METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN HORSES AND HORSE HANDLERS IN KADUNA AND ZARIA, NIGERIA.

BY

Aliyu ABDULKADIR, DVM (2008)

MSc/VET-MED/01464/2010-2011

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTERS OF SCIENCE IN VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE

DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE, AHMADU BELLO UNIVERSITY, ZARIA.

September, 2014

DECLARATION

I declare that the work in this project thesis entitled **METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN HORSES and HORSE HANDLERS IN KADUNA AND ZARIA, NIGERIA**, has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, under the supervision of Dr. J. Kabir and Dr. M. Bello.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project thesis was previously presented for another degree or diploma at this or any other institution.

Name of Student	**Signature**	**Date**

CERTIFICATION

This thesis entitled **'METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* IN HORSES and HORSE HANDLERS IN KADUNA AND ZARIA, NIGERIA'** by Aliyu ABDULKADIR meets the regulations governing the award of the degree of MSc Veterinary Public Health and Preventive Medicine of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Dr J. Kabir	_____	_____
Chairman, Supervisory Committee	Signature	Date
Dr M. Bello	_____	_____
Member, Supervisory Committee	Signature	Date
Dr E. C. Okolocha	_____	_____
Head of Department	Signature	Date
Veterinary Public Health and Preventive Medicine		
Prof. A. Z. Hassan	_____	_____
Dean, School of Postgraduate Studies	Signature	Date
Ahmadu Bello University		

DEDICATION

To grandma Iya and my aunt Fatima.

ACKNOWLEDGEMENT

My special gratitude goes to my supervisors Dr. J. Kabir and Dr. M. Bello who offered tremendous support and took time out of there busy schedules to offer very useful advice on all aspects of this work from the study design to the laboratory work and final write up. My very profound special gratitude and thanks goes to Professor J.K.P. Kwaga for his support even though not a member of the supervisory team. My appreciation goes to all the laboratory technologists who were always available and supportive whenever there was need.

I would like to especially acknowledge DNAlabs for providing the facilities for the molecular work; Zakari Imam for his technical assistance, support, and provision of primers for the multiplex polymerase chain reaction; Sumayya, Gloria, and Dr. Mustapha for their technical assistance and support.

I would lastly like to acknowledge the enormous contributions of Sa'adatu Muhammad Babangida, Hadiza Muhammad Sani, Salma Muhammad, and Bareerah Saidu.

ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) is of great public health concern having become resistant to most commonly used antibiotics. They are associated with institutions such as hospitals, but are now increasingly becoming prevalent in community-acquired infections. The occurrence of MRSA in horses and horse handlers in Kaduna and Zaria, Nigeria was studied. Nasal swabs from 240 horses and 65 horse handlers were collected through a one-stage cluster sampling and analysed using standard microbiological tests and genotyping methods. Structured questionnaires were also administered to 65 horse handlers to assess for risk factors associated with MRSA colonisation. MRSA were found in 10% (24/240) and 6.2% (4/65) of the nasal swab samples collected from the horses and horse handlers respectively. Of the 44 *S. aureus* isolates tested for antibiotic susceptibility, 54.6% (24/44) were resistant to penicillin and oxacillin, 47.7% (21/44) to erythromycin, 34.1% (15/44) to tetracycline, 22.7% (10/44) to amikacin and gentamicin, 6.8% (3/44) to sulphamethoxazole+trimethoprim, 2.3% (1/44) to ciprofloxacin, and 2.3% (1/44) were resistant to chloramphenicol. Multidrug Resistance was found in 74.8% (21/28) of the MRSA isolates. SCC*mec* types I, III, and IV were found in both the horses and the horse handlers while only one horse was found to carry SCC*mec* V. Risk factors identified for the horse handlers included being a Veterinarian, recent exposure to antimicrobial agents, hospital visitation, and personal hygiene. Risk factors for the horses from this study were allergy and wound management. MRSA-carriage by horses and horse handlers alike as well as MDR (multidrug resistant) strains of *S. aureus* have been established from this study, which is of public health concern.

TABLE OF CONTENTS

Title	Page
Title page	I
Declaration	II
Certification	III
Dedication	IV
Acknowledgement	V
Abstract	VI
Table of contents	VII
List of figures	XII
List of tables	XIII
List of plates	XIV
List of appendices	XV
Abbreviations	XVI

CHAPTER 1: INTRODUCTION

1.1 Background of Study----------1

1.2 Statement of Research Problem----------3

1.3 Justification----------4

1.4 Aim----------5

1.5 Objectives ----------5

CHAPER 2: LITERATURE REVIEW

2.1 History of *Staphylococcus aureus* ----------6

2.2 Importance of MRSA----------9

2.3 General Description of *S. aureus* ----------10

2.3.1 Staphylococcal cell envelope----------11

2.3.2 *Staphylococcus aureus* genome----------12

2.4 Classification of MRSA ----------13

2.4.1 Staphylococcal cassette chromosome *mec* elements----------15

2.5 Laboratory Detection and Identification----------18

2.5.1 Pathogenesis and virulence factors----------20

2.6 MRSA in Horses----------23

2.7 Antibiotic Resistance----------25

2.8 Emerging Methicillin Resistant *Staphylococcus aureus*----------28

2.8.1 Mechanism of methicillin resistance----------29

2.8.2 Risk factors associated with nosocomial MRSA----------31

2.8.3 Epidemiology of MRSA in community ----------32

2.8.4 Treatment and control of MRSA----------33

2.8.5 The typing of MRSA----------34

2.9 Mechanism of Genetic Variability of Bacterial Genome ----------34

2.10 Phenotyping Methods----------36

2.11 Genotyping Methods----------36

2.11.1 Pulsed field gel electrophoresis----------37

2.11.2 Multi-locus sequence typing----------37

2.11.3 Staphylococcal protein A sequence typing (*spa* typing) ----------37

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Area----------40

3.2 Study Design----------40

3.3 Sample Size Determination----------41

3.4 Sample Collection----------42

3.5 Materials Used----------42

3.5.1 Bacteriological culture media----------42

3.5.2 Sugars and Andrade peptone----------42

3.5.3 Antibiotic impregnated discs and their concentrations----------43

3.5.4 Other materials used----------43

3.6 Isolation of *S aureus*----------43

3.6.1 Brain heart infusion broth (BHI) ----------43

3.6.2 Columbia media with 5% sheep blood----------43

3.6.3 Baird Parker-egg yolk + tellurite media----------44

3.7 Biochemical Characterization----------44

3.7.1 Catalase test----------44

3.7.2 Coagulase test ---------- 44

3.7.3 Sugar fermentation----------45

3.8 Antibiotic Sensitivity Testing----------45

3.9 PBP2a Latex Agglutination Test----------47

3.10 Genotyping----------47

3.10.1 DNA extraction----------47

3.10.2 Multiplex polymerase chain reaction (M-PCR) ----------48

3.11 Identification of Risk Factors Associated with MRSA in Horses and Horse Handlers -----49

3.12 Data Analysis----------49

CHAPTER 4: RESULTS----------59

4.1 Isolation and Identification of MRSA from horses and horse handlers----------51

4.2 Antibiotic Susceptibilities of the MRSA Isolates from the Horses and Horse Handlers-----54

4.3 PBP2a latex agglutination test to confirm methicillin resistance----------58

4.4 SCC*mec* Typing----------60

4.5 Risk Factor Analysis----------62

CHAPTER 5: DISCUSSION----------63

CHAPTER 6: CONCLUSION

6.1 Conclusions----------67

CHAPTER 7: RECOMMENDATIONS

7.1 Recommendations----------68

References----------69

Appendices----------84

LIST OF FIGURES

Figure	Title	Page
Figure 2.1	Three dimensional structure of *S. aureus* peptidoglycan	12
Figure 2.2	The time line of the emergence of antibiotic resistance in *S. aureus*	27
Figure 2.3	The mechanism of β-lactam resistance in *S. aureus*	30
Figure 4.1	Resistance levels of various antibiotics for the MRSA isolates from horses and horse handlers.	56

