

Identification of methicillin-resistant *Staphylococcus aureus* in horses using conventional and molecular techniques

BY

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DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of Master of Science (Animal/Human/Ecosystem Health) has not been previously submitted by me for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.

Signed:



Date: 12 April 2016

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IDENTIFICATION OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* IN HORSES USING CONVENTIONAL AND MOLECULAR TECHNIQUES

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ABSTRACT

Staphylococcus aureus is a coagulase-positive, Gram-positive, coccal bacterium. It is one of the leading causes of both skin and invasive infections. It plays an important role in diagnostics and treatment due to its ability to develop resistance to antimicrobial drugs. Methicillin-resistant *Staphylococcus aureus* or MRSA is an important nosocomial pathogen in both humans and animals due to its resistance to all β -lactam antimicrobial agents. Colonization of MRSA in horses poses a great concern. This is considered an important risk factor for development of staphylococcal related diseases in horses admitted to veterinary hospitals. Colonized horses can also be a source of zoonotic MRSA infections. Methicillin-resistant *Staphylococcus aureus* detection based on a PCR reaction is commonly used and various types of PCR-based assays were developed to assist in early detection of MRSA. The main aim of the study was to compare the currently used conventional microbiological techniques with a published multiplex PCR assay targeting the *mecA*, *spa* and *pvl* genes for the rapid and accurate identification of MRSA in horses admitted to the Onderstepoort Veterinary Teaching Hospital, University of Pretoria. A total of 50 isolates, which consists of isolates from horses and their immediate environment, were included in the study of which 94% (n=47) were shown to be infected with methicillin resistant *Staphylococcus aureus* using conventional microbiological techniques. The remaining three gave inconsistent results. Their isolates were obtained, DNA was extracted and subjected to the multiplex PCR assay. The PCR results indicated that both the *mecA* and *spa* genes were present in 72%

(n=36) of these isolates, indicative of MRSA strains. In 20% (n=10) of the isolates, only the *spa* gene could be detected; suggesting that these cannot be classified as being methicillin resistant. The *pvl* gene could not be detected in any of the isolates tested. A total of four isolates (8%) yielded results that were inconsistent with being MRSA using molecular identification. Overall there was a good correlation between genotypic analysis by PCR and phenotypic determination using *S. aureus* species identification and susceptibility testing methods. The multiplex PCR assay had a detection limit of 2.18×10^8 colony-forming units (cfu)/ml. This detection limit is higher compared to other published molecular identification techniques used for *Staphylococcus aureus* but sensitive enough for the accurate detection of MRSA in overnight cultured isolates. Results suggest that the current PCR assay could be used as a supplementary diagnostic method in the routine diagnosis for rapid, sensitive, and specific detection of *S. aureus* and its associated antibiotic resistance genes in equine samples.

INTRODUCTION

Staphylococcus aureus is a coagulase-positive, Gram-positive, coccal bacterium. It is one of the leading causes of both skin and invasive infections in humans (Weese 2010, Maddox et al. 2010). Clinical symptoms in humans include abscesses, various pyogenic infections including endocarditis, septic arthritis and osteomyelitis. There is also as risk of food poisoning caused by the production of an enterotoxin and toxic shock syndrome where the toxin enters the bloodstream and resulting in toxæmia. *Staphylococcus aureus* is a major cause of hospital acquired pneumonia, septicaemia and surgical wound infections in humans. Kawasaki syndrome resembles toxic shock syndrome in humans and is caused by super antigens of *S. aureus* (Levinson 2006). It may be a potential pathogen in animals that can cause pyogenic conditions of which the major one in livestock is mastitis in cattle, sheep and goats. *Staphylococcus aureus* is also responsible for tick pyaemia of lambs; chronic pyogranulomatous inflammation (botryomycosis) that can occur in horses, cattle and pigs; folliculitis and furunculosis in horses, goats and sheep; pyoderma in goats, piglets and cattle; staphylococcal dermatitis in sheep; polyarthritis in young animals; impetigo or subcorneal pustular dermatitis of piglets and dermatitis of the udder in goats (Henton 2004). It plays an important role in clinical diagnostics and treatment due to its ability to develop resistance to antimicrobial drugs. Methicillin-resistant *S. aureus* or MRSA is an important nosocomial pathogen in both humans and animals due to its resistance to all β -lactam antimicrobial agents (Weese, Rousseau 2005, Vincze et al. 2014). MRSA is associated with increased morbidity, mortality and healthcare costs. It was traditionally considered a hospital associated pathogen although it has expanded to community associated infections (Verkade et al. 2014).

Methicillin was developed in the 1960s to treat the influenza-like pandemic of penicillin-resistant *S. aureus* infections. Outbreaks were reported in Australia, the United States and the United Kingdom in the late 1950s. Resistance to methicillin was observed in strains of *S. aureus* only 6 months after its appearance on the pharmaceutical market (Tokateloff et al. 2009). It has since become an even more important pathogen that needs to be detected as early as possible in order to prevent further nosocomial spread, either from human to human, from human to animal or from animal to human. Methicillin-resistant *Staphylococcus aureus* is a very important pathogen in humans and is also an emerging concern in veterinary medicine and animal production (Weese 2010, Loncaric et al. 2014).

In the Netherlands during 2003, MRSA emerged in pigs and cattle and was then classified as livestock-associated MRSA (LA-MRSA). Healthcare guidelines were adapted so that all human patients that came in contact with live pigs or cattle were screened for MRSA upon hospital admission. Transmission of healthcare-associated MRSA (HA-MRSA) between patients and their household members were described in several studies with transmission rates up to 36%. It has also been suggested that incomplete cooking of contaminated meat may cause transmission of MRSA (Verkade et al. 2014).

Suspected transmissions between humans and small animals or horses have been documented several times in the past. Evidence of increased infection rates in horses, dogs and cats has been described in recent decades (Vincze et al. 2014). Equine MRSA infections were documented from Asia, North America, Australia and Europe. Even though there are limited data available for the prevalence of MRSA in horses, the predominant infection site in horses seems to be wounds and surgical sites (Walther et al. 2009). A study on the colonization of MRSA in nasal swabs from horses done in Ontario and New York showed a prevalence of 4.7% in horses on local farms. Hospitalization and prophylactic use of penicillin are viewed as a risk factor for an increase in the prevalence of multi-drug resistant commensal *S. aureus* in horses. The epidemiology of MRSA in horses is poorly understood and the lack of information regarding virulence and risk factors for resistance development of *S. aureus* poses a problem. Genetic typing may give us insight into the molecular composition of MRSA strains from different species (Walther et al. 2009).

There are quite a few reported incidences of MRSA infections in horses worldwide (Weese, Rousseau 2005, Tokateloff et al. 2009, O'Mahony et al. 2005, De Martino et al. 2010a, Bergstrom et al. 2012a, Axon et al. 2011, Busscher, van Duijkeren & Sloet van Oldruitenborgh-Oosterbaan 2006, Burton et al. 2008). It also seems that a portion of healthy dogs, cats, rabbits, cattle, pigs, poultry and horses carry MRSA (Weese 2010, Weese et al. 2005, Slater 2005). Companion animals, like horses, have been reported to be carriers of MRSA without showing clinical signs (Weese, Lefebvre 2007, De Martino et al. 2010b). This is referred to as the colonization of MRSA in its host.

The colonization of MRSA in animals poses not only a risk for transmission to other animals but also the risk of transmitting it to humans. This may lead to disease in susceptible animals and humans (Weese, Rousseau 2005, Weese et al. 2006). There is

clear evidence that MRSA can be spread between animals and humans and vice versa (Weese 2010). In a report published in 2008 following the European conference on MRSA, it was concluded that MRSA is a growing problem in both animals and humans and that urgent attention is required from both veterinary and public health sectors (Anonymous2008).

Colonization of MRSA in horses represents a big concern. This is considered a risk factor for development of disease in horses admitted to veterinary hospitals. Colonized horses can also be a source of zoonotic MRSA infections (Weese, Lefebvre 2007). As an example, a 24-h-old thoroughbred foal was admitted to a veterinary teaching hospital in February 2004 for evaluation and treatment of acute renal failure and septicaemia. Part of the infection control protocol included the taking of nasal swabs for screening of MRSA. The Foal Watch program was part of the disease management in the admitted foal. The Foal Watch consisted of personnel, mainly university students, providing 24 h nursing care to individual foals in 4 h shifts. The shifts involved students sitting in direct contact with the foals for the whole period of the shift. The foals were often partially on the students lap in order to provide proper restraint, feeding and provision of nursing care. On day 6 of hospitalization, the presence of MRSA isolated from the nasal swab was reported. MRSA-induced arthritis and omphalophlebitis had developed in the foal in the interim. Numerous Foal Watch personnel reported skin lesions seven days after the foal was admitted. Foal Watch students were contacted and asked to report to the University of Guelph Student Health Services for evaluation of skin lesions, if present. Nasal and groin swabs were collected and screened for MRSA. Three individuals were diagnosed with MRSA skin infections. A further 10 of 103 individuals that were screened, were found to be colonized by MRSA (Weese et al. 2006).

Polymerase chain reaction (PCR) assays have been available for quite a few years and revolutionized the approach to pathogen detection in diagnostic pathology laboratories. It enabled early detection of many pathogens resulting in effective treatment of infected individuals. MRSA detection based on a PCR reaction is also commonly used and various types of PCR-based assays were developed to assist in early detection of MRSA (Anderson, Weese 2007, Eigner et al. 2005, Buhlmann et al. 2008). In some cases a PCR based method that shows good results with human specimens may yield poor results with animal specimens (Anderson, Weese 2007). This created the need for a study to compare a published PCR-based method for MRSA (Larsen, Stegger & Sorum 2008) and the

conventional identification techniques for MRSA to evaluate the correlation and optimize the PCR-based test to enable early detection of MRSA in animal samples.

The sooner MRSA is detected, the sooner one can make informed decisions regarding infection control measures to be taken in the case of positively identified horses. This represents the main justification for optimizing a PCR-based screening method for MRSA detection in horses admitted to equine hospitals. Rapid results from a PCR-based test will assist in deciding whether a horse should be admitted to the hospital or treated as an out-patient.

The main aim of the study was to compare the current conventional microbiological techniques used for identification of MRSA strains from horses with a previously described multiplex PCR assay (Larsen, Stegger & Sorum 2008) for the rapid and accurate identification of MRSA in horses admitted to the Onderstepoort Veterinary Teaching Hospital, University of Pretoria.

LITERATURE REVIEW

1. General characteristics

Staphylococcus spp. is classified in the family *Micrococcaceae*. They are Gram-positive cocci and responsible for suppurative diseases (abscesses, mastitis, pyoderma etc.) in animals and humans (Coetzer, J.A.W. & Tustin, R.C. 2004). It is estimated that 20-30% of the human population are carriers of *Staphylococcus aureus* (Plata, Rosato & Wegrzyn 2009). There are 35 recognized species but only *S. aureus*, *S. epidermidis*, *S. intermedius* and *S. hyicus* are significant in livestock diseases. They are 0.8-1µm in diameter, non-motile, facultatively anaerobic, catalase-positive and oxidase-negative (Coetzer, J.A.W. & Tustin, R.C. 2004, Plata, Rosato & Wegrzyn 2009). In exudate, they form cluster-like organizations similar to a bunch of grapes. White to yellow-orange, butyrous colonies can be seen on conventional agar media (blood tryptose agar, nutrient agar). Pigment production is due to the production of carotenoid pigment. Alpha and beta haemolysins produced by certain staphylococcal species e.g. *S. aureus* may cause a zone of haemolysis on blood agar plates (Coetzer, J.A.W. & Tustin, R.C. 2004, Plata, Rosato & Wegrzyn 2009)

They form part of the normal flora on the skin and mucous membranes of healthy humans and animals. They are also, to a lesser extent, environmental bacteria. Risk factors for lesions in the skin and mucous membranes include trauma, immune status, disturbances of the normal flora and whether or not the specific species produces certain toxins and enzymes (Coetzer, J.A.W. & Tustin, R.C. 2004).

