (i.e. *S. aureus* was present in the sample but the presumptive characteristics which manifested were of another species present and so *nuc* testing produced a negative result, more simply put, the medium's prediction was correct, but it was based on the biochemical activity of the wrong organism). Inclusion of this fifth category is to show the biological context of this data. However, to calculate the Odds Ratio (OR), for statistical purposes, these cases must be grouped with false negatives.

True State: *S. aureus* predetermined as **Absent**

- False positive (type I error) – A positive presumptive with negative *nuc*
- True negative – A negative presumptive and *nuc*. This also included plates with no growth, i.e. complete and successful selection took place.

Sensitivity was calculated for each medium as the proportion of true positives out of the total number of samples where *S. aureus* was predetermined as present. Similarly, Specificity was calculated for each medium as the proportion of true negatives out of the total number of samples where *S. aureus* was predetermined as absent. To demonstrate the difference in results error rates, the χ^2 value was calculated using IBM® SPSS® Statistics, version 24. Odds ratios were calculated for each medium, considering false false positives and false negatives together. Crosstabulation oriented the True State of *S. aureus* as the baseline factor and the medium's ability to provide a true or false result as the outcome, i.e. when using the formula OR = (a*d)/(b*c), a = number of True Positives, b = the number of False Negatives, c = the number of True Negatives and d = the number of False Positives (Szumilas, 2010).

3.4. Results and Discussion

Of the 56 samples tested, 23 had *S. aureus* present and 33 absent (True State). The graph below shows the results of selection with both media.

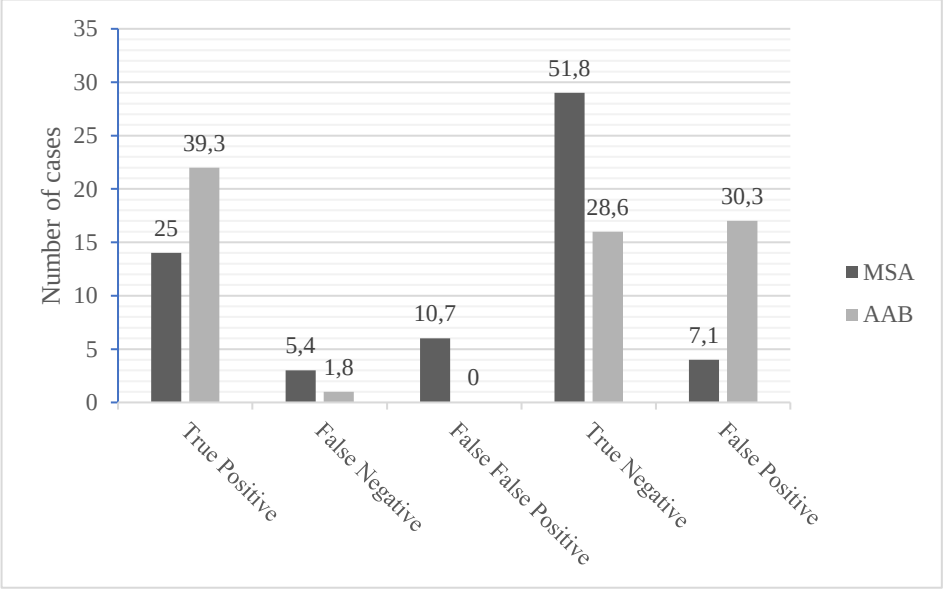

Figure 3.2: Histogram to show frequencies of selection results. Percentages (%) are shown above each bar (MSA – Mannitol Salt Agar; AAB – HiCrome™ Aureus Agar Base)

To truly understand the implications of these results, they must be discussed according to the medium's presumptive accuracy and medium's sensitivity with *nuc* confirmation:

Without the use of *nuc* for confirmation (based only on presumptive medium results), MSA was 58.3% accurate in its presumptive predictions of *S. aureus* being present (percentage of true positives out of all presumptively present cases). The remaining 41.7% (type I error) was attributable to the medium's inbility to eliminate competition masking *S. aureus* 60% of the time (% of false false positives out of erroneous presumptively present cases) and its inability to differentiate between isolates 40% of the time (% false positives out of all erroneous presumptively present cases). AAB was 56.4% accurate in its presumptive predictions *S. aureus* being present, with all error being attributable to a lack of differential capabilities in the medium (100% of error due to false positives), with no incidences of *S. aureus* being masked by competition. On this score, AAB already proves its capabilities as a more powerful selective medium than MSA.

This is reiterated when ***nuc* is used in conjunction with the medium** to confirm isolates as *S. aureus*. MSA was found to be 60.9% sensitive while AAB was 95.7% sensitive. Specificities were calculated as 87.9% for MSA and 48.5% for AAB. Low rates of specificity for AAB are purely related to poor differential ability on its own, due to *S. pseudointermedius* being present more often in canines than *S. aureus* and presenting similar colony morphology (Hoekstra & Paulton, 2002). Differential capabilities can always be adjusted with the addition of differentiating reagents such as egg yolk emulsion to test lipase activity or blood to test haemolytic ability or genetic testing. However, those differential tests are only as accurate in predicting true state as the selective step before it is sensitive enough to detect all existing isolates of interest. As proved here, AAB is clearly more appropriate.

To prove this, each medium's accuracy in predicting the true state of *S. aureus* presence, with confirmation with *nuc*, was calculated. MSA incorrectly predicted the true state of *S. aureus* 9 times (16% error; 84% accurate) and AAB only made 1 incorrect prediction (1.8% error; 98.2% accuracy). The χ^2 value calculated suggested that AAB was significantly more accurate ($p < 0.01$) in predicting the true state of *S. aureus* from nasal swabs of dogs than MSA. It is also more sensitive and more selectively powerful than MSA. More evidence was that MSA allowed the growth of fungal colonies (figure 6.3.3, see appendix) in 17.9% of cases, all of which were scored as presumptively absent. 10% of cases were false negative as *S. aureus* was in fact present in the sample but remained undetected due to fungal overgrowth.