LIST OF TABLES

Table	Title	Page
Table 2.1	SCC*mec* classification	17
Table 3.1	Antibiotic discs used and break points for each based on CLSI, 2010.	46
Table 3.2	Primers used in the multiplex SCC*mec* PCR and the resulting amplicon sizes for SCC*mec* types I-V.	49
Table 4.1	Prevalence of MRSA isolated by category of horses	52
Table 4.2	Prevalence of MRSA in horse handlers by category of horses.	53
Table 4.3	Antimicrobial resistance of MRSA isolates from horses and horse handlers	55
Table 4.4	Antibiotic resistance patterns of *S. aureus* isolates (n=44).	57
Table 4.5	PBP2a latex agglutination test using PBP2A latex agglutination test kit (Oxoid, United Kingdom)	59
Table 4.6	SCC*mec* typing result based on method described by Boye *et al.,* 2009.	61

PLATES

Plate	Title	Page
Plate 1	Gel electrophoresis of multiplex PCR for 10 MRSA isolates	62
Plate 2	Gel electrophoresis of multiplex PCR for 18 MRSA isolates	63
Plate 3	Colonies of *Staphylococcus aureus* on Baird Parker Egg Yolk with tellurite agar.	90
Plate 4	Sugar fermentation after 24hours incubation showing colour changes with pink being positive while the cloudy yellow being negative	91
Plate 5	Antibiotic sensitivity testing on Mueller Hinton agar showing zone of inhibition	92
Plate 6	PBP2a latex agglutination test	93

LIST OF APPENDICES

Appendix I Composition of media----------89

Appendix II Flow chart outline showing the isolation and characterisation of MRSA----92

Appendix III Plates of isolation, characterisation and sensitivity test----------93

Appendix IV Results of cultural isolation and biochemical characterisation----------97

ABBREVIATIONS

AP-PCR; arbitrarily primed polymerase chain reaction

bp; base pairs

CA; community associated

CC; clonal complex

ccr; cassette chromosome recombinases

CDC; Centers for Disease Control and Prevention

CMRSA; Canadian methicillin resistant *Staphylococcus aureus*

CoNS; coagulase negative *Staphylococci*

DNA; deoxyribonucleic acid

eBURST; electronic based upon related sequence type

EDTA; ethylene diamine tetra acetic acid

EMRSA; epidemic methicillin resistant *Staphylococcus aureus*

HA; hospital associated

HCW; health care worker

ICU; intensive care unit

IgG; immunoglobulin G

IS; insertion sequence

ISS; integration site sequence

IV; intravenous

LTA; lipoteichoic acid

MGEs; mobile genetic elements

MIC; minimum inhibitory concentration

MLEE; multi locus enzyme electrophoresis

MLST; multi locus sequence typing

MLVA; multi locus variable number tandem repeat analysis

MRSA; methicillin resistant *Staphylococcus aureus*

MSCRAMMs; microbial surface components recognizing adhesive matrix molecules

MSSA; methicillin susceptible *Staphylococcus aureus*

orf; open reading frame

PBP; penicillin binding protein

PCR; polymerase chain reaction

PF; plasmid fingerprinting

PFGE; pulsed field gel electrophoresis

PVL; Panton valentine leukocidin

SCC; staphylococcal cassette chromosome

Spa; staphylococcal protein A

ST; sequence type

TT; thymine dimers

UK; United Kingdom

USA; United States of America

VISA; vancomycin-intermediate *Staphylococcus aureus*

VNTR; variable number tandem repeat

VRSA; vancomycin-resistant *Staphylococcus aureus*

VSSA; vancomycin-sensitive *Staphylococcus aureus*

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Staphylococcus; meaning **t**he "golden grape-cluster berry," and also known as "golden staph" and *Oro staphira*) is a facultative anaerobic Gram-positive cocci bacterium. It is frequently part of the skin flora found in the nose and on skin, and in this manner about 20% of the humans are long-term carriers of *Staphylococcus*. (Kluytmans *et al.,* 1997)

Staphylococcus aureus can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils (furuncles), cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia, and sepsis. It is still one of the five most common causes of nosocomial infections, often causing postsurgical wound infections. Each year, some 500,000 patients in American hospitals contract a staphylococcal infection. (National Institute of Health, United States, 2007)

Methicillin-resistant *S. aureus*, (MRSA) and often pronounced "*mer*-sa", is one of a number of greatly-feared strains of *S. aureus* which have become resistant to most antibiotics. MRSA strains are most often found associated with institutions such as hospitals, but are becoming increasingly prevalent in community-acquired infections. A recent study by the Translational Genomics Research Institute (2007) showed that nearly half (47%) of the meat and poultry in U.S. grocery stores were contaminated with *S. aureus*, with more than half (52%) of the isolates

resistant to antibiotics. There is increasing evidence that MRSA has transited from a nosocomial infection to a community-associated infection and now has been associated with productive livestock, pet animals, and horses (Cuny *et al.,* 2010). Animals on which MRSA was isolated include companion animals from the United Kingdom (Rich *et al.,* 2005) and Germany (Strommenger *et al.,* 2006), poultry ((Persoons *et al.,* 2009: Nemati *et al.,* 2008), cattle from Pakistan (Farzana *et al.,* 2004), Korea (Moon *et al.,* 2007), pigs from the Netherlands (Huijsdens *et al.,* 2006), Denmark (Guardabassi *et al.,* 2007), and horses from Japan (Anzai *et al.,* 1996), Northern America (Weese *et al.,* 2005), United Kingdom (Baptiste *et al.,* 2005), Austria (Cuny *et al.,* 2006), Germany (Walther *et al.,* 2006), and Ireland (O'Mahony *et al.,* 2005)

MRSA infections in horses associated with wide dissemination of a particular clonal lineage were documented in Canada and since 2005 emergence of MRSA in domestic animal species have been reported with increasing frequencies (Voss *et al.*, 2005: Moodley *et al.*, 2006: Wulf *et al.*, 2006). An example of animal-to-human transmission was demonstrated by Weese *et al.,* (2005) who identified that one MRSA strain was primarily involved in colonizing horses and the people that worked with them including farm veterinarians, veterinary technicians, farm laborers and managers. The study sampled 107 people who were in contact with the horses, and 13% of the people tested positive for MRSA colonization. All of the people that were colonized had reported contact with a colonized horse and had an isolate that was indistinguishable from the colonized horses. Although a recent visit to a human hospital is considered a major risk factor for becoming colonized, none of the colonized humans reported a recent visit to a human health care setting (Johnson *et al.,* 2003: Klevens *et al.,* 2007). Of 972 horses that were sampled 4.7% were identified as being colonized with MRSA. The horses that were colonized were located on

different farms and these farms had short-term populations, which may have contributed to the rate of colonization.

1.2 Statement of Research Problem

In the 1950s, it was recognised that most strains of *S. aureus* produced penicillinase, making them resistant to penicillin. Methicillin was developed in order to solve the problem of penicillin resistance. *S. aureus* is a well-known pathogen of humans and methicillin resistance in this bacterial species presents a threat to human and animal health. Originally methicillin resistant *S. aureus* (MRSA) was a nosocomial pathogen but in the 1990s, MRSA spread into communities initiating worldwide concern. It is a serious human and animal health issue that is evolving from a problem that was confined to hospitals to a much more general concern and certainly deserves attention.