Since the molecular identification of MRSA is not available in all laboratories, phenotypic tests remain the method of choice. The main disadvantage of phenotypic tests is the variable results that are obtained despite genetic homogeneity due to variable culture conditions that may include temperature and osmolarity of the medium. These heterogeneous phenotypes can complicate the rapid detection of MRSA with conventional methods. Conventional identification techniques include oxacillin and cefocitin disk diffusion tests, oxacillin agar screening and latex agglutination (Alipour, Ahmadi & Javadi 2014).

2. History of MRSA

The resistance to methicillin was discovered in the 1960s due to the acquisition of the *mecA* gene by *Staphylococcus aureus* (Loncaric et al. 2014, Deurenberg et al. 2007b). For many years methicillin resistant *S. aureus* or MRSA was seen as a pathogen in healthcare units and was thus called healthcare-associated MRSA (HA-MRSA). In the late 1990's it emerged into communities where exposure to proper healthcare were lacking. This led to community-associated MRSA (CA-MRSA) (Loncaric et al. 2014). *Staphylococcus* organisms are among the most resistant non-spore forming organisms. They are relatively heat resistant and are destroyed at a temperature of 60°C for 30 minutes. They tolerate common disinfectants better than other bacteria and are generally resistant to many of the commonly used antibiotics (Henton 2004).

The first isolation of MRSA occurred in 1961 in the UK (Deurenberg et al. 2007b). This strain (NCTC10442) harboured the staphylococcal cassette chromosome *mec* (SCC*mec*) type I gene. During 1982 another MRSA strain (N315) was isolated in Japan with SCC*mec* type II present (Deurenberg et al. 2007b). Following the rapid spread of these two strains, isolation of a third strain occurred in New Zealand during 1985 (Deurenberg et al. 2007b). This third strain harboured the SCC*mec* type III. MRSA strains with the SCC*mec* type IV were spreading during the 1990s. Only in the 21st century MRSA with SCC*mec* type V was described in Australia (Deurenberg et al. 2007b). The cassette's origin might be unknown but it is believed that the SCC*mec* may be from staphylococci other than *S. aureus*. *Staphylococcus sciuri* is the ancestor for harboring the penicillin-binding protein 2a (PBP2a) gene since there is an 87.8% homology of amino-acids found in a penicillin-binding protein (PBP) from *S. sciuri*. These strains were all susceptible to methicillin. Resistance to methicillin was only observed upon growing the isolates in the presence of methicillin. This was due to an increase in the transcription rate of the gene *mecA* subsequent to a point mutation in the promoter. The introduction of the *mecA* homologue in a methicillin-sensitive strain of *S. aureus* (MSSA) resulted in MRSA. An epidemic MSSA strain and an isogenic MRSA strain were isolated from a neonate. This individual had no contact with any MRSA strains. The *mecA* gene was identical to that found in *Staphylococcus epidermidis* from the same patient. Horizontal *in vivo* transfer of the gene between the two species led to MRSA isolation from the neonate who never had contact with MRSA (Deurenberg et al. 2007b).

3. MRSA in animals and humans

Staphylococcus aureus is a commensal and pathogenic bacterium. Colonization increases the risk of infections due to the carrier acting as a reservoir until immunity is compromised and an active infection can occur. Usually the same strain carried as a commensal is the causative agent for disease (Plata, Rosato & Wegrzyn 2009).

Methicillin resistant *S.aureus* (MRSA) has emerged in a variety of animal and human populations through different mechanisms. The close contact between animals and humans facilitate the potential exposure of animals to human organisms (Weese 2010). MRSA transfer between animals and humans gained specific interest in recent years. The most common strain identified is that of MRSA multi locus sequence type (ST) 398 belonging to the clonal complex (CC) 398. It is the most common strain found in livestock and is also known as livestock associated MRSA (LA-MRSA) (Loncaric et al. 2014). Antimicrobial use in animals is presumed to be a facilitator for the spread of MRSA. Mechanisms, risk factors and other information about MRSA emergence in animals are poorly understood. Exposure of household pets to MRSA was bound to happen with an increase in community associated MRSA (CA-MRSA) in humans. A study done by Hanselman et al. in 2009 demonstrated that concurrent colonization of people and their pets with indistinguishable methicillin susceptible *S. aureus* is not uncommon (Hanselman et al. 2009). The potential for MRSA transmission between humans and their pets cannot be excluded (Weese 2010).

The first case of MRSA in animals was reported from cases of mastitis in dairy cattle during 1972 in Belgium (Devriese, Damme & Fameree 1972). Sporadic observations in other animals, including postsurgical wound infections in horses were reported. The clonal complex CC398 was associated with livestock and became known as the livestock-associated MRSA (LA-MRSA) strain. It is predominantly an asymptomatic nasal colonizer. MRSA CC398 is prevalent as a nosocomial pathogen in veterinary clinics, more specific in those for horses in Austria, Belgium, Germany, the Netherlands, Switzerland and United Kingdom (Cuny et al. 2016).

MRSA has been isolated from most domestic animals. *S. aureas* lineages from human origin such as CC1, CC8, CC22 and CC45 can be associated with MRSA in horses, cats and dogs. Livestock strains of MRSA however belong to non-human *S. aureas* lineages such as

CC398, CC97, CC9 and CC151 (Loncaric et al. 2014). MRSA is an emerging equine pathogen. Initially, MRSA in horses were reported as sporadic infections normally originating in a veterinary hospital setup. However, increased reports of MRSA in private practices and the community are seen (van Duijkeren et al. 2010). There are interesting comparisons and differences between MRSA in horses and household pets. The first report of a MRSA outbreak in horses was reported 1999 at a veterinary hospital in the USA (Cuny et al. 2016) . It was followed by a description of clusters of MRSA infections in equine hospitals in Canada and in Central Europe a few years later (Cuny et al. 2016). Colonization of a small percentage of healthy horses with MRSA was identified. The same were true for pets. Most studies report colonization of up to 11% of horses on farms or upon admission to veterinary hospitals. The figure may be higher in specific groups and one farm reported 43% colonization of horses with MRSA (Weese 2010).

A risk factor for colonization before admission and becoming colonized during admission to veterinary hospitals include antimicrobial exposure. Other factors that can result in colonization upon admission include previous colonization of the horse, previous identification of colonized horses on the farm, admission to neonatal intensive care units and admission to a service other than surgery. Most colonized horses do not develop clinical infections. However, the colonization of the horse at the time of hospital admission is a risk factor for disease development. MRSA colonized horses also pose a risk for transmission to other horses, humans and potentially other animal species (Weese 2010).

MRSA colonization in horses is transient. In most cases the decolonization occurs naturally in the absence of reinfection. The strains involved with MRSA in horses differ from the strains found in household pets. Most reports for MRSA in horses involved ST8 or related strains, more specific the USA500/CMRSA-5 clone, *mecA*-carrying clonal complex (CC) 8 and CC398 (Weese 2010, Haenni et al. 2015). These are human epidemic clones but are currently not frequently found in people. Their prevalence in horses is disproportionate suggesting that the strain may be more adept than others for survival in horses. Less frequently encountered human clones found in horses includes USA100/CMRSA-2 and ST398. There are different origins of MRSA sources of infection and routes of transmission in the horse population. The ST8 strain originally from human origin may be horse adapted and endemic due to its predominance in horses and horse personnel. Sporadic infections may be the result of more common human epidemic clones (Weese 2010, van Duijkeren et al. 2010). Van den Eede et al (2009) and Witte et al (2007) described colonization and infection of horses with MRSA ST398 (Van den Eede et al. 2009, Witte et al. 2007) which is

normally associated with livestock infections and colonization. This strain transmission could be the result of veterinarians working on farms that has livestock and horses (van Duijkeren et al. 2010).

Outbreaks of clinical MRSA infections may occur and sporadic infections or outbreaks are the most common. This is true for both veterinary hospitals and in the community where a wide range of opportunistic infections may accompany the MRSA infection. Joint incision and skin or soft tissue infections are frequently seen in community-onset cases whereas surgical site infections are predominantly seen in hospitalized horses (Weese et al. 2005, van Duijkeren et al. 2010). Pneumonia, metritis, omphalophlebitis, sinusitis, sepsis, invasive device infections, osteomyelitis, tenosynovitis and mastitis are other possible infections that can be seen. The severity of the disease is quite variable. Clinical signs seen can be mild and superficial to aggressive and life threatening. There is no evidence to suggest that MRSA infections have a different clinical presentation than methicillin sensitive *S. aureus* (MSSA) infections. Risk factors for infection with MRSA need further investigation as opposed to studies involving risk factors for colonization. There is clear evidence of interspecies transmission of MRSA between horses and humans resulting in infections or colonization. This is especially true for horse owners and equine veterinarians where a higher incidence of MRSA colonization is seen. One study reported figures as high as 13% of colonization in horse owners with the presence of one colonized person on every farm that had one or more colonized horses with an indistinguishable strain between humans and horses (Weese et al. 2005).

Veterinary personnel are exposed to an occupational risk of horse contact. The strains identified in veterinary personnel for equine and food animal contact have been identified as ST8 or related strains. It suggests an equine origin due to the low prevalence of this strain in human populations. MRSA colonization of veterinary personnel especially equine veterinary personnel can be limited by proper hand hygiene. There are limited reports of the zoonotic potential of MRSA in veterinary personnel but an occupational risk is present (Weese 2010). There is mutual transmission between humans and horses where MRSA is a nosocomial problem in horse clinics (Cuny et al. 2016).

4. Molecular epidemiology of MRSA SCCmec

The presence of the *mecA* gene is responsible for the resistance of *S. aureus* to methicillin. The *mecA* gene encodes the 78-kDa PBP2a (Cuny et al. 2010, Deurenberg et al. 2007a,

Turlej, Hrynewicz & Empel 2011). The PBP in the cell wall of the organism is the target for β -lactam antibiotics causing a disruption in the synthesis of the peptidoglycan layer which ultimately leads to the death of the organism. The β -lactam antibiotics cannot bind to the PBP2a and thus the synthesis of the peptidoglycan layer and cell wall synthesis can continue. The MecI and the trans-membrane β -lactam-sensing signal transducer MecRI are responsible for the regulation of the *mecA* gene. The MecI represses the transcription of both *mecA* and *mecRI-mecI* when there are no β -lactams present. A metallo-protease becomes active in the presence of a β -lactam through the MecRI being cleaved autocatalytically. This metallo-protease cleaves MecI that is bound to the operator region of *mecA*. This leads to the production of PBP2a through the transcription of the *mecA* gene (Deurenberg et al. 2007b).

The *mecA* gene is 2.1-kb in size. It is located on a mobile genetic element known as the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) (Deurenberg et al. 2007b, Turlej, Hrynewicz & Empel 2011). There are 5 types of SCC*mec* and they range in size from 20.9-66.9-kb (fig 1.)