Odds Ratio (OR) for MSA was calculated as 0.215 suggesting that MSA is 4.65 ($= 0.215^{-1}$) times more likely to accurately detect the absence of *S. aureus* than its presence. With the 95%

confidence intervals of 0.056 and 0.82 (not spanning across 1.0) this makes this observation statistically significant ($p < 0.05$). This reiterates the poor sensitivity calculated for MSA (60.9%), concluding that MSA is not suitable for detecting *S. aureus* from dog nares as it under-represents the data. AAB had an OR of 23.375, suggesting that AAB is 23.375 times more likely to accurately detect *S. aureus* presence than its absence. As the 95% confidence intervals did not span across 1.0 (2.81 to 194.18), this observation is considered statistically significant ($p < 0.05$). This reiterates AAB's poor specificity calculated

Using the medium used as the baseline factor and the ability to accurately detect *S. aureus* presence (only) as the outcome, an OR of 0.071 is calculated, suggesting that AAB is 14.08 ($= 0.071^{-1}$) times more likely to detect *S. aureus* in dog nare samples than MSA. This is also statistically significant ($p < 0.05$) by the confidence intervals (0.008 and 0.62) and is reiterated by the highly significant ($p < 0.01$) χ^2-value calculated previously that suggested the outcomes of the two media are significantly different.

Previous studies comparing media for *S. aureus* showed very different results in terms of MSA accuracy/sensitivity (96.5% as demonstrated by Kim & Oh, 2010, compared to 60.9% as demonstrated here), suggesting these sensitivity results are dependent on the type of sample used (inoculated food compared to environmental dog nare samples). This proves that the context of the sample is required just as much consideration when making the choice of what isolation medium to use as the organism of interest.

Many studies have included mannitol salt agar as the only selective medium for the isolation of *S. aureus* from environmental samples, with other media or biochemical tests, like growth on Columbia Blood agar or a positive coagulase test as means of differentiation only, not selection. Studies by Kottler *et al*. (2010), Hanselman *et al*. (2009), Boost *et al*. (2008) and Middleton *et al*. (2005) used MSA as the selective agent for *S. aureus* sampled from dog nares and all might have underestimated prevalence rates by as much as 60% as a result (MSA sensitivity was 60.9%). Here it has been proven that MSA is not reliable to isolate all *S. aureus* present, depending on the competition levels within a sample. With an under-estimation of prevalence can come and underestimated risk of a population as a reservoir for disease.

One limitation to this investigation is that the sample size was possibly too small and retesting this hypothesis that MSA is not selectively powerful enough to detect all *S. aureus* and remeasuring the sensitivity of MSA with a greater sample size may draw more insight on the reliability of selective media for environmental samples. Also, the microbial competition in

the nares of a dog obviously do not represent all cases of swabs from animals as every species is host to different niches which they allow for.

However, the underestimation of by 60% is in close (but not complete) agreement with the results seen in Chapter 3 of this dissertation compared to those studies done previously. While prevalence rates previously range between 8 and 17% in studies that used MSA, 35.4% was detected when using AAB (<50%). However, there are too many uncontrolled variables (year, region, sampling criteria, veterinary healthcare standards etc.) between the datasets for this to be a guaranteed fact.

3.5. Conclusions

For prevalence studies, selective media must carefully be considered depending on the type of biological sample that will be used, or data may be underestimated, and particularly for the delicate nature of *S. aureus* in dogs, MSA is not sensitive enough to provide accurate population data, allowing for too much microbial competition to mask *S. aureus*. Potassium tellurite media, like AAB, are more suitable for selection but require additional levels of differentiation. While *nuc* testing is always accurate (assuming no contamination), if the medium was not able to select for *S. aureus* present in the sample, genetic methods are of no use.

3.6. References

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CHAPTER 4

ASSESSMENT OF VIRULENCE AND ANTIMICROBIAL RESISTANCE GENES AND OBSERVED ANTIMICROBIAL RESISTANCE PATTERNS IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM DOGS IN DURBAN

4.1. Abstract

Dogs can carry pathogenic *S. aureus* asymptomatically and act as sources for reinfection to humans. However, low prevalence of *S. aureus* in companion animals have caused them to be overlooked as potential nurseries for antibiotic resistance. Nasal swabs were taken from 113 dogs visiting a local Veterinary Hospital in Durban of which 35% were found to carry Methicillin Susceptable *S. aureus*. No cases of Methicillin Resistant *S. aureus* were observed. Prevalence of virulence and antibiotic resistance genes were estimated. Kirby-Bauer Disc diffusion was used to detect resistance to 9 classes of antibiotics. The most notable findings were 12.5% tetracycline resistance attributable to *tetK* (12.5% of isolates) and *tetM* (2.5% of isolates) ($p < 0.01$); 15% of samples carried immune evasion clusters (IEC) carried by βC-ϕ's; 7.5% of isolates were linezolid and vancomycin resistant (LR-VRSA) not attributable to resistance genes *cfr* and *vanA* respectively and were not induced by veterinary practices. This is of great concern as LR-VRSA has never been detected before in animals or outside of India and it is clear that some isolates are surviving beyond treatment, hidden in reservoir populations, like dogs. This is a huge concern for medical and veterinary practices alike. The high prevalence of *S. aureus* and the presence of LR-VRSA conclude that dog populations of Durban pose more of a threat as an overlooked reservior for potentially dangerous *S. aureus* than considered before.

Keywords: *Staphylococcus aureus*; LR-VRSA; Antibiotic Resistance Genes; Antibiotic Susceptibility; Virulence Genes; Dog

4.2. Introduction

Staphylococcus aureus is a Gram positive, ubiqutous bacterium that can be both commensal (of no harm to its host) or pathogenic (disease causing) and will present in 80% of all humans throughout their lives (Kluytmans *et al.*, 1997) and is commonly understood to be a zoonotic

microbe (Public Health Agency of Canada, 2011). In its virulent or pathogenic form, it can cause several diseases with varying severity, as a result of infection such as infective endocarditis ~~(infection of the membrane lining the inside of the heart)~~, osteoarticular infections ~~(infection of joints)~~, pleuropulmonary infections ~~(infection of the membrane lining the lungs and thorax)~~, infections of skin and soft tissue and also severe infections involving internalised devices such as catheters, IV drips and other devices (Kluytmans *et al*, 1997) (Liu, 2009) (Tong *et al*, 2015). It is also widely understood that *S. aureus* is transmitted between people through contact (Koenig *et al*., 2016) (Jacobs, 2014) which poses a serious threat to healthcare facilities such as hospitals and clinics as any strain of *S. aureus*, especially methicillin-resistant *S. aureus* (MRSA), may be passed on by asymptomatic staff and visitors to ill or even immuno-compromised patients.