The problem of resistance associated with *S. aureus* and how resistant strains of the bacteria are spreading outside of the hospital setting is a major concern of public health workers and it is this aspect that has caused some attention to be given to animals. Antimicrobial agents, including penicillin, erythromycin, and tetracyclines, are widely used for treating *S. aureus* and other infections (White *et al.,* 2003: Aarestrup *et al.,* 2000: Tanner, 2000). The extensive use of antimicrobial agents in animal husbandry contributes to the selection of drug-resistant strains. Recently, isolation of MRSA from animals has been reported at an increasing rate (Kwon *et al.,* 2006: Wulf *et al.,* 2006: Kwon *et al.,* 2005: Voss *et al.,* 2005: Lee, 2003: Seguin *et al.,* 1999).

There are limited data about the prevalence of MRSA in the horse populations. Most studies were based in animal hospitals. The epidemiology of MRSA in horses is poorly understood and there is also lack of information regarding the virulence and resistance determinants associated with MRSA strains from clinical specimens from equine patients. MRSA epidemiology may follow other patterns than those known in human medicine, and detailed molecular epidemiologic studies are required to address such potentially significant contrasts.

Recent studies have indicated that veterinarians have a higher risk of becoming colonized with MRSA, which may cause subsequent infections (Anderson *et al.,* 2007: Moore-kier *et al.,* 1999). Moodley *et al*. (1999) demonstrated that 4.0% of Danish veterinary practitioners were colonized with MRSA, which was a high prevalence when compared to professionals not exposed to animals, with only 0.7% (Moore-keir *et al.,* 1999). In another study, comparing colonization rates, it was found that 4.6% of veterinary personnel were colonized as compared to 1% of the general population (Wulf *et al.,* 2006). It was initially hypothesized that MRSA was only transmissible from infected or colonized humans to animals (Milheirico *et al.,* 2007: Weese *et al.,* 2006); current studies have suggested that transmission is also possible from animals to humans (Anderson *et al.,* 2007: Weese *et al.,* 2006: Moore-kier *et al.,* 1999).

1.3 Justification

The presence of MRSA in animals and humans worldwide is of both veterinary and public health concern, and it strongly suggests there are interspecies transmissions, animal to human and human to animal. The epidemiology of MRSA in pets may take a parallel course to that of humans (Vitale *et al.,* 2006). Failure to detect and treat infected or colonised cases can result in recurrent MRSA colonisation or infections in humans (Weese *et al.,* 2005). Hence, it must be

emphasised that there is a need to understand the epidemiology of MRSA in animals, similarly in humans also, so as to prevent the spread of MRSA in both sets of populations.

The African data on *S. aureus*, particularly on antibiotic susceptibility, are extremely limited (Taiwo *et al.,* 2005: Ako-Nai *et al.,* 1991), although methicillin-resistant *S. aureus* (MRSA) has disseminated in African countries. Between 1996 and 1997, the prevalences of MRSA, determined in eight African countries, were relatively high in Nigeria, Kenya, and Cameroon (21 to 30%) and were below 10% in Tunisia and Algeria, although in Algeria this rate increased to 14% (Kesah *et al.,* 2003: Ramdani-Bouguessa *et al.,* 2006). In Nigeria MRSA was first reported in pet animals in 1972 and since then no such research has been carried out in live animals to isolate and characterize MRSA. Hence the need to establish updated information and base line data that will be useful for directing future studies and research in this area, thus serve as a guide for specific and targeted control measures.

Horses unlike food animals are transported internationally for breeding, racing, polo etc. these movements within and into Nigeria are thought to contribute to the spread of a horse specific strain/horse adapted clone of MRSA, thus it is pertinent to ascertain and confirm if such a strain is in circulation and spreading within Nigerian horses and horse handlers.

Research questions;

1. Do horses and horse handlers harbour MRSA in their anterior nares?
2. Are the MRSA of horses and horse handlers similar?
3. Are the MRSA of horses and horse handlers multidrug resistant?
4. What risk factors are associated with MRSA carriage in horses and horse handlers?

1.4 Aim

The aim of this work was to study the occurrence of methicillin resistant *Staphylococcus aureus* (MRSA) in horses and horse handlers in Kaduna state

1.5 Objectives

- To isolate and characterise MRSA from horses and horse personnel in the study area.
- To determine the antibiotic sensitivity profiles (antibiograms) of the isolates.
- Determine possible co carriage of similar isolates by horses and their handlers.
- Identify risk factors associated with MRSA occurrence in horses and horse personnel.

CHAPTER TWO

LITERATURE REVIEW

2.1 History of *Staphylococcus aureus*

Staphylococcus aureus (SA) has been a plague of mankind since the dawn of history. In fact, an outbreak of staphylococcal skin disease may even be mentioned in the Bible. The book of Exodus, chapter 9, recounts the sixth plague of Egypt, when Moses and Aaron were commanded by God to take two handfuls of soot from a furnace and then scatter the ashes skyward. The Egyptian men and livestock developed festering eruptions known as *shhin*, a term translated as "boils," a festering malady that was extremely difficult to heal (Chambers, 2001).

The modern recognition of *S. aureus* dates back to the late 19th century. Sir Alexander Ogston, a Scottish surgeon and early advocate of antisepsis, first described the organism in 1881 as a bacterial cause of "acute suppuration." He named the organism, "*Staphylococcus pyogenes aureus*, taking clues from its microscopic morphology, purulent nature, and tendency to form golden colonies on plated media (Cookson *et al.,* 2003). For much of the next half-century, this bacterium remained a notable cause of severe morbidity and death among patients. During World War I, post-influenza staphylococcal pneumonia occurred in young, healthy military personnel producing "dirty salmon-pink anchovy sauce" colored sputum, ultimately leading to "cherry-red indigo-blue cyanosis" and rapid progression to death (Chambers, 2001). Skin and soft tissue infections also frequently progressed to sepsis without effective antibiotic therapy to halt their spread. In 1941, 82% mortality was documented for patients who were treated for *Staphylococcus aureus* septicemia in the pre-antibiotic era (Binh *et al.,* 2006). The survival rate in the over-fifty population in their sample was only 2% (Chickering *et al.,* 1999).

The introduction of penicillin G in 1941 was revolutionary, and mortality rates due to staphylococcal infections dropped precipitously. However, nonsusceptible strains of *S. aureus* were described almost immediately, and resistance to chloramphenicol, erythromycin, and the tetracyclines also emerged within the next decade (*Morbidity Mortality* Weekly Report, 1999). Within 5 years, approximately 50% of *S. aureus* isolates expressed resistance to penicillin via production of the beta-lactamase enzyme. Vancomycin, a glycopeptide antibiotic, was introduced in 1956, and methicillin, the first semisynthetic antistaphylococcal penicillin, was introduced in 1961, both in an attempt to combat penicillin-resistant *S. aureus* (Francis *et al.,* 2005). However, the availability of these agents did not stem the tide of resistance.

The first case of methicillin resistant *Staphylococcus aureus* was described in the United Kingdom in 1961 (Francis *et al.,* 2005), and MRSA was widespread in Europe by the 1970s and in the U.S. by the late 1980s (Chickering *et al.,* 1999). The decade of the 1990s saw nosocomial MRSA rates almost double from approximately 30% in 1990 to nearly 57% in 2000. Currently, nosocomial MRSA rates approach 60% or more in many areas of the United Kingdom (Fridkin, 2001).

The increasing prevalence of MRSA among *S. aureus* strains resulted in a significant increase in the utilization of vancomycin. By the mid-1990s, *S. aureus* strains with reduced susceptibility to vancomycin began to emerge (Figure 2.2). In 1997, the first *S. aureus* strains possessing reduced susceptibility to vancomycin, known as VISA (vancomycin intermediate *Staphylococcus aureus*), were documented in Michigan and New Jersey in patients on peritoneal dialysis who were administered vancomycin for prolonged duration (Moran *et al.,* 2006).