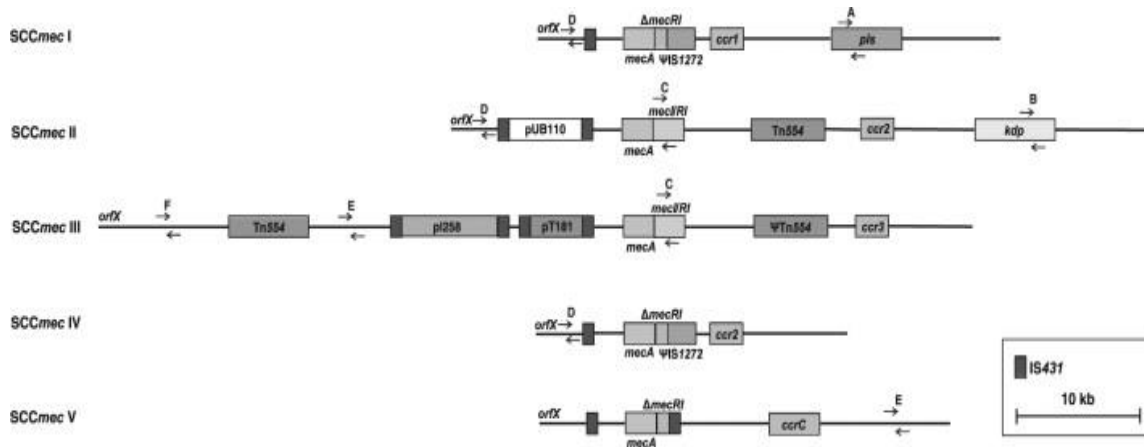


Figure 1: Schematic arrangement of SCC*mec* types I–V. The major elements of the five SCC*mec* types (*ccr* genes, *IS431*, *IS1272*, *mecA*, *mecI/RI*, *orfX*, *pI258*, *pT181*, *pUB101* and *Tn554*) are shown, as are the six loci (A–F) used for SCC*mec* typing according to the method of Oliveira *et al* (Deurenberg et al. 2007b).

Only SCC*mec* types I, IV and V are responsible for β -lactam resistance while the SCC*mec* types II and III are responsible for multi-resistance. SCC*mec* types II and III contain additional drug resistance genes on integrated plasmids known as *pUB110*, *pI258* and *pT181*. A transposon, *Tn554*, also carries additional drug resistance genes. Plasmid *pUB110* carries the *ant(4')* gene and codes for resistance to kanamycin and tobramycin. Plasmid

pI258 is responsible for penicillin and heavy metal resistance while plasmid pT181 codes for resistance to tetracycline. The *ermA* gene carried on transposon Tn554 codes for resistance to macrolide type antibiotics, lincosamide and streptogramin. Other resistance genes may be present in *S.aureus* but does not form part of the SCCmec. These genes can be carried on other parts of the chromosome and on plasmids. Insertion sequences are also found in SCCmec e.g. IS431. Regulatory genes that regulate *mecA* transcription are found on the SCCmec e.g. Δ *mecRI* on SCCmec types I, IV and V or *mecRI* and *mecl* on SCCmec types II and III. These genes are found in *mec* complexes. There are currently 5 major classes of *mec* complexes namely class A-E (Deurenberg et al. 2007b).

All SCCmec elements share a similar backbone structure. The backbone structure consists of (i) *mec* complex, (ii) *ccr* gene complex which includes the cassette chromosome recombinase (*ccr*) genes and (iii) three regions bordering the *ccr* and *mec* complexes known as joining (J) regions or junkyard regions (Turlej, Hrynewicz & Empel 2011). Within the SCCmec elements, cassette chromosome recombinase (*ccr*) genes are located. These genes are responsible for encoding *ccr* that are used for integration or excision from the chromosome at a specific site. The genes are designated as follows:

- *ccrA1* and *ccrB1* in SCCmec type I
- *ccrA2* and *ccrB2* in SCCmec types II and IV
- *ccrA3* and *ccrB3* in SCCmec type III
- *ccrA4* and *ccrB4* in SCCmec type IV of MRSA strain HDE288
- *ccrC* in SCCmec type V

The SCCmec elements are also divided in regions and include the J1 region, ranging from the chromosome's right junction to the *ccr* genes; the J2 region, ranging from the *ccr* genes to the *mec* complex and finally the J3 region between the *mec*-complex and left extremity of the SCCmec. Other methicillin-resistant coagulase-negative staphylococci may also carry the SCCmec e.g. methicillin-resistant *Staphylococcus epidermidis* (Deurenberg et al. 2007b).

5. Modes of MRSA transmission

Households are a potential transmission point for MRSA and other staphylococci of veterinary and human clinical importance. This includes methicillin-resistant *Staphylococcus pseudintermedius*, *Staphylococcus schleiferi* and other coagulase-negative staphylococci.

Strain characteristics such as biofilm production and the presence of Panton-Valentine leukocidin have been implicated in persistent colonization in households. Increased prevalence of MRSA in household pets has also been reported specifically in dogs and cats. Speculation that the human epidemic is driving the veterinary epidemic is adopted by some researchers but transmission can occur in both ways between humans and many species of pets and food animals (Davis et al. 2012, Ward et al. 2014). Staphylococci are able to survive on environmental surfaces and this increases the likelihood of strains establishing populations that persist in household environments. As discussed previously, non-aureus staphylococci may play a role in horizontal transfer of genes e.g. genes involved in resistance. This is applicable to household settings as well (Davis et al. 2012).

5.1 Environmental survival of staphylococci

Organisms in the genus *Staphylococcus* may survive in dry environments for periods of at least a week to three months or longer (Davis et al. 2012, Bergstrom et al. 2012b). Factors aiding this survival include dust composition, temperature, humidity, surface material and strain which all might affect the longevity in households. Colonized and infected people and pets may shed bacteria into the environment via direct contact with surfaces, shedding of skin cells with adherent bacteria, aerosol discharge by cloud carriers and gastrointestinal routes. The main routes of transmission for *S. aureus* and other staphylococci are direct person-to-person contact although indirect routes of transmission might include environmental exposure through aerosols, settled dust, fomites and eating or handling contaminated meat (Davis et al. 2012, Petinaki, Spiliopoulou 2012).

There are various ways of staphylococcal introduction into households and they include human, pet and environmental pathways. Pathways where people are involved may include health care settings such as hospitals, home care and home nursing facilities, networks like travel, and communities such as day-care centers, schools and gyms. Animal and environmental pathways include veterinary health care settings, environments such as beaches and agricultural sites, food and animal communities like dog parks and dog day care centres. The household environment has generally been unrecognized as a reservoir for *S. aureus* because the focus was mainly on hospital and community transmission. Positive home environments have been implicated as a persistent source of recolonization of individuals after decolonization therapy. Results of a case-control study showed that household contamination with community acquired MRSA strain USA300 was associated with re-infection of patients suggesting household contamination has consequences for clinical disease. Households have high-intensity contact between the same individuals

resulting in transmission dynamics being different for a household setting compared to public settings (Davis et al. 2012).

5.2 Veterinary staphylococci

Staphylococcus aureus is a main concern in human populations and can spill over into pet populations but *S. pseudintermedius* and *S. schleiferi* are predominant in animals. Coagulase-positive staphylococci like methicillin-resistant *S. pseudintermedius* are a concern for skin infections in dogs and cats. Infections of people are rare but pet owners and veterinarians can become colonized. Usually the same strain is shared between humans and their pets. In an occupational cohort of veterinary dermatologists and their pets, four of 171 households had related staphylococcal strains in both people and pets. One strain was MRSA and three strains were that of methicillin-resistant *S. pseudintermedius*. An additional seven households had related strains in their pets only (Davis et al. 2012).

In one study done by Laarhoven et al. in 2011, the concurrent environmental contamination, human colonization and animal colonization with staphylococci were investigated (Laarhoven et al. 2011). Sixteen households were visited bi-annually and assessed for staphylococcal species previously exposed to methicillin-resistant *S. pseudintermedius*. Some animals were colonized with the same genetic strain but many were intermittently colonized or became negative. Household environments were positive even in the absence of concurrent human or animal colonization. This is a concern for recolonization of pets and people who were naturally cleared or cleared after treatment (Davis et al. 2012).

Pets are a source for transmission of resistant pathogens. Loeffler et al. (2005) reported the spread of the United Kingdom HA-MRSA strain, EMRSA-15, between pets, staff and environmental surfaces using PFGE (Loeffler et al. 2005). O'Mahony et al. (2005) also reported the spread of the same strain of MRSA recovered from pets and staff in veterinary clinics in Ireland (O'Mahony et al. 2005). HA-MRSA strain ST5-II was identified and reported in dogs and hospitalized humans in Korea in a study done by Kwon et al in 2006 (Kwon et al. 2006). These examples of transmission clearly indicated the transmission from animals to humans and vice versa. Furthermore a clone associated with community associated infections, and of hospital origin, was identified among dogs, cats and humans (Petinaki, Spiliopoulou 2012).

The majority of MRSA isolates identified in pets are clones causing human infections. It follows the epidemiology of the specific geographical area. Veterinary staff are colonized or infected with strains related to the animals in their care (Petinaki, Spiliopoulou 2012).

5.3 Horizontal genetic transfer in household staphylococci

MRSA, methicillin-resistant *S. pseudintermedius* and other staphylococci share the *SCCmec*. A case of genetic transfer has been reported where the *SCCmec* element from *S. epidermidis* to methicillin-susceptible *S. aureus* during antimicrobial treatment of a patient colonized with both, resulted in an MRSA strain. Many *SCCmec* elements of methicillin-resistant *S. pseudintermedius* are of a distinct type. Sometimes it is a combination of elements from *S. aureus* and other staphylococci. At least one *SCCmec* (type V) is largely homologous to the type in MRSA. In one case study a dog was colonized with methicillin-resistant *S. pseudintermedius*. This dog was not colonized with MRSA even though it was living with an MRSA case-patient (Davis et al. 2012).

In the presence of multiple staphylococcal species, horizontal transfer of resistance genes from *SCCmec* may occur simultaneously within the household microbial community. It must be mentioned that in one study it was shown that *SCCmec* types were different in methicillin-resistant *S. pseudintermedius* isolated from pets and methicillin-resistant coagulase-negative staphylococci isolates from patients in the same household. This suggests that there is no common source of *SCCmec* for household staphylococci (Davis et al. 2012).

Previous treatment with antimicrobials e.g. fluoroquinolones and cephalosporins, are associated with later colonization or infection with methicillin-resistant staphylococci in both people and animals. The potential for selections of drug resistance by antimicrobial use in *S. pseudintermedius* and potentially other staphylococci is of great concern. Widespread use of antimicrobials in kennels promotes methicillin-resistant *S. pseudintermedius* in dogs. When animals are infected with methicillin-resistant *S. pseudintermedius* or MRSA it could require the use of antimicrobials more commonly used in people and this may expose the household microbiome to these drugs. The household microbiome must be investigated for resistance genes. This includes the quantification of genetic transfer for drug resistance and other virulence genes (Davis et al. 2012).

Veterinary staff in equine hospitals are colonized or infected by strains that are of animal origin. However, these strains are not related to the strains found in non-veterinary staff in

the same region. MRSA strains from horses and horse handlers are different from those strains spread in the human population or pets (Petinaki, Spiliopoulou 2012).

5.4 Intervention strategies

The cleaning strategies in a hospital environment have been studied for the effective eradication of environmental staphylococci but decontamination strategies for households is under-studied. Household contamination poses a problem for health care workers in terms of colonization and recolonization. Household cleaning strategies include gaseous ozone, replacement of carpets, commercial steam cleaning, general disinfection of hard surfaces, replacement of mattresses and low temperature laundering of clothes (Davis et al. 2012, Lakdawala et al. 2011). In a hospital setting laundered clothing may become recontaminated within hours if exposed. This suggests more frequent laundering of clothes in households (Davis et al. 2012).

Lakdawala et al (2011) did a study on the effectiveness of low temperature laundry of healthcare workers' clothes. Nurses in the United Kingdom are expected to laundry their own uniforms at home. Most domestic laundry machines have a low-temperature washing cycle. The conclusion of the study was that laundry in a domestic environment with water temperatures of 60°C for 10 minutes is effective in reducing the bio-burden, including MRSA, by 7-logs or with the addition of a biological detergent or non-biological detergent on experimentally contaminated pieces of material. Ironing is sufficient in cases where uniforms become recontaminated with low numbers of predominantly gram negative bacteria (Lakdawala et al. 2011).