There is some uncertainty about the rates of transfer of *S. aureus* from dogs to humans and humans to dogs. A cross-sectional study done by Boost *et al*., (2008) showed that there is definitely transfer between owner and dog. However, it was shown that there was a high association of transfer between pet and owner and whether the owner worked in healthcare, suggesting that human to dog transfer is more likely. However, there is still potential risk to humans as a number of other cases (Manian, 2003; van Duijkeren *et al*., 2005) found that the family dog was a source of reinfection to immuno-compromised individuals who with multiple rounds of antibiotic treatment, continued to become reinfected with MRSA. It was only after the dogs had undergone treatment did the reinfections cease.

Virulence in *S. aureus* is generally tailored to human immune systems (Boost *et al*., 2008) and as such virulent strains can remain asymptomatic in carrier populations of other species (such as dogs). Unfortunately, *S. aureus* has demonstrated resistance to all antibiotic classes used against it, acquisition of such caused by changes in its genetic code, either by mutations caused by over-exposure to the antibiotics or by acquiring whole resistance genes via horizontal gene transfer (Leclercq, 2002; Campion *et al.*, 2004; Courvalin, 2006; Yoon *et al.*, 2008; Ramirez & Tolmasky, 2010; Tsubakishita *et al*., 2010; Endimiani *et al*., 2011; Liu *et al*., 2011; Lim *et al.*, 2012; Long *et al.*, 2014; Banik *et al.*, 2015). With cases of Multi Drug Resistant *S. aureus* on the rise (Hiramatsu *et al*., 2014), it is important to assess the prevalence of virulent, resistant *S. aureus* in asymptomatic carriers, especially those like dogs which are in constant contact with humans and especially in countries like South Africa which have high rates of immuno-compromised individuals due to diseases such as HIV, AIDS and others like diabetes (Pillay *et al*., 2016).

The purpose of this study was to detect the presence of antibiotic resistant genes and other virulence genes as well as the antibiotic susceptibility profiles of isolates, thus assessing the risk dogs, as a population, can have as carriers for *S. aureus*.

4.3. Materials and Methods

Sample collection

Between October 2016 and June 2017, samples were taken from 113 dogs (100 pets in for various degrees of veterinary care, 13 were strays left with the clinic to undergo primary care in order to bring the animals to adoption appropriate health) housed at a veterinary hospital located in Westville, Durban, which caters to pets from inland Durban between Berea and Pinetown. Samples were from the nares or any lesions present on the animal's body. They were collected using sterile cotton swabs dipped in autoclaved deionised water, then just the bud of the swab was placed in a sterile 15ml **For safety:** all animals were handled by veterinary staff. Animals infected with other severe zoonotic diseases as well as parvovirus were not sampled.

Enrichment

Into each 15ml tube, 5ml of autoclaved peptone water was added and incubated at 37°C for 18-24hrs before selection.

Selection

Sigma-Aldrich's HiCrome^{TM} Aureus Agar Base (AAB) supplemented with the equivalent of 3.5% potassium tellurite was used in place of the traditional Mannitol Salt Agar as it is more suitable for environmental samples and is more effective at limiting the growth of fungal spores likely to be picked up on a nare's swab. Onto 60mm plates of AAB, 100µl of peptone water culture was spread plated and then incubated at 37°C for 18-24hrs. If plates were overgrown, a second spread plate was performed using a 10^{-1} or 10^{-2} dilution of peptone water, as necessary. Very dark black colonies were picked up and isolated on a second 60mm AAB plate using the 4-way streak method and then incubated at 37°C for 36-48hrs (the extended incubation time was to allow for a greater cell mass of any slower growing MRSA isolates).

DNA isolation

Individual colonies from the 4-way streak plates were picked up using an inoculation loop and broken up into 500µl sterile water at 4°C. Then added was 500µl of 24:1 chloroform and

isoamyl alcohol. Tubes were vortexed and centrifuged at 10000xg for 5 min. Approximately 350 to 400µl of the top aqueous layer was removed and placed into a new tube where it could be stored at -20°C for up to 2 months or 5 freeze-thaw cycles (Ruiz-Barba *et al*., 2005).

PCR detection of relevant genes

Listed below in table 4.1 are the primers used for the detection of each gene or gene set. Only isolates positive for the thermonuclease gene *nuc* were tested for the presence of virulence and antibiotic resistance genes and susceptibility to antibiotics. All samples were run against a negative water control and a neutral species control using *S. aureus* strain ATCC 25923.

Table 4.1: Primers used in this study for antibiotic resistance and virulence genes in conventional PCR

Target gene	*Primer sequence*	*Product size*	*Reference*
nuc	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	270	Brakstad *et al*., 1992
mecA	F: AAAATCGATGGTAAAGGTTGGC R: AGTTCTGCAGTACCGGATTTG	310	Ghanbari *et al*., 2016
Cfr (hot start)	F: TGAAGTATAAAGCAGGTTGGGAGTCA R: ACCATATAATTGACCACAAGCAGC	746	Kehrenberg & Schwarz, 2006
*ermA**	F: GTTCAAGAACAATCAATACAGAG R: GGATCAGGAAAAGGACATTTTAC	421	Ghanbari *et al*., 2016
*ermB**	F: CCGTTTACGAAATTGGAACAGGTAAA GGGC R: GAATCGAGACTTGAGTGTGC	359	
*ermC**	F: GCTAATATTGTTTAAATCGTCAATTCC R: GGATCAGGAAAAGGACATTTTAC	572	
tetK	F: TATTTTGGCTTTGTATTCTTTCAT R: GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski *et al*., 2000
tetM	F: ACAGAAAGCTTATTATATAAC R: TGGCGTGTCTATGATGTTCAC	171	Aminov *et al*., 2001
vanA	F: GGGAAAACGACAATTGC R: GTACAATGCGGCCGTTA	732	Dukta-Malin *et al*., 1995
fnbA	F: GATACAAACCCAGGTGGTGG R: TGTGCTTGACCATGCTCTTC	191	Mongodin *et al*., 2002
fnbB	F: GGAGCGGCCTCAGTATTCTT R: AGTTGATGTCGCGCTGTATG	201	
hlb	F: GTGCACTTACTGACAATAGTGC R: GTTGATGAGTAGCTACCTTCAGT	309	Moraveji *et al*., 2014