The first case of vancomycin resistant *Staphylococcus aureus* (VRSA) was subsequently documented in Michigan in 2002 in a patient administered vancomycin for almost 6 weeks.

Subsequent studies have documented progressive overall decreasing vancomycin susceptibility among clinical isolates of *Staphylococcus aureus* (i.e., "susceptibility creep") as well as the emergence of heteroresistant *S. aureus* strains (hetero-VRSA). These strains form subpopulations of vancomycin resistant daughter cells in a larger population of more susceptible *S. aureus* strains which may be selected out in the presence of vancomycin therapy (*National Nosocomial Infections Surveillance* Report, 2004). MRSA has also become a prominent pathogen outside the healthcare setting. In the late 1980s, reports surfaced of MRSA cases occurring in the community in patients without exposure to hospitals or nursing homes. These cases affected younger individuals, ethnic minorities, and often involved severe skin or soft tissue infections. Outbreaks were associated with certain higher risk groups, including individuals who used drugs, participants in close contact sports (Kazakova *et al.*, 2005), and residents living together in crowded conditions, such as inmates (Malcolm, 2011), military recruits (Craig *et al.*, 2004), and disabled individuals in group homes (O'Sullivan and Keane, 2000). In the late 1990s, four paediatric deaths attributable to "community acquired MRSA" (CA-MRSA) was documented involving previously-healthy children (Ochoa *et al.,* 2005). CA-MRSA infections differ from healthcare-associated MRSA infections in a number of ways. CA-MRSA infections are predominantly skin and soft tissue infections, are often susceptible to other non-β-lactam antimicrobial drugs, and carry a type IV or V staphylococcal cassette chromosome (SCC) with the *mec*A gene (Seybold *et al.,* 2006). In contrast, healthcare-associated MRSA infections are found at multiple body sites, are usually multidrug resistant, and carry the SCC*mec* types I, II and III. In the United States, 2 major clones of CA-MRSA have been identified by pulsed-field gel electrophoresis (PFGE) and designated USA300 and USA400 by the CDC. Toxin expression of the CA-MRSA strains carry the intracellular toxin Panton-Valentine leukocidin (PVL), which

is known for pore formation on polymorphonuclear cells of the host. In addition, the USA300 clone appears to be emerging as the predominant strain of MRSA for the United States in both community and hospital-acquired settings, with outbreaks of skin and soft tissue disease, community and hospital-acquired pneumonia, and bacteremic syndromes (CDC, 2002). CA-MRSA rates in some areas of the United States now exceed 75% of the strains, especially in the pediatric population (Wenzel, 2004). Thus, trends in the second half of the current decade include the increasing role of MRSA as a community-acquired pathogen, the re-emergence of surgical debridement as a therapeutic modality in managing *Staphylococcus aureus* skin and soft tissue infection, and the waning use of vancomycin, as other Gram positive antibiotic agents take a more prominent role in the treatment of MRSA skin infections, pneumonia, bacteremia, and endocarditis.

2.2 Importance of MRSA

Since the 1990s, MRSA has become a concern in people who have not been hospitalized or recently had invasive procedures; the strains that cause such infections are called community-acquired or community-associated MRSA (Lee, 2003). Community associated MRSA first appeared in high-risk populations such as intravenous drug users, people in nursing homes, and people who were chronically ill, but they are now reported even in healthy children (Duquette *et al.,* 2004). Until recently, these strains were susceptible to many antibiotics other than beta-lactams; however, resistance seems to be increasing, and multiple antibiotic resistant strains have started to emerge (CDC, 2005).

MRSA can be transmitted between people and animals during close contact (Duquette *et al.,* 2004). The pig-associated lineage MRSA CC398 is a particular concern. This lineage, which

apparently emerged in pigs between 2003 and 2005, has spread widely among swine in some locations (Cuny *et al.,* 2010). It was first recognized as a zoonosis in the Netherlands, where the scarcity of human hospital-associated MRSA strains allowed CC398 infections to be recognized (van Rijen *et al.,* 2008). Since that time, methicillin-resistant CC398 has been detected in a number of countries in Europe, some herds in North America, as well as among pigs in Singapore. In some locations, large numbers of swine are colonized asymptomatically with CC398, and asymptomatic carriage is common among people who work with these animals (Catry *et al.,* 2010). Clinical cases have also been reported in humans (Aspiroz *et al.,* 2010). In addition to pigs, which seem to be the reservoir hosts for CC398, this lineage has also been detected in a variety of other domesticated animals, as well as rats living on pig farms (Loeffler *et al.,* 2010). Veal calves have been reported to carry CC398 at high prevalence on some farms (Catry *et al.,* 2010)

MRSA outbreaks in horses suggest that this organism might be an emerging problem in the equine population with both nosocomial and community-acquired MRSA infections being reported in horses (Weese *et al.,* 2005). MRSA carriage, sporadic clinical cases and/or small outbreaks also occur in other species including dogs, cats, pet birds, cattle, zoo animals and marine mammals. MRSA isolates other than CC398 can be shared between animals and people in close contact (Lee, 2003). Most strains in pets seem to originate from humans, although their prevalence in healthy dogs and cats is usually low, clinical cases as well as asymptomatic carriage have been reported (Duquette *et al.,* 2004). During outbreaks in veterinary hospitals, kennels and other facilities, carriage rates in small animals have been as high as 20%. Concerns have also been raised about the ability of pets to transmit MRSA back to people, particularly

those who are immunosuppressed, chronically ill, or unusually susceptible for other reasons (Duquette *et al.,* 2004).

2.3 General Description of *S. aureus*

S. aureus is a member of the *Staphylococcaceae* family (Firmicutes), Gram-positive facultative anaerobes that exhibit a coccal morphology, and are non-motile and non-sporulating. Its cells are 0.5-1.5μm in diameter and forms grape-like clusters. It is catalase and coagulase positive and some strains produce capsules. On blood agar, colonies of *S. aureus* appear golden (caused by staphyloxanthin, a membrane-bound carotenoid) surrounded by haemolytic zones (Wieland *et al.*, 1994). In addition, *S. aureus* strains secrete various extracellular virulence factors such as coagulase and enterotoxins. Despite the fact that *S. aureus* is a normal component of the microbiota of the nasal passages, skin and mucous membranes of humans and animals, it is the cause of several important diseases (Brown *et al.*, 2005: Humphreys, 2002: Bergey *et al.*, 1994: Marshall and Wilmoth, 1981).

2.3.1 Staphylococcal Cell envelope

The cell walls of Gram-positive bacteria differ from those of Gram-negative bacteria. The *S. aureus* cell wall is a multilayered structure (20-40 nm), comprising a copolymer of peptidoglycan and teichoic acid. Peptidoglycan represents about 50% of cell wall by weight and is a polymer consisting of repeating units of sugars of 1, 4 β-linked N-acetylglucosamine and N-acetylmuramic acid. The glycan chains are cross-linked by tetrapeptide chains (L-alanine, D-glutamine, L-lysine, and D-alanine) bound to N-acetylmuramic acid and a pentaglycine bridge that links tetrapeptide chains on adjacent glycan strands. This cross-linking is catalysed by the transpeptidase (TPases) activities of penicillin binding proteins (PBPs) (Fig. 2.1) (Stapleton and

Taylor, 2002: Lowy, 1998). Another major cell wall component is teichoic acid, an anionic polymer that consists of repeating alditol phosphate groups covalently linked to the muramic acid residue of peptidoglycan.