Effectiveness of household detergents like dishwashing liquid in the presence of food residue is questionable even though *S. aureus* might be susceptible to detergents in a laboratory environment. Sodium hypochlorite (2.1%) with a degreaser is effective against *S. aureus* on kitchen items. A combination of chlorine and quaternary ammonium-based detergents can be used for disinfection of multiple sites in the kitchen, bathroom and pet-associated areas. Some strains of *S. aureus* may contain mutations that can cause resistance to these detergents. Further to the above mentioned practices, good hand hygiene will contribute to less contamination of household sites (Davis et al. 2012).

Some households might require a combined effort of household decontamination and treatment of animals and humans. When people or animals are diagnosed with a

staphylococcal infection or colonization, household cleaning and laundering of human and animal bedding is recommended (Davis et al. 2012).

Decolonization of people includes short term treatment with the topical nasal antibiotic mupirocin and chlorhexidine (Davis et al. 2012, Gurieva, Bootsma & Bonten 2012). The use of topical mupirocin in combination with isolation precautions can reduce hospital associated MRSA disease incidence by 69.6% (Gurieva, Bootsma & Bonten 2012). This can temporarily eradicate MRSA but long-term effectiveness is still unclear. Exposure within the household or other places of human or animal contact may lead to recolonization (Davis et al. 2012). In a healthcare setting, decolonization of staff can be an effective intervention to control MRSA among patients (Gurieva, Bootsma & Bonten 2012).

Implementing barrier precautions for human patients with MRSA is the basis for intervention strategies and sometimes in combination with decolonization of carriage. The efficacy of patient isolation to control nosocomial spread of MRSA in high endemicity areas still needs further investigation. Healthcare staff is an important risk factor in nosocomial spread in a healthcare facility. As mentioned previously, proper hand hygiene is considered the key intervention to limit direct contact transmission. In cases where healthcare workers become colonized with MRSA e.g. in the nose or injured skin, they are a constant source of MRSA transmission. Proper hand hygiene will not clear persistent carriage (Gurieva, Bootsma & Bonten 2012).

Patient decolonization is more effective than patient isolation. Decolonization of persistently colonized healthcare workers is only effective if the patient acquisition of MRSA by colonized healthcare workers is a considerable proportion (e.g. >50%) (Gurieva, Bootsma & Bonten 2012).

If there is a persistent infection of dogs and cats with *S. aureus* and MRSA, one should consider effective treatment in conjunction with decolonization of people since pet owners are most probably the source. Pet infections should be handled on a case-by-case basis under veterinary supervision. Pet owners must be informed of good hygiene and wound care practices limit the environmental contamination and spread to people. Decolonization of pets without drug treatment is common. Transmission to people and the environment can be achieved by temporary contact isolation e.g. sleeping in a crate on a surface that can be disinfected or laundered rather than in bed with people. Contact precautions like social distancing and good hygiene practices for households with MRSA-positive pets are

recommended. In serious cases pets might have to be included in treatment regimens for the whole household (Davis et al. 2012).

The occurrence of infected horses can be costly and can result in suffering of affected animals. Factors that influence the impact of a disease include, number of animals infected, morbidity and mortality rates, treatment costs, ability to prevent further outbreaks or spread of the disease, loss of performance potential of infected animals, limits placed on movement of horses onto and off affected premises, zoonotic potential of the disease, public relations issues e.g. loss of confidence in the treatment facility when owners bring their animals for treatment and potential for litigation. The main purpose of a proper biosecurity program is to limit or prevent the spread or exposure of animals in the hospital to infectious agents (Traub-Dargatz et al. 2004).

Treatment decisions for horses infected with MRSA should be based on the infection severity and location of infection as well as antimicrobial susceptibility of the MRSA strain. *In vitro* antimicrobial susceptibility results are reliable. Fluoroquinolone susceptibility is frequently reported *in vitro* for horses infected with MRSA. The unpredictable response that fluoroquinolone treatment has in humans despite *in vitro* susceptible results should be considered in equine treatment strategies. It is important to base the treatment strategy on the antimicrobial susceptibility result in conjunction with infection site (ability of drug to penetrate the site), infection character (presence of organic debris), patient age and patient health status (e.g. renal compromise). It is important that the drug can reach the infection site and reach therapeutic levels without being inactivated by organic debris. Chloramphenicol treatment is currently the drug of choice for treating MRSA in horses. This is due to its efficacy, cost, oral route of administration and safety. Trimethoprim-sulfonamide resistance is common in horse isolates but when a susceptible isolate is present it is an excellent drug combination. Aminoglycoside may be effective but resistance is common. Local therapies like the topical application of mupirocin or fusidic acid could be effective in treating superficial infections. There is a concern developing resistance if these topical agents are used. Antiseptic solutions (e.g. 0.2% chlorhexidine digluconate or 1% acetic acid) may be useful. Adjunctive therapy can include surgery, joint lavage, or fluid therapy can be successful but depends on the location and severity of the infection (Weese 2009).

Rifampicin has been used as a systemic treatment for decolonization of people and animals. This approach is limited due to rifampicin resistance in staphylococci after treatment and its toxic effects. Routine use of rifampicin in the decolonization of pets is not recommended.

Effective treatment of canine pyoderma or decolonization of pets can be achieved using a 2% or 4% chlorhexidine scrub or shampoo. The use of topical and systemic antimicrobial drugs, avoiding β -lactams, based on individual culture and sensitivity reports are recommended under supervision of a veterinarian. Overuse or misuse of antimicrobial drugs can lead to drug resistance and for this reason it is recommended to use initial topical therapy such as chlorhexidine and tris-EDTA for decolonization of pets since no definitive studies for decolonization of pets exist. Veterinary intervention strategies are complicated due to the high prevalence of multidrug resistance among isolates of methicillin-resistant *S. pseudintermedius* (Davis et al. 2012).

6. Molecular typing

In order to have effective strategies against MRSA, it is necessary to have a thorough understanding of the dissemination and the epidemiology of MRSA. Various molecular typing techniques have been developed to assist in this area and include; pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), SCC*mec* typing and *spa* typing which involves the variable tandem repeat region of protein A (Deurenberg et al. 2007b, Cuny et al. 2016).

6.1 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis is still considered to be the gold standard when it comes to typing MRSA isolates. For the purpose of studying outbreaks and hospital-to-hospital transmission, PFGE is considered the most discriminative typing method. The purified chromosomal DNA is digested with restriction enzyme *Sma*I and the resultant fragments then separated by agarose gel electrophoresis. The patterns seen on the gel are interpreted using a system developed by Tenover et al. (1995). It involves the patterns being analyzed with the Dice coefficient and unweighted pair-group matching analysis settings (Tenover et al. 1995). A lot of effort and research has been done in order to create harmonious PFGE protocols and standardized nomenclature. The success of PFGE is limited when judged in accordance with its reproducibility, speed and cost of analysis. Drawbacks of this method are that due to the strict adherence to standardized protocols, common databases have only been done at national level and attempts to produce a common nomenclature have not been successful at international level (Deurenberg et al. 2007b).

6.2 Multilocus sequence typing

Multilocus sequence typing is an excellent tool when it comes to the investigation of the MRSA evolution. Seven *S. aureus* housekeeping genes are the basis for this technique. It involves the sequence analysis of 0.5-kb fragments from *arcC*, *aroE*, *glpG*, *gmk*, *pta*, *tpi* and *ygiL*. Each housekeeping gene is assigned distinct alleles according to a different sequence in the genes. Accordingly, each isolate is defined by the alleles of the seven genes. The result is an allelic profile or sequence type for each isolate. An example is the Iberian clone with the MLST profile 3-3-1-12-4-4-16. This isolate is defined as the ST247. Nomenclature of MRSA strains involves both the ST and SCC*mec* type e.g. ST247-MRSA-I. This means that it is the Iberian clone harboring the SCC*mec* type I. *Staphylococcus aureus* strains with 5 of the 7 housekeeping genes having the same sequence are grouped in the same single clonal complex. The ST with the largest number of single-locus variants is considered to be the ancestor of each clonal complex. Subgroup founders are defined as single-locus or double-locus variants of a founder of a clonal complex. The subgroups are usually prevalent in a population and may have subsequently diversified to produce its own set of single-locus and double locus variants. MLST is laborious and very time consuming (Deurenberg et al. 2007b).

6.3 SCC*mec* typing

There are currently four ways to characterize the SCC*mec*. The first method was developed by Oliveira and de Lencastre (2002) and involves a multiplex PCR for SCC*mec* types I-IV. In this PCR the *mecA* and six different loci on the SCC*mec* are detected (Oliveira, de Lencastre 2002). Another method is available where parts of the structure of the *mec* complex and *ccr* genes are amplified using PCR. The drawback is the inconsistency between these two methods when the same SCC*mec* type of MRSA was characterized. A real-time PCR has also been developed where the SCC*mec* types I-IV are characterized according to the *mec* complex and *ccr* genes. A multiplex PCR method for characterization of SCC*mec* type I-IV has also been developed by Zhang et al (2005). This method involves the detection of the *mecA* and a single locus on the SCC*mec* (Zhang et al. 2005). A single method for the classification of the SCC*mec* would be useful since all the previously mentioned methods involve detection of different structural properties for the SCC*mec*. Chongtrakool suggested in 2006 a universal method where the *ccr* genes are indicated by a number and the *mec* complex is indicated by an upper case letter (Chongtrakool et al. 2006). This method lead to SCC*mec* type 1A (type I), type 2A (type II), type 3A (type III), type 2B (type IV) and type 5C (type V). The difference in the J1 and J2-J3 regions are further

indicated as e.g. SCC*mec* type 2B.2.1 (type IVb). The *ccr* genes and J regions are numbered in chronological order according to their discovery (Deurenberg et al. 2007b).

6.4 *spa* Typing

The X region of the *S.aureus* protein A (*spa*) gene consists of a number of mainly 24-bp repeats. Its diversity is mainly due to deletions and duplications of repeats. In rare cases it may contain point mutations. Frenay et al (1996) developed a single-locus sequence typing method for this polymorphic region (Frénay et al. 1996). The fact that *spa* typing only involves sequencing of a single-locus makes it a very simple method compared to MLST. The discriminatory power however, lies between that of PFGE and MLST. With *spa* typing it is possible to investigate the molecular evolution as well as hospital outbreaks of MRSA. This is in contrast to MLST that only investigates the clonal evolution of MRSA. An added advantage of *spa* typing is that researchers may use their own sequencing platforms and analyze the resulting sequence chromatograms using dedicated software thus making decentralized typing of the *spa* gene accessible to most laboratories and not just to reference laboratories. Common nomenclature and comparisons with excellent quality of data are made possible with this method. There are two major systems for nomenclature used worldwide and this was described by Koreen et al. (2004) and Harmsen et al. (2003) (Harmsen et al. 2003, Koreen et al. 2004). The fact that there are two major systems for nomenclature makes it difficult to compare published data for *spa* typing due to differences in the two systems. Analysis of *spa* sequences in Europe mainly revolves around Ridom Staph Type software. Individual laboratory results for *spa* typing are synchronized via the internet with the central *spa* server found at web address <http://www.spaserver.ridom.de>. The server is curated by European SeqNet.org. This initiative ensures a universal nomenclature and public access to data. Up to date, the *spa* server database contains >1200 *spa* types. This consist of a combination of 100 *spa* repeats from >13000 isolates from 36 countries around Europe. The continuous collection of *spa* typing data for infection control purposes and developing electronic early-warning algorithms for the automatic detection of MRSA outbreaks in regions or hospitals endemic for MRSA but with heterogeneous *spa* types gives this method a further advantage over other methods. Several *spa* types can be found within one ST as determined by MLST. They do however remain within an assigned clonal cluster. A new clustering algorithm has been implemented into StaphType based upon repeat patterns (BURP). Cluster analysis based on *spa* types is made possible in this manner but future studies are recommended to determine its usefulness and compatibility with clonal complexes established by MLST (Deurenberg et al. 2007b).