** Genes are in a multiplex reaction*

In each reaction, 12.5µl of ThermoFisher's DreamTaq® Green PCR master mix (2x) or Promega's GoTaq® Hot Start Green master mix (2x) (as indicated) was used with 4µl of the extracted DNA supernatant. Primers synthesised by Inqaba Biotech were used in the concentrations mentioned below and the reactions were made up to 25µl using sterile nuclease free water. References for these conditions are provided in table 4.1. Each primer was used in the following concentration: 4µM for *nuc* and *mecA*; 3µM for *ermA-C*; 1.25µM for *tetM* and *vanA*; 1µM for *cfr*, *fnbA*, *fnbB* and *hlb*; and 0.5µM for *tetK*. Cycling conditions for *nuc* were initial 94°C for 4 minutes, then 40 cycles of 94°C for 1 minute, 56.6°C for 30 seconds and 72°C for 90 seconds and then a final 72°C for 3 minutes and 30 seconds. For *mecA*: initial 94°C for 4 minutes, then 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute and then a final 72°C for 5 minutes. For *cfr*: initial 94°C for 4 minutes, then 34 cycles of 94°C for 1 minute, 48°C for 2 minutes and 72°C for 3 minutes and then a final 72°C for 7 minutes. For *ermA-C*: initial 94°C for 10 minutes, then 35 cycles of 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute and then a final 72°C for 10 minutes. For *tetK*: initial 94°C for 4 minutes, then 35 cycles of 94°C for 1 minute, 50°C for 1 minute and 72°C for 90 seconds and then a final 72°C for 5 minutes. For *tetM*: initial 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and then a final 72°C for 7 minutes. For *vanA*: initial 94°C for 2 minutes, then 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute and then a final 72°C for 10 minutes. For *fnbA* and *fnbB*: initial 94°C for 3 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds and then a final 72°C for 7 minutes. For *hlb*: initial 94°C for 7 minutes, then 35 cycles of 94°C for 1 minute, 58°C for 1 minute and 72°C for 1 minute and then a final 72°C for 7 minutes.

Gel electrophoresis

All products were run through 1.5% agarose gels stained with ethidium bromide at 80V until the loading dye provided by the master mix was ~1cm from the bottom. A representative image is provided below (Figure 4.1)

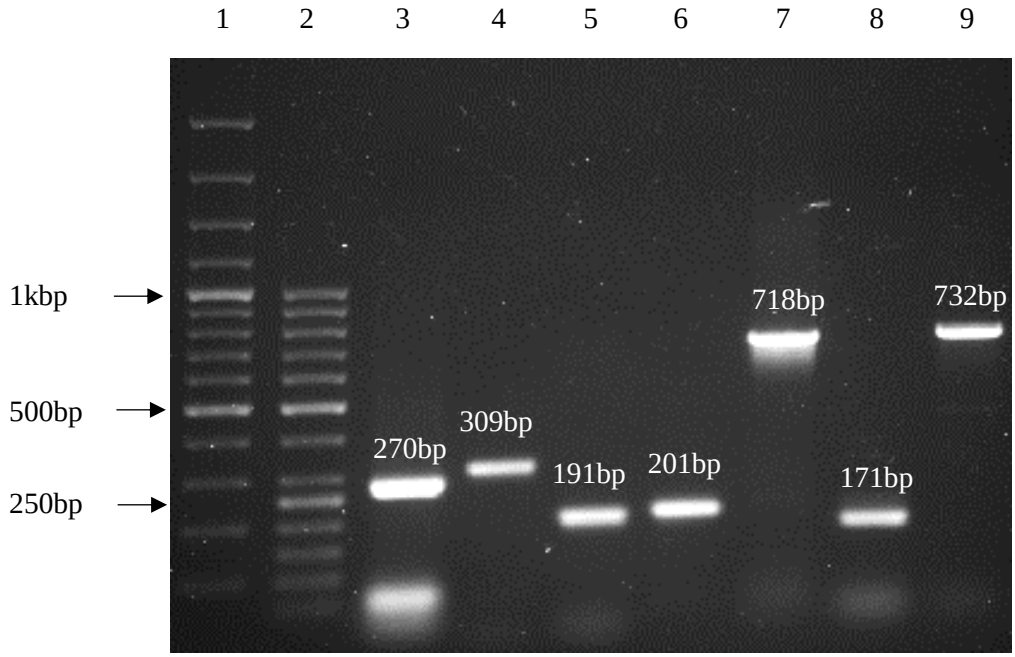

Figure 4.1: Virulence and Antibiotic resistance genes detected in *S. aureus*. (Lanes: 1 – 100bp ladder; 2 – 50bp ladder; 3 – *nuc*; 4 – *hlb*; 5 – *fnbA*; 6 – *fnbB*; 7 – *tetK*; 8 – *tetM*; 9 – *vanA*)

Antibiotic susceptibility

Susceptibility was tested using the Kirby-Bauer Disc Diffusion method. Samples were grown in Brain Heart Infusion broth overnight at 37°C and then adjusted to the 0.5 McFarland standard at 600nm. 100µl of broth was spread plated onto 10mm plates of Mueller-Hinton Agar, onto which 5 antibiotic discs were placed (2 plates needed to for all 10 antibiotics). Clindamycin and Erythromycin discs were placed no more than 15mm apart so to test for inductive resistance to clindamycin. All other discs were placed 25mm apart and away from the edge (Figure 6.1 and 6.2 in the appendix). Concentrations of antibiotics used are given in table 4.2 below. Despite vancomycin disc testing not being recommended by the Clinical and Laboratory Standards Institute, teicoplanin accurately represents vancomycin, as in *S. aureus* resistance is controlled by the same genes (CLSI, 2015). Here, they have been used in conjunction with each other.