In some *S. aureus* strains the cell wall is coated with an extracellular polysaccharide capsule. More than 90% of clinical strains of *S. aureus* can produce polysaccharide capsules that are usually thin (<0.05µm) and consist of aminouronic acid sugars and fucosamine (Seaman *et al.*, 2004: Wright and Novick, 2003). Serotyping has identified 11 serotypes, with serotypes 5 and 8 being responsible for about 75% of human infections. Most MRSA strains are serotype 5 (Lowy, 1998).

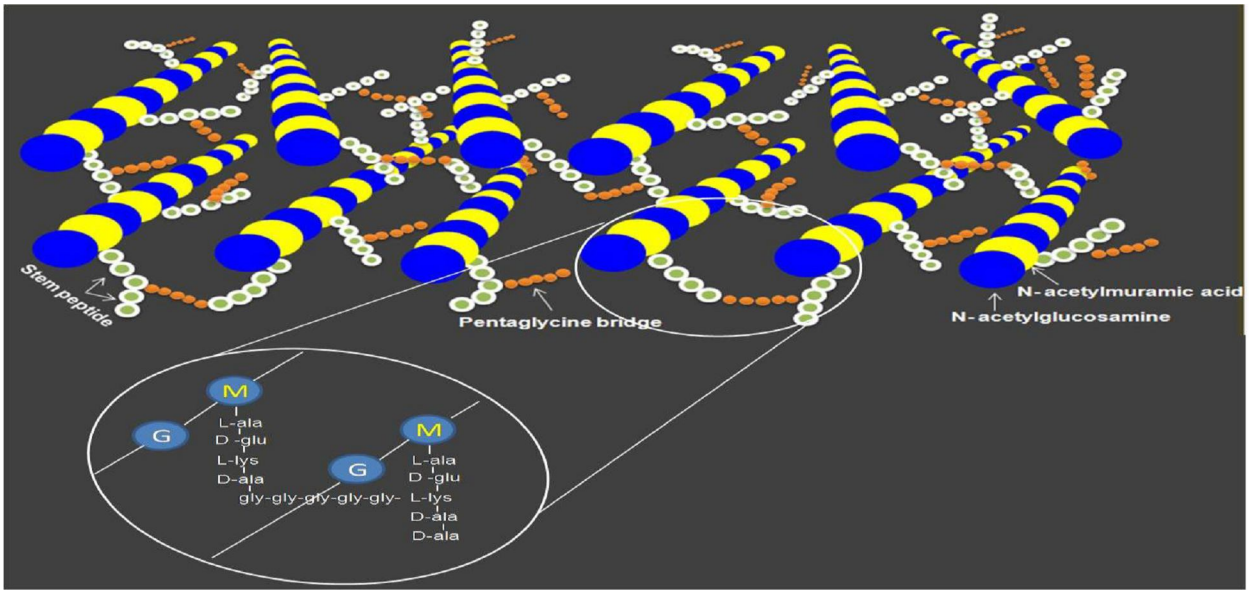


Figure 2.1 Three-dimensional structure of *S. aureus* peptidoglycan**,** consisting of repeating units of 1, 4 β-linked N-acetylglucosamine and N-acetylmuramic acid with tetrapeptides and cross-linked by pentaglycine Bridge. (Adapted from Tomasz, 2006, and Todar, 2002).

2.3.2 *S. aureus genome*

The genome of *S. aureus* has been extensively studied and currently 18 staphylococcal genomes have been completely sequenced (which include MRSA, MSSA, VRSA strains) and a further 28 genomes are currently being sequenced. This has provided an extraordinary glimpse into this so-called "super genome" and has led to a significant increase in our knowledge of the structure and functioning of *S. aureus* (Zahrani, 2011).

The staphylococcal genome is a closed circular molecule of double-stranded (ds) DNA of between 2.7 – 3.0Mbp in size, encoding 2509 to 2892 open reading frames (ORFs). It is composed of two domains called the core genome and the accessory genome. The core genome is inherited from the ancestors and is highly conserved in all staphylococcal species (Shittu *et al.*, 2007). The core comprises approximately 75% of *S. aureus* chromosome and it is highly conserved between strains (Lindsay and Holden, 2004). It contains all the housekeeping genes that are required for essential cell functions, such as DNA replication, proteins synthesis and core metabolism etc. The genome includes a wide variety of genes encoding functions that contribute to virulence, such as toxins, exoenzymes and capsule biosynthetic cluster. The battery of virulence genes is highly strain variable (Shittu *et al.*, 2007).

The second domain, comprising of ~25% of the staphylococcal genome, is the accessory genome. The accessory genome mostly consists of mobile genetic elements (MGEs) that encode variety of non-essential components required for growth and survival. These elements include pathogenicity islands, bacteriophages, chromosomal cassettes, genomic islands, plasmids and transposons. Many of these elements encode virulence and antibiotic-resistance determinants that are transferred horizontally between strains of clinical importance (Shittu *et al.*, 2007: Lindsay and Holden, 2004). There is preliminary evidence to indicate that some of MGEs move among

isolates at a high frequency whereas others move if only rarely. Although, the transfer mechanisms are not fully understood, valuable information has been obtained about how *S. aureus* causes infection from the characterisation and identification of MGEs. Several studies suggest that certain virulence and antibiotic-resistance determinants are associated with particular strains and types of infection (Lindsay and Holden, 2004).

2.4 Classification of MRSA

The nomenclature of MRSA strains is not completely standardized; there are at least three different genetic techniques currently in use for classification (Khanna *et al.,* 2008). For this reason, an isolate can have multiple names. The genetic techniques currently used to classify and name MRSA isolates include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and DNA sequencing of the X region of the staphylococcal protein A gene (Weese *et al.,* 2005). Phage typing was used at one time to differentiate MRSA (and *S. aureus*) isolates, but PFGE became the method of choice in the late 1990s. Widely accepted names based on PFGE included the Archaic, Brazilian, Berlin, Iberian and New York–Tokyo clones, but some groups of organisms were given vague names such as 'PFGE type A,' which varied between laboratories. The U.S. Centers for Disease Control and Prevention (CDC) eventually established a nomenclature system, based on PFGE patterns that were common in the USA, listing eight original isolates, USA100 to USA800 (Tenover *et al.,* 2007). Similarly, common PFGE fingerprint clusters were identified in Canada, and named CMRSA1 through CMRSA10 (Golding *et al.,* 2008) A third designation, by EMRSA (epidemic MRSA) types originated in the U.K., with the initial designation of the most prevalent strain in England and Wales in the early 1980s as "*the* EMRSA." (Cookson and Phillips, 1988). This strain eventually became EMRSA1,

when EMRSA2 through EMRSA14 were identified (Kerr *et al.,* 1990). Additional strains have since been recognized. Sometimes, similar or indistinguishable MRSA isolates have more than one name. For example, CMRSA10 is indistinguishable from USA300, CMRSA2 resembles USA100, and CMRSA8 resembles EMRSA15 (Golding *et al.,* 2008). Some isolates, notably the livestock associated strain ST398, are not typeable by PFGE (Catry *et al.,* 2010).

MLST and spa tying have become popular recently (Tenover *et al.,* 2009). The naming convention for MLST types is sequence type (ST) followed by a number (e.g., ST398), while spa types are "t" followed by a number (e.g., t011) (Cuny *et al.,* 2010). PFGE and MLST typing usually sort isolates into similar clusters. MLST is also used to group MRSA into clonal complexes (e.g., CC398), which contain genetically related ST types (Catry *et al.,* 2010). The clonal complex contains organisms of the same ST type (i.e., CC8 contains ST8 isolates), but it can contain some related isolates that belong to other ST types. One or two clonal complexes tend to predominate in an area (Cockfield *et al.,* 2007).