7. Laboratory diagnostic methods

7.1 Oxacillin Disk Diffusion

Oxacillin disk diffusion (ODD) is the traditional method for detection of MRSA and recently cefoxitin disks have been reported to yield accurate results for predicting MRSA presence. In the ODD test, a sterile swab is dipped into a *S. aureus* suspension that meets McFarland standard suspension of 0.5. The suspension is inoculated onto Mueller-Hinton agar. Oxacillin disks of 1 µg concentration are applied in a sterile manner. The plates are incubated at 35°C for 24 h. After incubation the plates are observed and an inhibition zone of <10 mm in diameter indicates oxacillin resistance (Alipour, Ahmadi & Javadi 2014).

7.2 Cefoxitin Disk Diffusion

Cefoxitin disk diffusion (CDD) is based on the ODD method except for the use of cefoxitin disks instead of oxacillin disks. The concentration of the cefoxitin disks is 30 µg. Mueller-Hinton agar plates are inoculated with a *S. aureus* suspension equal to a McFarland standard suspension of 0.5. Cefoxitin disks (30 µg) are applied and the plates are incubated at 37°C for 18 h. Zone diameters are measured and inhibition zones ≤21 mm indicates oxacillin resistance (Alipour, Ahmadi & Javadi 2014).

7.3 PBP2 Latex Agglutination

Commercial kits for PBP2 latex agglutination e.g. Mastalex™ MRSA kit are available (Alipour, Ahmadi & Javadi 2014). The Mastalex™ MRSA kit relies on a sensitive and rapid slide agglutination assay. The kit detects PBP2a which is present in MRSA by means of latex sensitized with monoclonal antibody directed against PBP2a. Latex particles are coated with monoclonal antibodies to PBP2a which is extracted from suspected colonies. The agglutination reaction is monitored until it is visible with the eye.

7.4 Molecular Identification

Molecular identification of MRSA involves the extraction of the DNA. The extracted DNA is then amplified with a polymerase chain reaction assay. Primers for the *mecA*, *spa* and *pvl* genes are used and visualized with gel electrophoresis. There are three genes of interest in molecular identification of MRSA. They are the *mecA* gene, the *spa* gene and the *pvl* gene. The presence of the *mecA* gene is indicative of a methicillin resistant *Staphylococcus aureus* and the presence of the *spa* and *pvl* genes are useful in typing of the organism. Various typing methods are available and have been described in previous sections. In 2007 the *spa* typing replaced pulsed-field gel electrophoresis at the Statens Serum Institut (Copenhagen,

Denmark) as the main typing technique of MRSA (Larsen, Stegger & Sorum 2008). DNA-based identification and typing methods, as described in earlier sections, have shown to be more specific in distinguishing resistant from susceptible strains of *Staphylococcus aureus* compared to phenotypic identification methods. Pulsed-field gel electrophoresis remains the “gold standard” but it is costly, time-consuming and technically complex. (Saei, Ahmadi 2012).

As mentioned previously, the *mecA* gene is responsible for the resistance properties of *S. aureus* by encoding the gene responsible for penicillin-binding protein (PBP2a). The synthesis of the peptidoglycan layer in the cell wall can continue due to β -lactam antibiotics that cannot bind to the PBP2a. The presence of the *mecA* gene is thus used for disguising the resistant strains of *S. aureus* from those strains that are sensitive to methicillin.

Amplification of a hypervariable DNA region and digestion with endonuclease could be implemented as a preliminary screening method since it is rapid, simple and highly discriminatory (Saei, Ahmadi 2012). Protein A is a staphylococcal bacterial cell wall-associated molecule encoded by the *spa* gene. Protein A binds the constant region (Fc) of immunoglobulin G and impairs opsonisation by serum complement and phagocytosis by polymorphonuclear leukocytes. The *spa* gene is known to be heterogeneous in nature and thus suitable for interstrain variability (Saei, Ahmadi 2012). It is composed of approximately 2150-bp. Functionally distinct regions have been identified and are known as Fc binding region, X-region and a sequence responsible for cell wall attachment at the C-terminus. The X-region is polymorphic with a varying number of 24-bp repeats. Different strains of *S. aureus* have different numbers and sequences of individual repeats resulting in strain classification based on the fragment size. The number of repeats in the X-region of the *spa* gene is sufficiently stable to be used for epidemiological typing (Saei, Ahmadi 2012).

Community-acquired methicillin-resistant *S. aureus* (CA-MRSA) is responsible for causing disease in healthy individuals. The molecular basis for the advanced virulence is poorly understood but there is a strong association between CA-MRSA infections and the presence of an exotoxin, Pantone-Valentine leukocidin (PVL), which is encoded by the *pvl* gene. Increased strains with the *pvl* gene like pulsed-field types USA300 and USA400 lead to rapid emergence of CA-MRSA infections (Voyich et al. 2006). In young immunocompetent patients, *S. aureus* necrotizing pneumonia was associated with *S. aureus* strains that contained the *pvl* gene. Pantone-Valentine leukocidin is a staphylococcal synergohymenotropic exotoxin. It belongs to the pore-forming group of toxins. It causes lysis

of host defense cells (Genestier et al. 2005). Panton-Valentine leukocidin is considered a virulence factor that is important in the pathogenesis of *S. aureus* infections. At molecular level, the *pvl* gene is more prevalent in CA-MRSA compared to community-associated methicillin sensitive *S. aureus* (CA-MSSA) (Bocchini et al. 2006)(Bocchini et al. 2006).

For the purpose of molecular identification of MRSA the presence of the *mecA* gene and any of the variable size *spa* genes are necessary. The *pvl* gene is not a requirement before a strain can be classified as MRSA or MSSA. The *spa* and *pvl* genes are more commonly used for typing the organism in order to understand the epidemiology of a *S. aureus* infection.

MATERIALS AND METHODS

1. Sample collection

A total of 50 isolates (30 isolated from horses, 19 isolated from direct environment e.g. stables and one isolated from a rodent brain) were included in this study (Table 1). Of these, 47 were previously identified as methicillin resistant *Staphylococcus aureus* by the bacteriology laboratory of the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, Faculty of Veterinary Science. *Staphylococcus epidermidis* (Design Biologix culture field isolate), *Staphylococcus pseudintermedius* (Design Biologix field isolate), *S. aureus* (ATCC 6538) and *S. aureus* (ATCC 43300) were used as control organisms in this study. *Staphylococcus aureus* (ATCC 6538) was used as the negative control being sensitive to methicillin, *S. aureus* (ATCC 43300) was used as the positive control being resistant to methicillin, *S. epidermidis* was used as an additional control organism that does not contain any of the *spa*, *mecA* or *pvl* genes and *S. pseudintermedius* was used as an additional control that only contains the *mecA* gene.

Table 1: Origin of isolates

ORIGIN / SAMPLE TYPE	NUMBER OF ISOLATES
Equine:	30
Transtracheal aspirate	2
Incision swab	2
Nasal swab	23
Surgical wound swab	1
Urine	1
Exudate	1
Environment:	19
Stable	1
Stable floor and wall	1
Stable bars	3
Environmental sites	14
Other:	1
Rodent brain	1

2. Bacterial isolation and characterization

Isolates were provided as subcultures on blood agar plates by the bacteriology laboratory, DVTD. The isolates as well as the control organisms were sub-cultured upon receipt by using blood agar plates. The plates were inoculated and incubated at 37°C for 24 h to check the viability of all the cultures prior to storage at -80°C.

The freshly inoculated plates were removed from the 37°C incubator after 24 h and each sample was sub-cultured again by using one inoculation loop full of growth inoculated into 10 ml of tryptone soy broth. The broth cultures were incubated in a 37°C incubator for 48 h. The bottles containing the broth were visually inspected for growth after 48 h. It was thoroughly mixed before the broth cultures were stored in cryofreezer tubes at -80°C in aliquots of 2 ml for use. Cultures were stored without the addition of a cryofreezing agent e.g. glycerol.

3. Phenotyping

Retrospective results were obtained from bacteriology laboratory in the Department of Veterinary Tropical Disease at the Onderstepoort campus of the University of Pretoria. Isolates were not phenotypically tested again in this study. The phenotypic testing was previously done with swabs that were received being put in tryptone soy broth and incubated overnight. After incubation overnight a sample of the broth was inoculated on selective chromogenic agar plates and incubated overnight. The agar plates were visually inspected and suspected colonies were inoculated on blood and MacConkey agar plates to investigate the haemolytic properties of the suspected colonies. A staphylase test was done and inoculated on DNase with mannitol and purple agar with maltose. The isolates were then tested for antibiotic resistance using polymyxin B and oxacillin. If results were compatible with MRSA, the isolate was reported to be MRSA (personal communication Kapp, Jansen van Vuuren 2015).

4. DNA extraction

The DNA extraction was done as a two-step procedure using an initial enzymatic digestion followed by a final digestion of 10 min cooking at 99°C. This two-step procedure is an adaptation from the procedure used for coagulase-negative staphylococci as described by Alexopoulou (Alexopoulou et al. 2006). Samples were removed from -80°C storage and left at room temperature to thaw. The thawed samples were mixed thoroughly to get a

homogenous suspension of organisms and 200 µl were then transferred to a 200 µl PCR tube. It was centrifuged at 7000 rpm for 5 minutes (Eppendorf Centrifuge 5415R). The supernatant was carefully removed and the pellet washed with 200 µl nuclease free water followed by another centrifugation step at 7000 rpm for 5 minutes. The supernatant was carefully removed and discarded. The initial digestion step was started using the QuickExtract™ Bacterial DNA Extraction kit from Epicentre (United States of America). The pellet was resuspended in 100 µl of the QuickExtract™ Bacterial DNA Extraction Solution and 1 µl of Ready-Lyse Lysozyme Solution were added to the suspension and gently mixed in order to get proper dispersion of the lysozyme. The tubes were left at room temperature for 60 minutes. After 60 minutes, the tubes were put in a thermal cycler (Applied Biosystems, Veriti, 96-well) with a one-step cycle of 99.9°C for 10 minutes. After the 10 minutes the temperature was lowered to 4°C until the tubes were used for the amplification step.

5. Preparation of primer working stock solutions

Primer sequences were based on published data (Larsen, Stegger & Sorum 2008) and obtained from Inqaba Biotech and prepared as per instruction sheet received with each primer. The primers were stored at -20°C in aliquots of 20 reactions per tube. The *pvl* primers (*pvl-RP*, *pvl-FP*) were used at a concentration of 1 µM, the *mecA* primers (*mecA-p7*, *mecA-p4*) were used at a concentration of 0.45 µM and the *spa* primers (*spa-1514r*, *spa-1113F*) were used at a concentration of 0.18 µM (Larsen, Stegger & Sorum 2008). The primer sequences and expected band sizes are summarized in Table 2.

Table 2: Nucleotide primer sequences used for the amplification of the *mecA*, *spa* and *pvl* genes

PRIMER	NUCLEOTIDE SEQUENCE	AMPLICON SIZE (BP)
<i>mecA p4</i>	5'-TCCAGATTACAACCTTCACCAGG	162
<i>mecA p7</i>	5'-CCACTTCATATCTTGTAACG	
<i>spa-1113f</i>	5'-TAAAGACGATCCTTCGGTGAGC	165 - 600
<i>spa-1514r</i>	5'-CAGCAGTAGTGCCGTTTGCTT	
<i>pvl-FP</i>	5'-GCTGGACAAAACCTTCTTGGAATAT	80
<i>pvl-RP</i>	5'-GATAGGACACCAATAAATTCTGGATTG	

6. Optimized multiplex PCR conditions

PCR reaction tubes were prepared containing 25µl Multiplex PCR Master mix (Qiagen; HotStarTaq *Plus* Polymerase, 6 mM MgCl₂ pH 8.7, Ultrapure dATP, dCTP, dGTP, dTTP), 10 µl Q-solution (Qiagen), 5 µl DNA template (≤1 µg/50 µl reaction), 1 µM *pvl-RP* and *pvl-FP*, 0.45 µM *mecA-p4* and *mecA-p7*, 0.18 µM *spa-1514r* and *spa-1113f* and 1.68 µl nuclease free water for a total reaction volume of 50 µl (Larsen, Stegger & Sorum 2008). Amplification was performed in a Veriti 96-well thermal cycler (Applied Biosystems) with initial heat activation for 15 minutes at 94°C followed by 30 cycles of 30 seconds denaturation at 94°C, annealing for 60 seconds at 59°C, extension for 60 seconds at 72°C with a final extension of 10 minutes at 72°C. The temperature was lowered to 4°C until the PCR product was visualized. The PCR products were visualized using a 2% agarose gel. A total of 10 µl of PCR product was thoroughly mixed with 3 µl of loading dye and the gel was loaded with 10 µl per well. The gel was run at 75 V for 80-90 minutes.