Table 4.2: Antibiotic discs used in this study

Antibiotic Name	*Antibiotic Class*	*Disc Content (µg)*
Cefoxatin	Cephamycin	30
Ceftoroline	Cephalosporin	30
Ciprofloxacin	Fluoroquinolone	5
Clindamycin	Lincosamide	2
Erythromycin	Macrolide	15
Gentamicin	Aminoglycoside	10
Linezolid	Oxazolidinone	30
Teicoplanin	Glycopeptide	30
Tetracycline	Tetracycline	30
Vancomycin	Glycopeptide	5

Statistical Analyses

All statistical analyses were done using IBM® SPSS® Statistics, version 24. Correlation analyses using the Pearson's coefficient were conducted between genes detected and between genes and resistance profiles observed. Significant values were considered at the 95% confidence level (two-tailed) and highly significant values were considered at the 99% confidence level (two-tailed). All genes or antibiotic profiles with unanimous results were excluded from the correlation analysis.

4.4. Results and Discussion

Part 1: *Prevalence*

Out of 113 dogs sampled, 40 (35.4%) were positive for *S. aureus*. This is substantially higher that previous studies have indicated: 12.5% by Tarazi *et al*., (2015); 8.8% by Boost *et al*., (2008); 8.25% by Kottler *et al*., (2010). However, it is not clear if this is due to sampling biases (this study only used animals from one clinic, i.e. the clinic itself could be a source of infection, while other studies sampled from unrelated locations) or due to isolation procedures (this study used an isolation technique more appropriate for environmental samples while all others utilised mannitol salt agar (see chapter 4)).

Virulence

Table 4.3, shows the count of samples which tested positive or negative for each virulence gene.

Table 4.3: Presence and absence of virulence genes tested in studied samples

Gene	*Present(%)*	*Absent(%)*
hlb	34(85)	6(15)
fnbA	40(100)	0(0)
fnbB	40(100)	0(0)

Under normal circumstances, *hlb* should be detected in all *S. aureus* isolates. However, βC-φ (β-haemolysin converting bacteriophages) will splice themselves into the host's genome at a site that falls within this gene, interrupting the *hlb* gene. The majority of these prophages carry variations of the immune evasion cluster. In this population, 15% of samples are most probably carrying other virulence genes in the form of the immune evasion cluster (*scn*, *sak*, *chp*, *sea* and *sep*). It is difficult to compare this prevalence rate with other studies as there are no other studies which have measured this in strains isolated from dogs. However, van Wamel *et al*., (2006) detected 90% of clinically isolated strains (MRSA and MSSA alike) were carrying IEC clusters (all *hlb* negative samples had an IEC), while Cuny *et al*., (2015) detected 73% from MSSA alone from a much smaller sample size (n=15) and only from *S. aureus* strain CC398. From livestock associated MRSA (LA-MRSA) isolated from horses, they detected 6% IEC prevalence and in clinical MRSA 19%, all from *S. aureus* CC398 isolates. There is no data for generalised IEC prevalence in *S. aureus*. However, the data from this investigation still suggests that IEC virulence is present in dog populations and can pose a risk to humans who may contract *S. aureus* from their pets.

The genes *fnbA* and *fnbB* are in all isolated samples. This differs from numerous studies which suggest varied prevalence rates for these 2 genes. An Indian paper reported 85.5% rate for *fnbA* in clinical *S. aureus* isolates (Bhatty *et al*., 2013), whilst an Iranian paper reported 82.2% of MRSA isolates carried *fnbA* and 46.7% carried *fnbB* (Mirzaee *et al*., 2015). A second Iranian paper detected 56% and 46% *fnbA* and *fnbB*, respectively in MSSA clinical samples and higher rates (64% and 51%) in MRSA isolates (Ghasemian *et al*., 2016).These studies suggested varying *fnbA* rates but almost aggreable *fnbB* rates. Both completely disagree with the findings of this study. The variations seen are most probably due to the ancestry of *S. aureus* strains in

each region. This means that the dog populations of Durban carry highly virulent forms of *S. aureus* commonly.

Resistance Profiles

Table 4.4.1: Presence and absence of antibiotic resistance genes tested in studied samples (excluded genes had 100% absent cases)

Gene	*Present(%)*	*Absent(%)*
tetK	5(12.5)	35(87.5)
tetM	1(2.5)	39(97.5)
vanA	1(2.5)	39(97.5)

Table 4.4.2: Samples with observed resistance (excluded antibiotics had 100% susceptibility)

Antibiotic	*Susceptible(%)*	*Intermediate(%)*	*Resistant(%)*	***Total***
Linezolid	37(92.5)	0	3(7.5)	40
Tetracycline	34(85)	1(2.5)	5(12.5)	40
Vancomycin/Teichoplanin	37(92.5)	0	3(7.5)	40

Amongst the 40 isolates, no **β-lactam resistance**, i.e. methicillin or ceftaroline resistance was observed with no cases of *mecA* presence either (the causative for both resistance mechanisms). This is not in agreement with numerous studies carried out over the last 20 or so years, these studies stating prevalence rates between less than 1% and as much as 50% (Iowa State University, 2016; Reddy *et al*., 2016; Boost *et al*., 2008; Duquette & Nuttal, 2004). However, the majority of these studies demonstrate sampling biases, which prevent their data being comparible to this data, due to sampling criteria used, for example, Reddy *et al*., (2016) only sampled from dogs which suffered from recurrent pyodermic infections. Another recent study that does agree with 0% MRSA or *mecA* only sampled 8 *S. aureus* strains and thus 0% MRSA or *mecA* cannot accurately represent prevalence within the population sampled (Daley *et al*., 2016). Considering the criteria for sampling in this project included but did not distinguish between animals at risk of *S. aureus* carriage (such as postoperative pets, immuno-compromised individuals, individuals that had lengthly stays at veterinary hospitals etc.), it is not fair to assume that the data accurately represents general prevalence rates in all dogs in

Durban, as the population ranges in the 100 000's and a sample size of little over 100 individuals may not detect MRSA if its actual prevalence is less than 1% of less than 40% of the population of dogs. However, what can be interpreted from this data is that, regionally, the dogs of Durban are not at any concernable risk of MRSA contamination and as a result should not be considered a reservoir for MRSA infection in humans.