Spa typing is useful because it can provide better discrimination between isolates, compared to PFGE or MLST, for epidemiologic investigations. A single MLST type or PFGE type can contain several different spa types (Van den Broek *et al.,* 2009). A problem with spa typing is that unrelated lineages can sometimes contain similar spa types (Cuny *et al.,* 2010). For example, CMRSA5, CMRSA9 and CMRSA10 all contain the spa type t008. This may occur because spa typing focuses on a small region of the genome, and recombination might result in discrepancies with MLST and PFGE clustering. Additional genetic testing can resolve such discrepancies (Golding *et al.,* 2008). Isolates may be identified with a combination of tests for a more complete description. MRSA ST8 t064 SCCmecIV, for instance, is a genetic type that has been found in some horses (Cuny *et al.,* 2010).

2.4.1 Staphylococcal cassette chromosome *mec* (SCC*mec*) elements

The emergence of MRSA is the result of the acquisition of a *mecA* gene by the chromosome of an MSSA strain. The *mecA* gene is a methicillin-resistance determinant that is carried on a large (21- 67 kb) mobile genetic element called the staphylococcal cassette chromosome *mec* (SCC*mec*) element. SCC*mec* integrates into the staphylococcal chromosome at a specific-site (*att* BSCC) at 3□-end of *orfX*, close to the origin of replication (de Lencaster *et al.*, 2007: Ito *et al.*, 2007). The SCC*mec* element does not resemble other mobile genetic elements such as phages or transposons, but is instead more like a pathogenicity island that lacks virulence factors (Short and Enright, 2007). The origin of SCC*mec* is still unknown, but there is evidence that SCC*mec* can be horizontally transferred between staphylococcal species, and coagulase-negative staphylococci (CNS) are a potential source for SCC*mec* (Hanssen and Sollid, 2006).

According to their genetic structure and contents, SCC*mec* elements are categorised into several types and subtypes (IWG-SCC, 2009). Almost all SCC*mec* types shared common characteristics: (1) the elements carry the *mec* gene complex; (2) They contain a cassette chromosome recombinase (*ccr*) gene complex; (3) SCC*mec* elements integrate at the same site into chromosome (near the origin of chromosome replication); (4) They are flanked by incomplete inverted and direct repeat sequences that contain integration site sequence (ISS) and located at the integration site of chromosome; those repeat sequences are recognised by *ccr* recombinases during the excision and integration of SCC*mec* (Ito *et al.*, 2007: Hanssen and Sollid, 2006).

SCC*mec* elements are currently classified into various types and subtypes based on a combination of the class of the *mec* gene complex and the allotype of the *ccr* gene complex. In SCC*mec* of *S. aureus*, three classes of the *mec* gene complex have been described: class A has the intact *mec* regulon (*mec*A- *mecRI* - *mecI*) with insertion sequence IS*431mec* downstream of

mecA, whereas classes B and C1/C2 contain *mecA* regulation genes that are truncated by insertion sequence IS*431-mecA-me*cRI-IS*1272* and IS*431- mecA- mecRI-* IS*431*, respectively **(**IWG-SCC, 2009: de Lencaster *et al.*, 2007: Hanssen and Sollid, 2006: Ito *et al.,* 2003). Another essential component of SCC*mec* is the *ccr* gene complex. Two genes (*ccrA* and *ccrB*) with four allotypes have been identified. Another gene, *ccrC*, has been identified in only one of the allotypes. A summary of the classification of *ccr* genes is shown in Table 2.1. The *ccr* gene complex plays a key role in the integration and excision of SCC*mec* into the conserved *orfX* locus by encoding the recombinase proteins of the invertase/resolvase family (Ito *et al*., 2003). Although the remaining regions of the SCC*mec* element carry antibiotic-resistance determents, they are called junkyard regions since they harbour the non-essential components. Three junkyard regions have been classified (J1-3) and variations in these regions are used to define the SCC*mec* subtypes (de Lencaster *et al.*, 2007**).**

Table 2.1. SCC*mec* classification (Adapted from IWG-SCC, 2009)

SCC*mec* types	***mec* gene complex**	***ccr* gene complex**	**Size**
I	B (IS*1272*- *mecA*- *mecRI*)	1 (A1B1)	34kb
II	A (IS*431*- *mecA*, *mecRI* - *mecI*)	2 (A2B2)	53-58kb
III	A (IS*431*- *mecA*, *mecRI* - *mecI*)	3 (A3B3)	67kb
IV	B (IS*431*- *mecA*- *mecRI* - IS*1272*)	2 (A2B2)	20-25kb
V	C2 (IS*431*- *mecA*- *mecRI*- IS*431*)[b]	5 (C)	28kb
VI	B (IS*431*- *mecA*- *mecRI* - IS*1272*)	4 (A4B4)	20-25kb
VII	C1 (IS*431*- *mecA*- *mecRI*- IS*431*)	5 (C)	28-30kb
VIII	A (*mecA*, *mecRI* and *mecI*)	4 (A4B4)	32kb

[b] Difference between C1 and C2, the orientation of IS*431*upstream of *mecA* is reversed in C2 *mec* gene complex.

Eight SCC*mec* elements have been identified according to the criteria in Table 2.1 (IWG-SCC, 2009). The size of SCC*mec* types I, II and III are 34kb, 53-58kb and 67kb respectively whereas type IV is 20-24kb and type V is 28kb. SCC*mec* types VI, VII and VIII are 20-25kb, 28-30kb and 32kb respectively (Plata *et al.*, 2009: Ito *et al.,* 2004: Rohrer *et al.*, 2003: Oliveira *et al*., 2002). Many antibiotic resistant genes and heavy metal resistance elements are encoded by SCC*mec* types II, III and VIII. These include integrated plasmids (pUB110 and pT181) and transposons (*Tn*554 and *IS*431). In contrast, type I, IV, V, VI and VII do not contain antibiotic resistant genes other than *mec*A (IWG-SCC, 2009: Plata *et al*, 2009: de Lencaster *et al*, 2007: Grundmann *et al*, 2006).

2.5 Laboratory Detection and Identification

S. aureus infections are diagnosed by culturing specimens from the affected site, while staphylococcal food poisoning is diagnosed by examination of the food for the organisms and/or toxins (United States Food and Drug Administration, 1992). On blood agar, they are usually beta-hemolytic, young colonies are colorless; older colonies may be shades of white, yellow or orange (Bottone *et al.,* 1984). Enrichment media, as well as selective plates for MRSA, are available. Biochemical tests such as the coagulase test are used to differentiate *S. aureus* from other staphylococci. *S. aureus* can also be identified with the API Staph Identification system. Detection of the organism in clinical specimens can vary, depending on the isolation method used (Brown *et al.,* 2005).

If *S. aureus* is isolated from an infection, genetic testing or antibiotic susceptibility testing should be done to identify MRSA (CDC, 2006). Fluoroquinolone-resistant *S. aureus* strains should, in

particular, be suspected of being MRSA (van Duijkeren *et al.,* 2004). Genetic tests to detect *mec*A, such as polymerase chain reaction (PCR) assays, are the 'gold standard' for identification (CDC, 2006). PCR methods to detect *mec*A in *S. aureus* are commercially available (Paule *et al.,* 2007). A latex agglutination test can be used to detect PBP2a (CDC, 2006). Antibiotic susceptibility tests such as the agar screen test, disk diffusion test, or MIC determination can also be used to identify MRSA (Lee, 2003). Most antibiotic susceptibility tests use oxacillin or cefoxitin, as methicillin is no longer commercially available in the United States (CDC, 2006). Antibiotic susceptibility testing has some drawbacks compared to the detection of *mec*A or PBP2a. Methicillin-susceptible and resistant subpopulations can co-exist *in vitro*; although the entire colony carries the resistance genes, only a small number of bacteria may express resistance in culture (CDC, 2006). The expression of resistance in phenotypic tests can also vary with growth conditions such as temperature (Lee *et al.,* 2004). In addition, some susceptibility tests can overestimate methicillin resistance; isolates that do not carry *mec*A (and thus, are not MRSA) can appear to be phenotypically resistant to methicillin (Lee *et al.,* 2004).