7. Analytical specificity of the multiplex PCR assay

To determine the analytical specificity of the multiplex PCR assay, 5 µl (≤1 µg/50 µl reaction) DNA of other *Staphylococcus* species, including *Staphylococcus intermedius* and *Staphylococcus chromogens* was subjected to the assay. In addition, DNA from other organisms that are frequently found in the upper respiratory tract of horses was tested for this purpose (Table 3).

In addition, to ensure the integrity of the DNA and to eliminate possible false negative results, universal primers fD1 and rP2 (Weisburg et al. 1991) were used to amplify the bacterial 16S rDNA. The PCR reaction conditions were as described above; the primers were added at a concentration of 20 µM/50 µl reaction.

8. Analytical sensitivity of the multiplex PCR assay

To determine the lower limit of detection of the multiplex PCR assay, a 10-fold dilution series was prepared and subjected to the multiplex PCR assay. In short, methicillin resistant *S. aureus* was used and put in 2 ml PBS to a saturated level. A dilution series were prepared with dilutions from 10⁻¹ to 10⁻⁴ using 1.8 ml PBS and 200 µl specimen from each previous dilution. A volume of 100 µl were taken from each dilution and inoculated on a blood agar plate with a spreading technique in order to yield countable single colonies. A volume of 1 ml

were taken from each dilution and centrifuged at 5000 rpm for 3 minutes. The supernatant was discarded and the pellet used for DNA extraction and DNA amplification as described above. The detection limit of PCR for each gene was defined as the lowest concentration (cfu/ml) yielding a positive result.

Table 3: Isolates used for determination of analytical specificity

Organism	Origin
<i>Staphylococcus epidermidis</i>	Design Biologix Field Isolate
<i>Staphylococcus pseudintermedius</i>	Design Biologix Field Isolate
Methicillin sensitive <i>Staphylococcus aureus</i>	ATCC 6538
Methicillin resistant <i>Staphylococcus aureus</i>	ATCC 43300
<i>Klebsiella pneumoniae</i>	ATCC 700603
<i>Escherichia coli</i>	Design Biologix Field Isolate
<i>Streptococcus equi</i> subsp <i>equi</i>	Univ. Pretoria Field Isolate
<i>Staphylococcus chromogens</i>	Design Biologix Field Isolate
<i>Proteus vulgaris</i>	ATCC 8427
<i>Pseudomonas aeruginosa</i>	ATCC 27853
<i>Streptococcus pneumoniae</i>	ATCC 6303
<i>Micrococcus</i> spp	Univ. Pretoria Field Isolate
<i>Streptococcus equisimilis</i>	ATCC 12388
<i>Streptococcus equi</i> subsp <i>zooepidemicus</i>	ATCC 700400
<i>Rhodococcus equi</i>	Univ. Pretoria Field Isolate

9. Comparison of the performance of the multiplex PCR assay in relation to a monoplex PCR format

The optimized DNA amplification procedure was followed as described above except that a single set of primers per PCR reaction was used (i.e. in a monoplex format). Methicillin resistant *S. aureus* (n=5) and methicillin sensitive *S. aureus* (n=5) isolates were randomly selected and subjected to the monoplex and multiplex PCR assays.

RESULTS

A multiplex PCR assay targeting the *mecA*, *spa* and *pvl* genes of *Staphylococcus aureus* was evaluated for use to accurately identify methicillin resistant *S. aureus* (MRSA) in horses admitted to the Onderstepoort Veterinary Teaching Hospital, University of Pretoria. Results were compared to those previously obtained by conventional microbiological techniques.

1. Conventional Bacteriology Results

The conventional microbiological identification results were obtained retrospectively from the bacteriology laboratory in the Department of Veterinary Tropical Diseases at the University of Pretoria. Isolates were classified as MRSA based on the isolate being β -haemolytic, a staphylase positive result and resistance to polymyxin B and oxacillin. From the 50 isolates received, 47 (94%) were identified as being MRSA while three isolates (6%) yielded questionable results. The phenotypic results are summarized in Table 4.

2. Molecular Identification

Of the 50 isolates tested for the presence of the *mecA*, *spa* and *pvl* genes, 36 (72%) isolates were identified as MRSA strains based on the presence of the *mecA* and *spa* gene (Larsen, Stegger & Sorum 2008). The *pvl* gene is often of interest as a stable marker for the community-acquired MRSA strains as described earlier (Vandenesch et al. 2003). All 36 isolates showed the presence of the *mecA* and *spa* genes. None of the 50 isolates tested showed the presence of the *pvl* gene. Methicillin sensitive *S. aureus* were identified in 10 of the isolates where the presence of the *spa* gene was observed but no *mecA* gene detected. Four of the isolates yielded results that show no relationship to *Staphylococcus aureus*. The results for the molecular characterization of 50 suspected MRSA isolates are summarized in Table 4.

Table 4: Phenotypic results vs. molecular results

Sample type	Isolate number	Phenotypic results				Multiplex PCR results		
		haemolytic properties	staphylase test	antibiotic resistance: polymyxin B	antibiotic resistance: oxacillin	<i>mecA</i>	<i>spa</i>	<i>pvl</i>
Equine:								
Transtracheal aspirate	1	β-haemolytic	+	R	R	+	+	-
	7	β-haemolytic	+	R	R	+	+	-
Incision swab	6	β-haemolytic	+	S	R	+	+	-
	14	β-haemolytic	+	R	R	+	+	-
Nasal swab	8	β-haemolytic	+	R	R	+	+	-
	10	β-haemolytic	+	ND	ND	+	+	-
	11	β-haemolytic	+	ND	ND	+	+	-
	12	β-haemolytic	+	ND	ND	+	+	-
	13	β-haemolytic	+	ND	ND	+	+	-
	30	β-haemolytic	+	R	R	-	+	-
	31	β-haemolytic	+	R	R	+	+	-
	32	β-haemolytic	+	R	R	+	+	-
	33	β-haemolytic	+	R	R	-	+	-
	34	β-haemolytic	+	R	R	-	+	-
	35	β-haemolytic	ND	R	R	-	+	-
	36	β-haemolytic	+	R	R	-	+	-
	37	β-haemolytic	ND	R	R	-	+	-
	38	β-haemolytic	+	R	R	-	+	-
	39	β-haemolytic	+	R	R	-	+	-
	40	β-haemolytic	ND	R	R	+	+	-
	41	β-haemolytic	ND	R	R	-	+	-
42	β-haemolytic	+	R	R	-	+	-	
43	β-haemolytic	+	R	R	+	+	-	
44	β-haemolytic	+	R	R	+	+	-	

	45	β -haemolytic	+	R	R	+	+	-
	46	β -haemolytic	ND	R	R	+	+	-
Surgical wound swab	47	β -haemolytic	ND	R	R	+	-	-
Urine	48	β -haemolytic	+	R	R	-	-	-
Exudate	49	α -haemolytic	+	R	R	+	-	-
Environment:								
Stable (13-7)	9	β -haemolytic	+	R	R	+	+	-
Stable floor and wall (13-12)	2	β -haemolytic	+	ND	ND	+	+	-
Stable floor and wall (13-28)	4	β -haemolytic	+	ND	ND	+	+	-
Stable floor and wall (13-2)	5	β -haemolytic	+	R	R	+	+	-
Stable bars (13-12)	3	β -haemolytic	+	ND	ND	+	+	-
Environmental sites	15	ND	+	ND	ND	+	+	-
	16	ND	+	ND	ND	+	+	-
	17	ND	+	ND	ND	+	+	-
	18	β -haemolytic	+	ND	ND	+	+	-
	19	β -haemolytic	+	ND	ND	+	+	-
	20	β -haemolytic	+	ND	ND	+	+	-
	21	β -haemolytic	+	ND	ND	+	+	-
	22	β -haemolytic	+	ND	ND	+	+	-
	23	β -haemolytic	+	ND	ND	+	+	-
	24	β -haemolytic	+	ND	ND	+	+	-
	25	β -haemolytic	+	ND	ND	+	+	-
	26	β -haemolytic	+	ND	ND	+	+	-
	27	β -haemolytic	+	ND	ND	+	+	-
	28	β -haemolytic	+	ND	ND	+	+	-
	29	β -haemolytic	+	ND	ND	+	+	-
Other:								
Rodent brain	50	β -haemolytic	-	R	R	-	-	-

ND = Test not done

R = Resistant to antimicrobial agent

S = Sensitive to antimicrobial agent

3. Validation of the DNA Extraction method

To ensure the integrity of the DNA and to eliminate possible false negative results, universal primers fD1 and rP2 were successfully used to amplify a ~1500 bp fragment (Weisburg et al. 1991) of the *S. aureus* 16S rDNA (Figure 2).

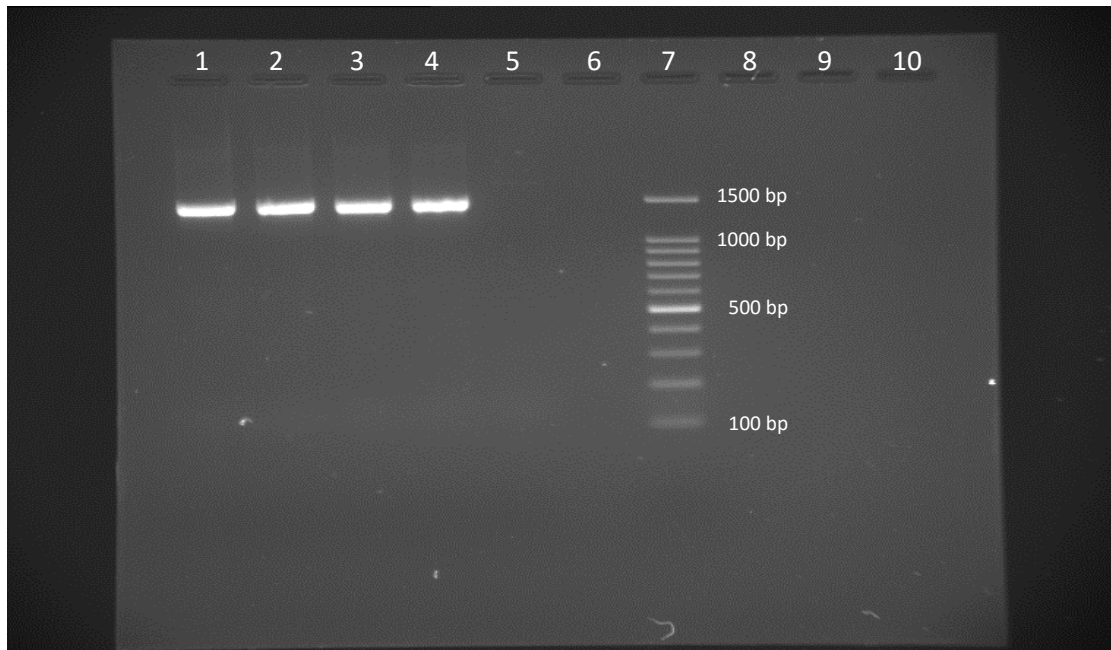


Figure 2: The presence of the eubacterial DNA using fD1 and rP2 primers. Lane 1: *S. epidermidis*; Lane 2: *S. pseudintermedius*; Lane 3: Methicillin sensitive *S. aureus*; Lane 4: Methicillin resistant *S. aureus*; Lane 5: Water; Lane 6: Not loaded; Lane 7: 100kb bp-Ladder

4. Analytical specificity of the multiplex PCR assay

To determine the analytical specificity of the multiplex PCR assay, DNA of other *Staphylococcus* species, as well as DNA from other organisms that are frequently found in the upper respiratory tract of horses (Table 3) was subjected to the multiplex PCR assay. None of the extracted DNA from these species cross-reacted with the primer pairs, showing that the primers were specific for identifying *S. aureus* isolates only (Figure 3).