Tetracycline resistance was found in 15% (2.5% intermediate, 12.5% absolute resistance) of isolates with all cases, except the intermediate case, being attributable to either *tetK* or *tetM*. One isolate was found to have carry *tetK* but did not express resistance. Higher rates of tetracycline resistance compared to other antibiotics is not surprising as tetracycline used to be the first line of antibiotic used for general respiratory infections in veterinary care (Guardabassi *et al*., 2004). It has since been made a second choice antibiotic due to its wide-spreading resistance (Maaland *et al*., 2013). There are very few recent studies (after 2010) that look at prevalence rates in dogs with *S. aureus* specifically (none in Africa). Boost *et al*., (2008) reported 29% resistance to tetracycline in *S. aureus* isolated from dogs, but incidence of *tetK* and *tetM* were not measured. *S. pseudointermedius*, the most prevalent *Staphylococcal* pathogen in dogs (Werckenthin *et al*., 2001) is capable of sharing resistance with co-infecting *S. aureus*, and such related resistance prevalence is not impossible. A study by Rubin *et al*., (2011) showed 34% of *S. pseudointermedius* carried by dogs exhibited resistance to tetracycline, in agreement to the results seen in the study by Boost *et al*., (2008). In conclusion, tetracycline resistance observed was not unexpected but the lower levels observed as compared to those in papers of 10 years previous to this study, are possibly due to the awareness of growing incidence of tetracycline resistance and the subsequent restrictive use in veterinary care.

Vancomycin resistance was observed in 3 cases (7.5%), all of which were also **resistant to linezolid** (Figure 6.2). This is relatively disturbing as linezolid is the first choice of antibiotic for use against vancomycin (glycopeptide) resistant strains of *S. aureus* (Balkhair *et al*., 2010; Loomba *et al*., 2010). While there have been reports of linezolid resistant *Enterococci* (LRE) in India, China and the UK (Auckland *et al*., 2002; Kumar *et al*., 2014; Tian *et al*., 2014) there a only a few isolated incidents of linezolid resistant *S. aureus* (LRSA) in cystic fibrosis patients that were exposed to linezolid for extended periods, on multiple occasions (Gu *et al*., 2013; Endimiani *et al*., 2011). Only very recently have LR-VRE been identified (Gupta, 2016), along with the emergence of LR-VRSA. Until now, no cases LR-VRSA have been reported outside of India, (Azhar *et al*., 2017; Kumar, 2016; Singh *et al*., 2014). This includes veterinary studies

as well. All strains were hospital acquired strains and are directly associated with antibiotic pressure (Mandal *et al*., 2017). Upon further communication with the head veterinarian at the Veterinary Hospital in Westville, it was deduced that these dual resistant strains had to have originated from a human patient, as linezolid has never been used on veterinary patients at that hospital due to the ethical implications it poses on humans, thus reaffirming the overlooked potential companion animals have as a reservoir for reinfection of *S. aureus* in humans.

As Kwa-Zulu Natal has the largest population of Indians outside of India, it is most probable that some of these strains were brought over by individuals visiting their families and that is why South Africa has seen this resistance before anyone else. It must be reitterated that this prevalence rate most likely does not represent the whole Durban population of dogs due to sampling biases, and the value of 7.5% rate of LR-VRSA relates to this data only, until such time as further studies with larger sample sizes of a more diverse nature are carried out. However, the shear existance of these strains, regardless of their prevalence should not be taken lightly. The presence of *cfr* (the only form of linezolid resistance that is transferable horizonatally) was not detected in these isolates suggesting mechanisms derived from mutations caused by over exposure to linezolid (Gu *et al*., 2012). None of the isolates carried *vanA*, suggesting that another *van* gene was present, not detected by the used PCR protocol. However, *vanA* was found in 1 sample that did not express vancomycin resistance (2.5%). This agrees with the findings of Azhar *et al*., (2017) who noted 3.1% of MSSA were vancomycin resistant. They too observed some non-*vanA* associated vancomycin resistance (22% of vancomycin resistant isolates).

Part 2: *Correlation*

Below in table 4.5 are the counts of various factor combinations observed in this study. Factors in *italics* indicate genes present (note: "*iec*" is not a gene but represents the presence of IEC as proved by a lack of and intact *hlb* gene). Factors in uppercase represent resistance observed. Resistance with a '*' represent intermediate resistance.

Table 4.5: Factor profiles observed

Profiles	***Cases Observed***
None	22
iec	4
iec, LZD, VA	2
LZD, VA	1
vanA	1
tetK, TE	4
tetK	1
tetM, TE	1
DA*, TE*	1
CIP*	1
CN*	1
E*	1

A Pearson's 2-tailed correlation detected significant relationships between factors (note: vancomycin/teicoplanin and linezolid resistance were considered a single factor (LZD-VA) as all instances were found in the same 3 samples). The most significant relationships ($p < 0.01$) were between *hlb* and LZD-VA, *tetK* and TE, and *tetM* and TE. No other significant relationships were observed. Relationships between TE and the two *tet* genes is not surprising as there was no incidence of tetracycline resistance not attributable to either *tet* gene. The relationship between *hlb* and LZD-VA should be considered with caution, as 3 individuals with only 2 with identical profiles, while statistically significant, do not necessarily represent what would be observed in a larger population.

4.5. Conclusions

The major limitation of this investigation was that the sample size was not appropriately large enough for the large number of factors being tested (40 positive samples being tested against 10 genetic and 9 antimicrobial factors) and did not span enough sample sources to give a clear indication of the reliability of the results seen as good representation of the total population of dogs in Durban. However, some notable findings were made. Even from similar type studies with similar sampling biases, a substantially larger number of *S. aureus* isolates were detected than previously stated in literature. This already suggests that dogs as a population are most

likely underestimated as a potential reservoir for *S. aureus*. This is motivation to draw up a meta-analysis which tests the statistical contributions that varying sampling factors may have on a dataset in this context (household vs clinic; surfaces, staff and tools in clinics; etc). As it stands, this data does suggest that dogs may be a greater reservoir for *S. aureus* for human infection than previously anticipated.

The detection of LR-VRSA reinforces this fact and also casts a very long shadow across the future of antibiotics. Up until now, even though no clinical cases of LR-VRSA have been observed outside of India, it is clear that some isolates are surviving beyond treatment, hidden in reservoir populations, like dogs. This is a huge concern for medical and veterinary practices alike. When patients are infected with already resistant species and are onto their second round of antibiotics, they should be isolated until the infection has been completely eliminated. This will reduce the possibilities of instances of isolates developing second resistances and surviving undetected.