Clones or strains of MRSA are differentiated using genetic tests such as pulsed-field gel electrophoresis, SCC*mec* typing, multilocus sequence typing, spa typing and other tests (Weese *et al.,* 2005). These techniques are mainly useful for epidemiological studies, such as tracing outbreaks. Some isolates may be untypeable by certain methods (Catry *et al.,* 2010). Notably, PFGE cannot identify strains belonging to CC398. PFGE and MLST typing tends to be congruent, but unrelated lineages can sometimes contain similar spa types (Cuny *et al.,* 2010: Golding *et al.,* 2008). Spa typing can distinguish isolates that are indistinguishable by MLST or PFGE (Catry *et al.,* 2010). A combination of methods may be necessary to identify a strain.

S. aureus infections, including colonization, are diagnosed by culture. MRSA can colonize more than one site, and the best site for detecting carriers among dogs and cats is unknown (Kottler *et al.,* 2010). Nasal and rectal sampling should both be done whenever possible. In swine, one study reported that nasal swabs detected most colonized pigs, but some animals carried MRSA in both locations, and a few carrier pigs (all weanlings) could only be found using rectal swabs (Khanna *et al.,* 2008).

2.5.1 Pathogenesis and virulence factors

Although one-third of mankind is asymptotically colonised by *S. aureus*, under the right conditions this versatile organism is able to cause a wide range of infections (Archer, 1998). The nature of those infections depends on several factors, such as the pathogenic characteristics of the strain, host susceptibility and the route of entry into the host. In addition, *S. aureus* infections vary in seriousness and outcome from minor skin infections such as superficial lesions (furuncles, boils) and wound infections, to life-threatening infections such as septicaemia, osteomyelitis, acute endocarditis and necrotising pneumonia (Lowy, 1998: Keane, 1992)

The ability of *S. aureus* to colonise the host and the capacity of this bacterium to exchange and obtain genetic information reflect its success as a versatile pathogen. This contributes to the fact that S. *aureus* strains can express a variety of virulence factors that play key roles in their spread and proliferation in its human and animal hosts. It is noteworthy that a virulence factor can be multifunctional in pathogenesis and multiple virulence factors may play the same function (Decker, 2008: Gordon and Lowy, 2008)

The virulence factors of *S. aureus* can be divided mainly into three clusters: cell surface-associated factors including cell surface-bound proteins (the MSCRAMMs; microbial surface components recognizing adhesive matrix molecules) and other surface-components

(polysaccharide capsule & cell wall peptidoglycan); extracellular enzymes including coagulase and staphylokinase, and toxins such as haemolysins, leukocidins and toxic shock syndrome toxin (TSST) (Novick, 2006: Wright and Novick, 2003).

Most *S. aureus* strains produce a capsular polysaccharide that contributes in virulence of *S. aureus*. The capsule plays vital role in the adhesion of bacterial cells to each other and to host tissues and medical equipment. In addition, the capsule inhibits phagocytosis and restricts the ability of antibiotics to reach the bacterial cell surface. Moreover, other cell wall components (*e.g.* peptidoglycan and lipoteichoic acid) have a role in *S. aureus* pathogenicity: peptidoglycan, for example, has endotoxin activity that stimulates macrophages to release cytokines (Lowy, 1998). Lipoteichoic acid (LTA) is thought to play an important role in septic shock and other detrimental host responses (Novick, 2006: Ferry *et al.*, 2005: Fournier and Philpott, 2005). Staphylococcal surface proteins contribute to the spread and virulence of *S. aureus*. These proteins, which include protein A, fibronectin binding proteins, fibrinogen binding proteins and collagen binding proteins are known MSCRAMMs. These proteins perform a wide spectrum of functions and many recent studies have shown that these surface proteins play essential roles in the ability of these bacteria to colonize host tissues (Wright and Novick, 2003: Lowy, 1998) by promoting adhesion to the surfaces of host cells and tissues. MSCRAMMs are covalently attached to the cell wall by sortase enzymes that recognise and cleave the Leu-Pro-X-Thr-Gly (LPXTG) motif (Mazmanian *et al.*, 1999: Schneewind *et al*., 1995). MSCRAMMs can also help the organism to evade the innate immune system and increase iron uptake (Foster, 2005). It appears that MSCRAMMs play a key role in the colonisation of prosthetic-devices and endovascular infections by, for example, assisting in the formation of biofilms. Moreover,

MSCRAMM protein A is a good example of immune evasion factor that binds to the Fc portion of immunoglobulin and subsequently prevents opsonisation (Gordon and Lowy, 2008).

During infection, *S. aureus* secretes a wide variety of extracellular enzymes and toxins that contribute either directly or indirectly to pathogenesis. Most, if not all *S. aureus* strains produce haemolysins, coagulases, nucleases, protease, lipases, hyaluronidase and collagenases (van Belkum, 2007). Coagulase is produced exclusively, among the staphylococci, by almost all strains of *S. aureus* (with the exception of a few strains of *S. intermedius*). Coagulase reacts with blood-prothrombin to form a staphylothrombin complex that can convert fibrinogene to fibrin. Although, coagulase covers the bacterium with fibrin to reduce its susceptibility to host defences, its contribution to pathogenesis is not clear (Wright and Novick, 2003: Lowy, 1998). In contrast, staphylokinase disassembles fibrin by its interaction with plasminogen to form plasmin (a serine protease) that, by virtue of its ability to digest fibrin clots, allows staphylococci to spread into deep host tissue (Bokarewa *et al.*, 2006). Other enzymes secreted by *S. aureus* include proteases such as serine protease V8 and lipases that breakdown the bactericidal fatty acids produced by infected cells. Many *S. aureus* strains produce hyaluronidase that can degrade the hyaluronic acid, a component of the extracellular matrix of host tissues, and enables the bacterium to spread through host tissues. Hyaluronidase is commonly referred as the spreading factor (Wright and Novick, 2003: Hynes and Walton, 2000). The DNA and RNA of host cells are degraded by nucleases. These enzymes almost produced by all staphylococcal strains cleave the phosphodiester bonds of both single and double stranded DNA and RNA (Wright and Novick, 2003). Finally, *S. aureus* strains can produce β-lactamases that are responsible for the resistance of β-lactam antibiotics (Lowy, 1998).

S. aureus produces many cytotoxins that are grouped according to their mode of action (Lowy, 1998). Haemolysins (alpha, beta, gamma and delta) are porin-like toxins that lyse a variety of host cells such as red blood cells (erythrocytes) and platelets. Another important pore-forming toxin is Panton-Valentine leukocidin (PVL) that structurally resembles alpha toxin. This cytotoxin mostly forms its pores in the outer membrane of mitochondria and kills neutrophils and macrophages. Fortunately, <5% of *S. aureus* strains produces PVL and its production is associated with cutaneous infections and, more recently, with necrotizing pneumonia (Decker, 2008: van Belkum, 2007: Wright and Novick, 2003). *S. aureus* also secretes enterotoxins (A-E, G-I), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin (A, B). These toxins are responsible for diseases such as food poisoning, toxic shock syndrome and exfoliative dermatitis (scalded skin syndrome) respectively (Wright and Novick, 2003). Enterotoxin and TSST represent pyrogenic toxin superantigens (PTSgs) and both play a role in derailing and over-stimulating components of the immune response (van Belkum, 2007).