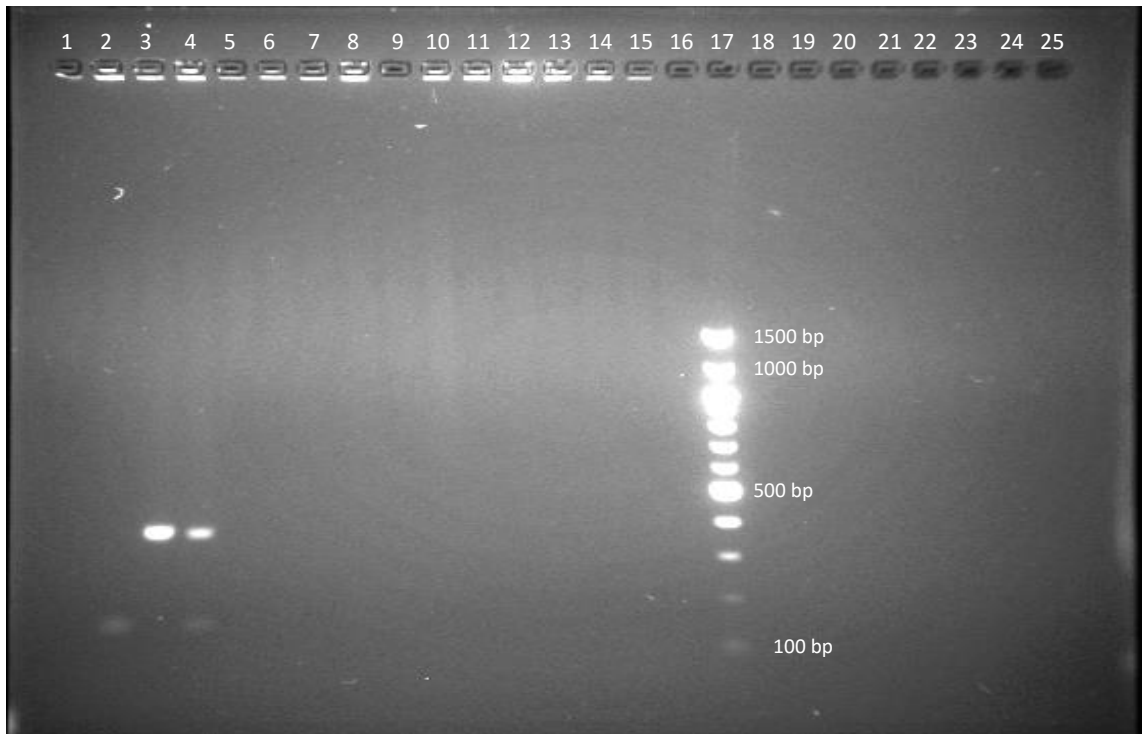


Figure 3: Analytical specificity results. Lane 1: *Staphylococcus epidermidis*; Lane 2: *Staphylococcus pseudintermedius*; Lane 3: Methicillin sensitive *Staphylococcus aureus*; Lane 4: Methicillin resistant *Staphylococcus aureus*; Lane 5: *Klebsiella pneumoniae*; Lane 6: *Escherichia coli*; Lane 7: *Streptococcus equi* subsp *equi*; Lane 8: *Staphylococcus chromogens*; Lane 9: *Proteus vulgaris*; Lane 10: *Pseudomonas aeruginosa*; Lane 11: *Streptococcus pneumoniae*; Lane 12: *Micrococcus* sp; Lane 13: *Streptococcus equismilis*; Lane 14: *Streptococcus equi* subsp *zooepidemicus*; Lane 15: *Rhodococcus equi*; Lane 16: Nuclease-free water; Lane 17: 100kb bp-ladder

5. Analytical sensitivity of the multiplex PCR assay

To determine the lower limit of detection of the multiplex PCR assay, a 10-fold dilution series was prepared and subjected to the multiplex PCR assay. The *spa* and *mecA* amplicons could reliably be obtained in the undiluted and at a dilution of 10^{-1} . This correlates to 2.18×10^8 colony forming units per milliliter. Since the *pvl* gene was not present in any of the test isolates or control isolates its detection limit cannot be determined in this study. The results for the undiluted and 10^{-1} diluted samples are depicted in Figure 4:

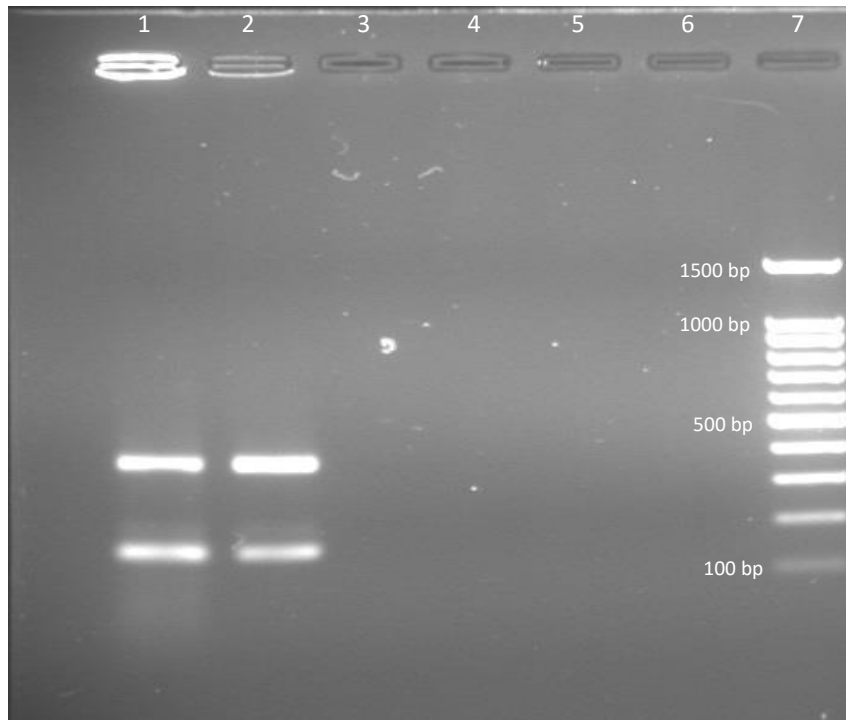


Figure 4: Dilution series of MRSA. Lane 1: Zero dilution; Lane 2: Decimal dilution 10^{-1} ; Lane 3: Decimal dilution 10^{-2} ; Lane 4: Decimal dilution 10^{-3} ; Lane 5: Decimal dilution 10^{-4} ; Lane 6: Nuclease-free water; Lane 7: 100kb bp-Ladder

6. Comparison of the performance of the multiplex PCR assay in relation to a monoplex PCR format

The results obtained from the multiplex PCR assay were in complete concordance with that of the *mecA*, *spa* and *pvl* PCRs being performed individually (monoplex). Results are shown in Figures 5 and 6:

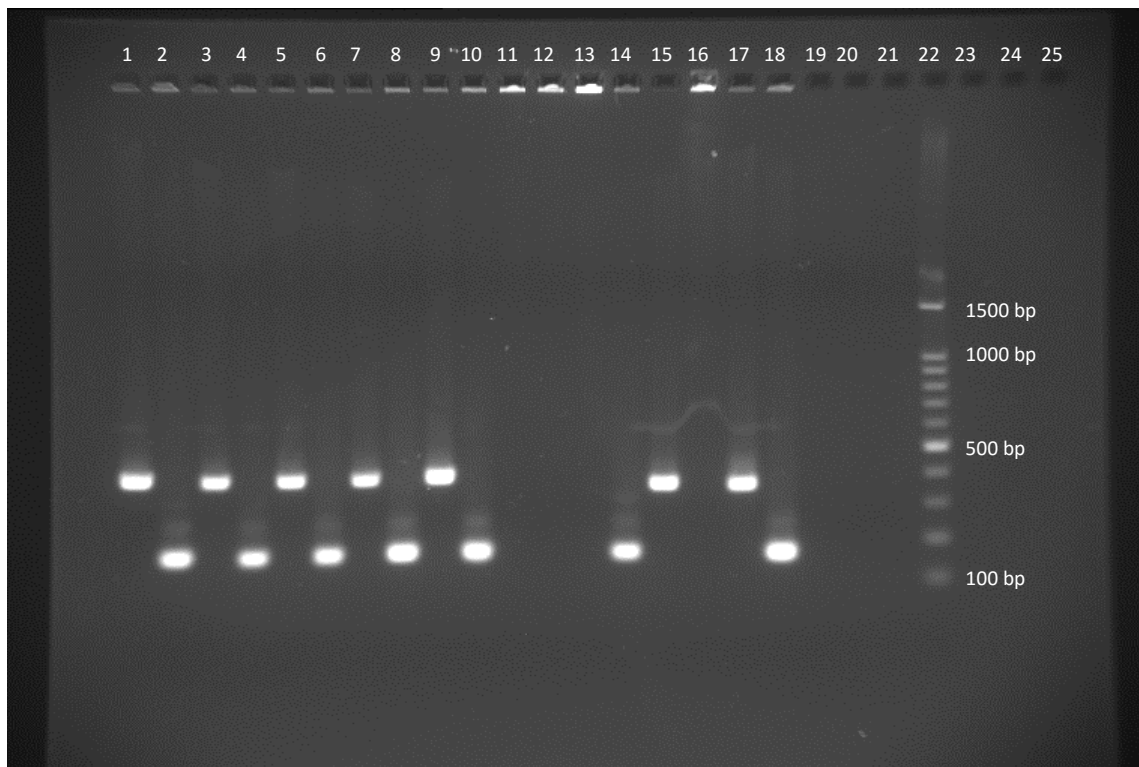


Figure 5: MRSA with presence of *spa* and *mecA* genes. The lanes should be interpreted in pairs e.g. Lane 1: MRSA with only *spa* primers and Lane 2: MRSA with only *mecA* primers etc. Lane 11: *Staphylococcus epidermidis* with *spa* primers; Lane 12: *Staphylococcus epidermidis* with *mecA* primers; Lane 13: *Staphylococcus pseudintermedius* with *spa* primers; Lane 14: *Staphylococcus pseudintermedius* with *mecA* primers; Lane 15: MSSA with *spa* primers; Lane 16: MSSA with *mecA* primers; Lane 17: MRSA with *spa* primers; Lane 18: MRSA with *mecA* primers; Lane 19: Nuclease-free water with *spa* primers; Lane 20: Nuclease-free water with *mecA* primers; Lane 22 100-kb-Ladder