Apart from this, campaigns should be started as updates to current measures in place to eduacate animal owners and veterinary staff about the risks of cross infections with this microbe specifically. Any registered breeders or adoption facilities should be required by law to inform new animal owners of the risks involved with cross-infenctions of *S. aureus* and the measures of prevention that can be taken. Booklets can then be handed to the new pet owners to take home for reference. Medical doctors and veternary specialists should consider pooling information of households by monitoring carrier status of both humans and animals living together biannualy (to monitor changes over flu season) and then appropriately sterilise individuals if status is found to be positive.

4.6. References

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CHAPTER 5: GENERAL CONCLUSIONS

Apart from, possibly, the anti-vaccination movement, there has been no doubt that the rise of multidrug resistant bacteria is the greatest threat to modern medicine to date, with cases of multidrug resistant *S. aureus* being reported all over the globe (Barua *et al.*, 2017; Carroll *et al.*, 2017; Deyno *et al.*, 2017; Saba *et al.*, 2017). While the major contributing factor is poor antibiotic etiquette on both the patient and doctors' sides, another major and overlooked factor is general hygiene practices. While no-one denies its importance for preventing the spread of dangerous pathogens, not many people commit to these practices vigorously, resulting in many pathogens enduring and remaining under the radar until someone becomes sick. Ultimately, this project aimed to gauge the extent of "under-the-radar" *S. aureus* in a population that is otherwise overlooked due to the fact that *S. aureus* remains asymptomatic in dogs, except in instances of co-infection. This project tested the extent of underestimating dogs as reservoir populations for *S. aureus* in two parts: by detecting isolates within the population and determining their pathogenic factors (virulence and antibiotic resistance) as a result of human interaction; and testing the methods previously employed to carry out the first part. Both yielded significant and agreeable findings.

5.1. Significant Findings

The prevalence study yielded 12.5% tetracycline resistance attributable to *tetK* (12.5% of isolates) and *tetM* (2.5% of isolates) ($p < 0.01$); 15% of samples carried immune evasion clusters (IEC) carried by βC-ϕ's; 7.5% of isolates were linezolid and vancomycin resistant (LR-VRSA) not attributable to resistance genes *cfr* and *vanA* respectively and were not induced by veterinary practices. Tetracycline resistance can be attributable, in some part, to veterinary practice as it is the first drug of choice against Gram-positive infections and are often administered in a preventative manner for fresh wounds and are second choice for respiritory infections (Maaland *et al.*, 2013; Guardabassi *et al.*, 2004). However, vancomycin and linezolid resistance (especially co-resistance) are definitely not associated with veterinary practices as veterinary medicine does not employ these last resort drugs which are both dangerous for the health of the animals and pose an unethical risk to human medicine. Thus, these strains were passed from human to animal, where they have remained asymptomatic and under-the-radar. The most troublesome information is that these strains have not been seen outside of India up

until now, suggesting that human individuals (probably owners of the animals) had contact with persons from India, carrying these strains. The large population of Indian decendants in Durban, Kwa-Zulu Natal, makes a good case for the potential bridge made for this pathogen that travels across the globe. This finding (LR-VRSA) in particular, and the high prevalence rate of *S. aureus* (35%) suggest that dogs as a popultion have been grossly underestimated as potential reservoir for *S. aureus*.

This is reitterated by the findings of the second part of this study which demonstrated that the methods which use MSA as the selective step for isolating *S. aureus* from dog nares specifically, when used to determine prevalence rates, is unreliable (not sensitve or accurate enough and is prone to type I errors due to poor selective power: "false false positives) and has possibly been underestimating the prevalence rates in studies which it was employed. AAB proved 14 times more likely to correctly detect *S. aureus* than MSA, in this context. This is an incredibly important finding as most studies up until now, some which are are still used as the standard for comparison (studies such as those done by Boost *et al*., 2008), all employed MSA as the method of isolation. The combination of results conclude that there has been a great underestimation of the potential dog populations pose as a reservoir for *S. aureus* and that this underestimation most probably has caused the casual manner in which humans acknowledge their hygiene in association with their pets.

5.2. Limitations

The greatest limitation seen in both parts of this study was that the sample population was not large or diverse enough to assure with 100% confidence, that these prevalence rates accurately represent the population of Durban pet dogs and the traits observed were definitely due to medical or veterinary practices (as discussed above). However, despite the small sample size, highly significant results ($p < 0.01$) were attained from the second study suggesting that, regardless of the sample size, it was definite that AAB was more accurate in this biological context (swabs from dog nares) than MSA. Precise error rates may not be truly representative and but testing a larger population size would make the results observed more significant.

5.3. Future Recommendations

With regard to this project and those that may stem from it, a larger and more diverse sample population must be used, including animals from different socio-economic areas, from households instead of clinics, strays and surrendered animals in organised facilities (strays that otherwise have no human contact may be safety risk for individuals involved and are unlikely to yield much valuable information as dog to dog *S. aureus* transferance is extremely rare) and should include potential erroneous factors (factors that skew the observed rates) such as testing the humans that are in contact with the animals (clinic staff, owners, individuals involved in the study) and the environments in which they are kept (cages, surfaces in clinics, x-ray equipment etc.). A study of that magnituted could make a powerful statistical argument for any significant findings (prevalence or correlation) that may be observed.

With regard to the general population and both medical and veterinary practices, these findings suggest that more attention needs to be paid to pets as reservoir populations of pathogens like *S. aureus*. Epidemiologically, these populations have been overlooked when surveillance of them has proven useful (in this study) in predicting the movement of resistant isolates on a global scale. Also, on a more individual civilian scale, the importance of maintaining certain hygiene practices with ones animals must be impressed upon as it is clear that forgoing such measures could be detrimental to the health of many people. Reminders to sterilise ones hands before leaving hospitals, clinics or doctors' rooms should be intalled and the general public, pet owners specifically, should be educated on the risks that not following these hygiene practices may have. It cannot be the responsibility of the medical community (a tiny part of the population) to maintain and remedy pathogens without the informed co-operation of the general population.