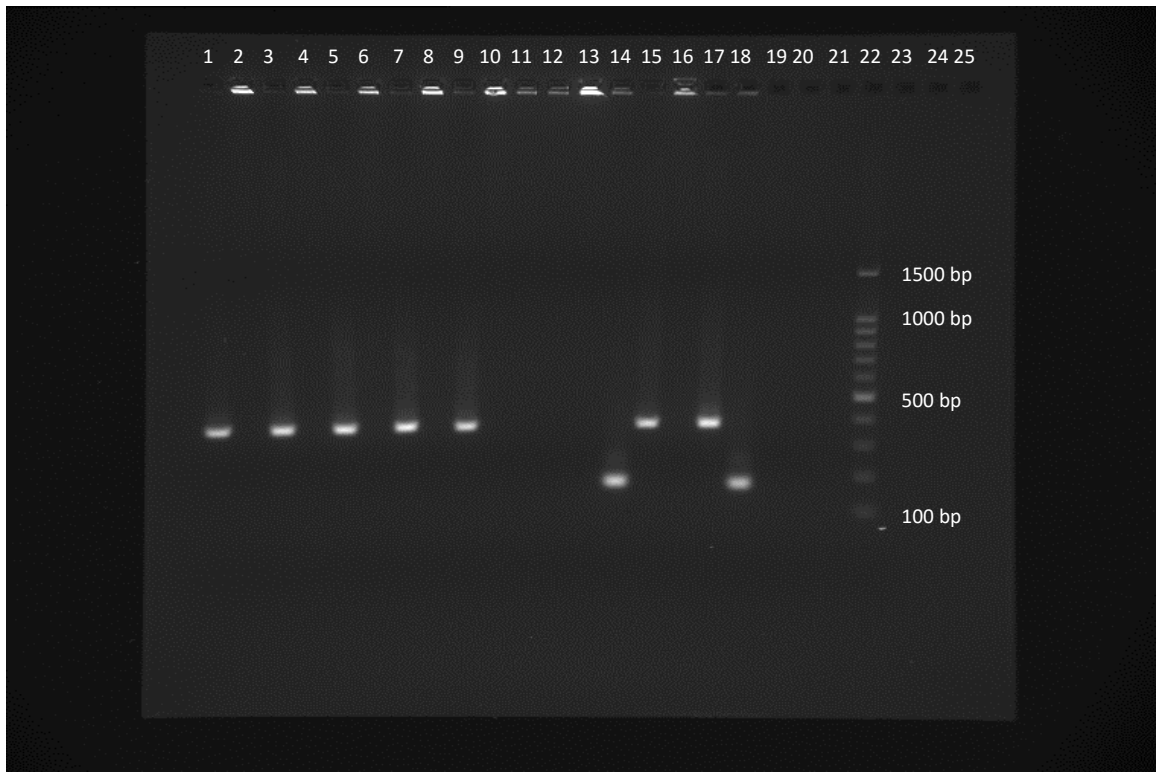


Figure 6: MSSA with the presence of only the *spa* gene. The lanes should be interpreted in pairs e.g. Lane 1: MSSA with only *spa* primer and Lane 2: MSSA with only *mecA* primer etc; Lane 11: *Staphylococcus epidermidis* with *spa* primers; Lane 12: *Staphylococcus epidermidis* with *mec A* primers; Lane 13: *Staphylococcus pseudintermedius* with *spa* primers; Lane 14: *Staphylococcus pseudintermedius* with *mecA* primers; Lane 15: MSSA with *spa* primers; Lane 16: MSSA with *mecA* primers; Lane 17: MRSA with *spa* primers; Lane 18: MRSA with *mecA* primers; Lane 19: Nuclease-free water with *spa* primers; Lane 20: Nuclease-free water with *mecA* primers; Lane 22 100-kb bp-Ladder

DISCUSSION

In this study, a multiplex PCR assay targeting the *mecA*, *spa* and *pvl* genes of *Staphylococcus aureus* was evaluated for use in the rapid and accurate identification of methicillin-resistant *S. aureus* (MRSA) in horses admitted to the Onderstepoort Veterinary Teaching Hospital, University of Pretoria. Results were compared to conventional microbiological identification results retrospectively obtained. A total of 50 isolates were included in the study of which 49 were of equine origin or isolated in the immediate environment of horses. One isolate was from a rodent brain specimen. Forty seven isolates were phenotypically identified as MRSA and three yielded inconclusive results.

Phenotypic testing is still widely used in various state and private laboratories as a means of identification of MRSA. This approach may yield inconsistent results due to operator variances and it is time consuming. A further drawback of phenotypic identification is the time taken from specimen collection until results become available. Bacteria such as *Escherichia sp.*, *Staphylococcus sp.* and *Streptococcus sp.*, can vary from 4-7 days before final results are available. For fastidious bacteria such as *Mycobacterium tuberculosis* it may take several months before final results including sensitivity tests become available.

Molecular techniques involve the genetics of a viable or non-viable biological system and have evolved in recent years to be one of the preferred means of identification. The main reason for this is the time from specimen collection to result availability. This can be anything from a few hours to a day or two. A further advantage is that it yields more specific and more sensitive results as the genetic material of a biological system, viable or non-viable, is investigated.

Various bacterial surface components and extracellular proteins play a role in the pathogenicity of *Staphylococcus aureus*. The precise role of single virulence factors in relation to an infection is still hard to determine (Melles, D.C., van Leeuwen, W.B., Boelens, H.A.M., Peeters, J.K., Verbrugh, H.A., van Belkum, A. 2006). During this study, the presence of the *mecA*, *spa* and *pvl* genes of *S. aureus* were investigated. As previously mentioned, the presence of the *mecA* gene and *spa* gene are indicative of a methicillin-resistant *S. aureus* and the presence of the *pvl* gene is useful in typing of the organism as it occurs in some MRSA isolates but not all MRSA isolates.

The *pvl* gene is more often found in community-associated MRSA (CA-MRSA) strains and it is distinguished from nosocomial MRSA by non-multidrug resistance and carriage of the type IV staphylococcal chromosome cassette element (SCC*mec* type IV). The *pvl* gene also plays a role in severe and invasive (soft tissue) infections but not with bacteraemia (Voyich et al. 2006, Melles, D.C., van Leeuwen, W.B., Boelens, H.A.M., Peeters, J.K., Verbrugh, H.A., van Belkum, A. 2006). The absence of the *pvl* gene in the tested isolates suggests that a nosocomial infection was present in the horses positively identified with MRSA infections. The result correlates with a study done by Weese et al. in 2000-2002 where nosocomial MRSA infections in horses and horse care personnel also showed the absence of the *pvl* gene (Weese et al. 2005)

The variable *spa* gene is also useful in the typing of MRSA and MSSA and in 2007 *spa* typing replaced pulsed-field gel electrophoresis at the Statens Serum Institut (Copenhagen, Denmark) as the main typing technique of MRSA (Larsen, Stegger & Sorum 2008).

The multiplex PCR requires at least 2.18×10^8 cfu/ml in order to yield interpretable results and thus an overnight culture would be the preferred starting material for a successful reaction. This detection limit falls within the specifications of the protocol required by QuickExtract™ Bacterial DNA Extraction kit from Epicentre (United States of America). The Qiagen® Multiplex PCR kit requires less than 1 µg DNA/reaction. Visible bands are still present using 2.18×10^8 cfu/ml. In a study performed in 2002, a detection limit of 10^5 cfu/ml was documented (Louie et al., 2002). A lower analytical sensitivity result was obtained in a study performed in 2015. This study used a multilocus fluorescence-based PCR assay with a detection limit of 4×10^3 cfu/ml (Huang et al. 2015). In another study the detection limit varied between 10^3 - 10^4 cfu/ml (Chandrashekhar et al. 2015). The analytical sensitivity of isolates used in this study was 2.18×10^8 cfu/ml. This is higher than expected compared to other molecular identification techniques.

There is no cross-reactions between primers in a multiplex PCR reaction compared to a monoplex PCR reaction. This will also save considerable time as only one PCR needs to be done instead of multiple monoplex reactions.

Organisms that are frequently found in the upper respiratory tract of horses include *Streptococcus equi subsp. zooepidemicus*, *Micrococcus spp.*, *Corynebacterium spp.*, *Staphylococcus intermedius*, *Staphylococcus aureus*, *Bacillus spp.*, *Streptococcus pneumoniae*, *Staphylococcus chromogens*, *Streptococcus equismilis*, *Pseudomonas*

aeruginosa, *Rhodococcus equi*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Streptococcus equi subsp. equi* (Mir et al. 2013). Other bacteria that is common in the upper respiratory tract of horses does not interfere with the PCR. An analytical specificity of 100% was achieved when other bacteria commonly found in the upper respiratory tract of horses were tested. This result correlates well with findings in a study performed in 1992 (Brakstad, Aasbakk & Maeland 1992). A 100% analytical specificity was also achieved by Huang in 2015 (Huang et al., 2015).

Molecular identification excluded the presence of the *mecA* in 10 specimens and thus it cannot be classified as being methicillin resistant since staphylococcal cassette chromosome *mec*, enables the expression of an additional penicillin-binding protein (PBP) 2a. This PBP2a substitutes an essential cross-linking step of the PBP2 in the presence of β -lactams and thus results in resistance to this group of antibiotics (Vincze et al. 2014). Another four specimens yielded results where the genus or species is questionable. Further investigation is suggested.

The results for this PCR based identification are available within 48-72 hours after the isolates are received. This is considerably less time compared to the 4-7 days needed for phenotypic identification.

Staphylococcus aureus is β -haemolytic organism. Even though the isolate from the rodent brain was phenotypically classified as MRSA it yielded a negative staphylase test and none of the required genes were present in the molecular identification. The identification of this isolate might need further investigation as it is highly unlikely to be *S. aureus*. From the three isolates that yielded inconclusive results with phenotypic testing one was α -haemolytic and two were β -haemolytic. Compared to the molecular profile of these specific isolates it can be concluded that it is highly likely to be other organisms and the identification needs to be investigated. The isolates lack the *spa* gene which is prominent in *S. aureus* strains.

β -haemolytic profiles were seen in the rest of the isolates except on three isolates where the phenotypic test was not done. All these isolates showed the presence of the *spa* gene which confirms the species to be *S. aureus*. Overall there was a good relation between polymyxin B and oxacillin resistance and the presence of the *mecA* gene. Eleven isolates yielded resistant results to these two antimicrobials but did not show the presence of the *mecA* gene which is an essential gene that encodes the penicillin-binding protein 2a that makes the organism resistant to β -lactam antimicrobials.

There was no correlation between the sample type/site versus the molecular resistance profile. Further typing of the *spa* gene might yield better epidemiological data related to specimen type/site and the resistance of the isolate. This study only used isolates that were retrospectively confirmed to be MRSA. Different results may be expected when previously untested isolates are used.

Molecular techniques for the identification of MRSA could be used as an additional confirmatory test when MRSA is phenotypically identified. This could assist in the infection control approach in a veterinary hospital situation.

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to obtain a rapid, more sensitive and robust method of identifying MRSA in horses in order to make quicker decisions regarding the treatment and handling of horses admitted to a veterinary hospital. This was achieved by molecular detection of the *mecA* and *spa* gene. The results were available faster and correlate well with the phenotypic identification methods.

Further investigation into the typing of the *spa* gene in these isolates is desirable but fell beyond the scope of this study. In order to investigate the presence of the *pvl* gene it is suggested that more isolates are tested and that the isolates are not limited to specimens from equine origin.

The implications of a questionable phenotypic result will greatly influence the decision making process in the veterinary hospital regarding infection control and for this reason it is recommended that any phenotypically identified MRSA be confirmed with a molecular identification method as well.

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APPENDIX



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	PCR-based screening for methicillin-resistant <i>Staphylococcus aureus</i> in horses admitted to a South African Veterinary Training Hospital
PROJECT NUMBER	V063-13
RESEARCHER/PRINCIPAL INVESTIGATOR	Mr. SJ Jansen van Vuuren

STUDENT NUMBER (where applicable)	970 149 83
DISSERTATION/THESIS SUBMITTED FOR	MSc

ANIMAL SPECIES	Horses	
NUMBER OF ANIMALS	To be reported	
Approval period to use animals for research/testing purposes	December 2013-July 2014	
SUPERVISOR	Prof. M van Vuuren	

KINDLY NOTE:

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

APPROVED	Date	28 October 2013
CHAIRMAN: UP Animal Ethics Committee	Signature	