5.4. References

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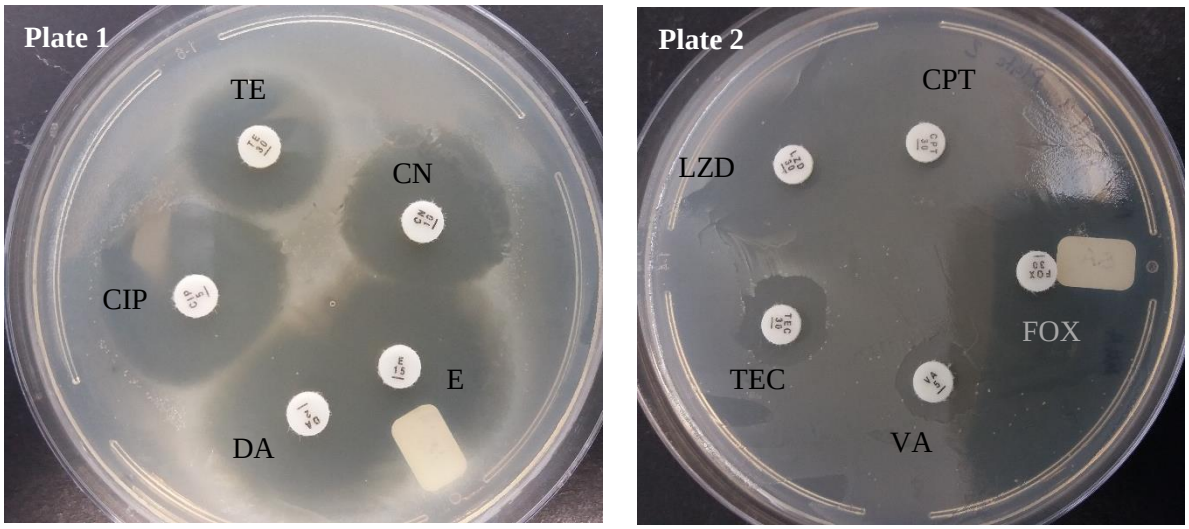
APPENDIX



Figures 6.1.1 and 6.1.2: Orientation of discs used for antibiotic susceptibility testing with a susceptible *S. aureus* control. (Plate 1: CIP – ciprofloxacin; DA – Clindamycin; E – Erythromycin; CN – Gentamicin; TE – Tetracycline. Plate 2: FOX – Cefoxitin; CPT – Ceftaroline; LZD – Linezolid; TEC – Teicoplanin; VA – Vancomycin)

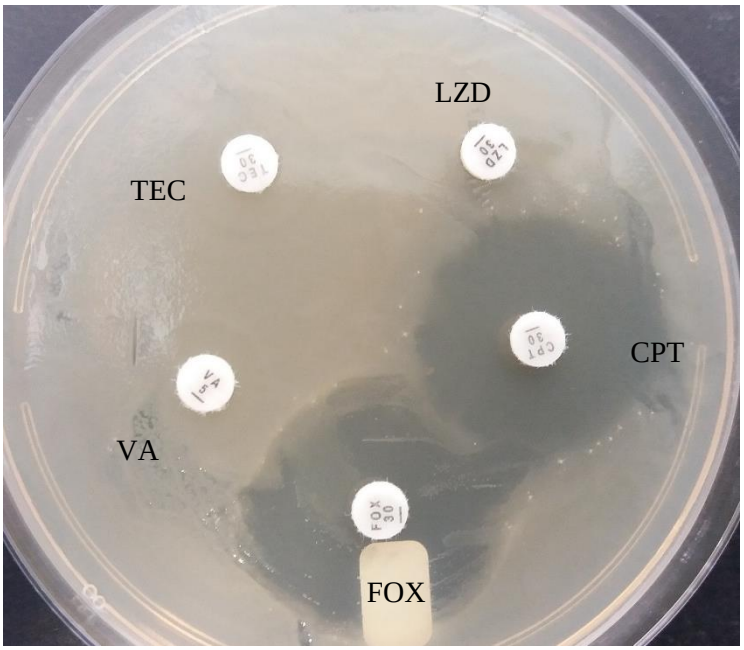


Figure 6.2: Observed resistance profile for LR-VRSA samples (FOX – Cefoxitin; CPT – Ceftaroline; LZD – Linezolid; TEC – Teicoplanin; VA – Vancomycin)

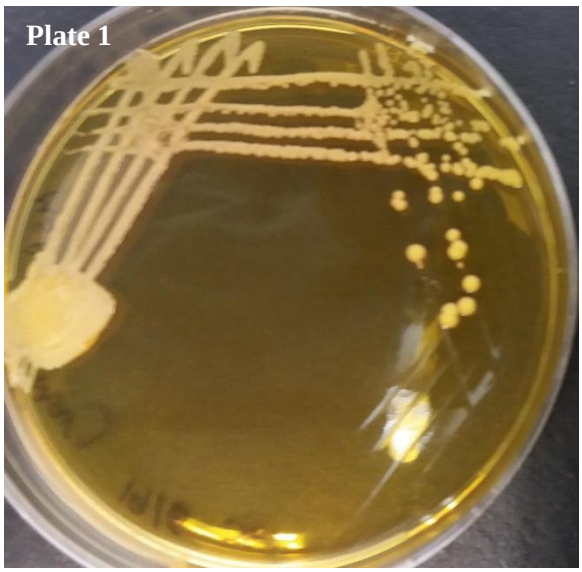


Figure 6.3.1 and 6.3.2: Presumptive *S. aureus* colony morphology on MSA (Plate 1) and AAB (Plate 2)

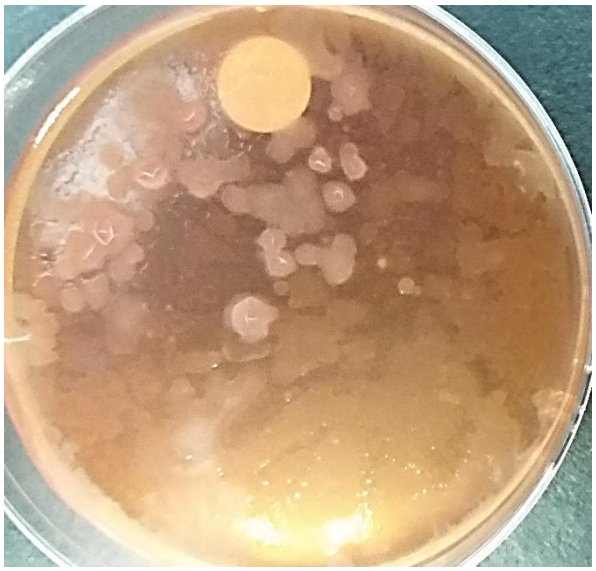


Figure 6.3.3: MSA plate showing mannitol reducing fungal colonies