Staphylococcal virulence factors are precisely regulated by a quorum-sensing system based on the accessory gene regulator (*agr*). In order to initiate colonisation of tissue sites, MSCRAMMs are expressed during exponential growth phase whereas secreted enzymes and toxins are produced later during stationary phase so as to aid the spread of the organism (Gordon and Lowy, 2008).

2.6 MRSA in Horses

There is evidence that some MRSA strains may be spreading in equine populations, and some MRSA in horses seem to be distinct from the common human strains. Shared isolates between humans and horses have been described in veterinary clinics, (Weese *et al.,* 2005) and an

elevated risk of MRSA was reported among equine practitioners at a veterinary conference (Catry *et al.,* 2010).

Some surveys in healthy horses report a low prevalence of MRSA carriage. In surveys conducted between 2004 and 2007, MRSA was not detected in any horses (samples of 100-500 healthy animals) in the Netherlands, Slovenia, or Canada (Catry *et al.,* 2010). Another survey reported that 1.3% of horses in western Canada carried MRSA in 2006 and 2007, although the colonization was often transient (Tokateloff *et al.,* 2009). In Ireland, carriage was detected in 1.7% of healthy horses in 2005-2006 (Abott *et al.,* 2010). One investigation of community-acquired infections among horses in North America found that these infections were clustered: colonization with MRSA was detected in 13% or 5% of the horses on two farms, but it was not found in any horses on eight other farms (Weese *et al.,* 2005).

MRSA is fairly common in equine clinical samples in some reports. In the U.S., one study reported that 14% of the *S. aureus* isolates submitted by veterinary teaching hospitals in 2001-2002 were MRSA; the positive samples originated from four horses, four dogs and a cat (Middleton *et al.,* 2005). Another study, conducted in 2006- 2008, detected MRSA in 42% of the clinical samples containing coagulase positive staphylococci from sick horses in the U.S. Midwest and Northeast (Lin *et al.,* 2010). In a study from Ireland, MRSA was isolated from approximately 5% of equine clinical samples between 2003 and 2006 (Abbott *et al.,* 2010). At one diagnostic facility in the Netherlands, the percentage of MRSA isolates in equine clinical samples increased from 0% in 2002 to 37% in 2008 (Catry *et al.,* 2010)

Outbreaks or clusters of clinical cases have been reported occasionally among horses at veterinary hospitals, and some studies suggest that MRSA may be an emerging pathogen in this species. At a veterinary teaching hospital in Ontario, Canada, MRSA was isolated from the nasal

cavity of 4% of horses during an outbreak with CMRSA5 (USA500; ST8-MRSA SCCmecIV) in 2000 (Weese *et al.,* 2005). Nosocomial colonization rates varied from 1% to 3.6% among horses admitted in 2002-2004, and clinical nosocomial infections occurred in 0.2%, with no increase over the 3 year period. In the same hospital, community-associated MRSA colonization was detected in 1.7% of equine admissions in 2002, 1.5% in 2003, and 5.7% in 2004 (Weese *et al.,* 2006). During an outbreak in 2003-2005 at a university veterinary hospital in Austria, the overall incidence was 4.8%. At a university equine clinic in the U.K., carriage was reported in 16% of the horses tested during an outbreak, and clinical cases occurred in 4% (Baptiste *et al.,* 2005). MRSA carriage has also been reported in 2.2% to 11% of horses presented at equine practices and university hospitals in Belgium, Germany (Berlin) and western Europe, but in less than 0.5% of horses tested at four equine clinics in Sweden (Catry *et al.,* 2010). Anecdotal reports suggest that MRSA infections are becoming more common in horses, including foals in neonatal intensive care units (O'Rourke, 2003).

Risk factors for MRSA infections in horses may include treatment with antibiotics, contact with carriers and previous hospital admission. Carriage of MRSA predisposes an animal to nosocomial infection while in the hospital. Surgery and orthopedic implants also seem to be associated with an elevated risk (Loeffler and Lloyd, 2010)

2.7 Antibiotic Resistance

Since the late 1940s, antibiotic resistance associated with hospital-acquired infections has emerged as a major problem worldwide. In this respect it is important to distinguish between bacterial strains acquired in hospitals from those acquired in the community, since the former are more likely to exhibit a greater range of antibiotic resistances than the latter (Zhang *et al.*, 2006). Penicillin resistant strains of *S. aureus* were first detected in the 1940s, shortly after this

antibiotic was introduced into clinical practice (Chambers and Deleo, 2009). This resistance was due to the production of β-lactamases that inactivate the antibiotic by hydrolyzing the β-lactam ring. The main mechanism of penicillin resistance is via the *blaZ-gene* which encoded β-lactamases (Rohrer *et al.*, 2003). Currently, 90% of clinical *S. aureus* isolates are penicillin-resistant and resistance is particularly prevalent in hospitals. Consequently, new generations of penicillins have been developed. The introduction of methicillin in the 1960s was followed rapidly by the emergence of methicillin-resistant isolates. Methicillin-resistance is not mediated by β-lactamases but by the production of an altered penicillin binding protein (PBP2a) that has a low affinity for β-lactam antibiotics (Johnson, 1999). The 1960s also saw the development of non-β-lactam antibiotics such as chloramphenicol, erythromycin, streptomycin and tetracycline. Although initially effective against *S. aureus,* nevertheless resistance against them developed rapidly. By 1976, resistance to kanamycin and gentamicin was reported and, by the early 1980s, multiresistant *S. aureus* strains were reportedly responsible for nosocomial outbreaks in many countries (Thomas and Archer, 1989).

Vancomycin and teicoplanin are glycopeptide antibiotics that have been the first choice for the treatment of serious nosocomial MRSA infections for the last fifteen years. Since the emergence of vancomycin-resistant enterococci, fully vancomycin resistant strains of *S. aureus* have been expected. The first vancomycin-intermediate resistant *S. aureus* (VISA) was reported in Japan in 1996. This isolate, designated Mu50, had an MIC for vancomycin of 8μg/ml (Hiramatsu *et al*., 1997). Thereafter, clinical cases from which VISA were isolated were reported in 1997 in USA, then France and later in the UK. All these reported strains were vancomycin-intermediate *S. aureus* (VISA) and were called glycopeptide-intermediate *S. aureus* (GISA) (Cui and Hiramatsu, 2003). The National Committee for Clinical laboratory standard (NCCLS) suggested the

definition for vancomycin-resistant terms to avoid any confusion. *S. aureus* strains that have a minimum inhibitory concentration (MIC) of 4 μg/ml or below for vancomycin are defined as vancomycin-sensitive *S. aureus* (VSSA), those that have an MIC of between 8-16 μg/ml as vancomycin-intermediate *S. aureus* (VISA), and those that have an MIC of 32 μg/ml or higher as vancomycin-resistant *S. aureus* (VRSA) (Srinivasan *et al.*, 2002). Although, VISA strains have been isolated, they are rare in most countries. However, 2002 witnessed the reporting of the first fully vancomycin-resistant *S. aureus* (VRSA) isolate from a renal dialysis patient in Michigan, USA (Fig.2.2) (Bartley, 2002). This strain had an MIC of >128 μg/ml and carried the vancomycin-resistance gene, *vanA* (Aires de Sousa and Lencaster, 2004: Chang *et al.*, 2003). Vancomycin acts on cell-wall peptidoglycan by binding to the carboxyl-terminal D-alanyl-D-alanine residues of peptidoglycan precursor, preventing PBPs from accessing their natural substrate. Vancomycin has to penetrate about 20 layers of peptidoglycan to reach the cytoplasmic membrane where the transglycosylation and transpeptidation reactions of PBPs take place. In the VISA strains Mu50 and Mu3, vancomycin-resistance is associated with a thickening of the cell wall caused by excessive activation of peptidoglycan synthesis. The vancomycin

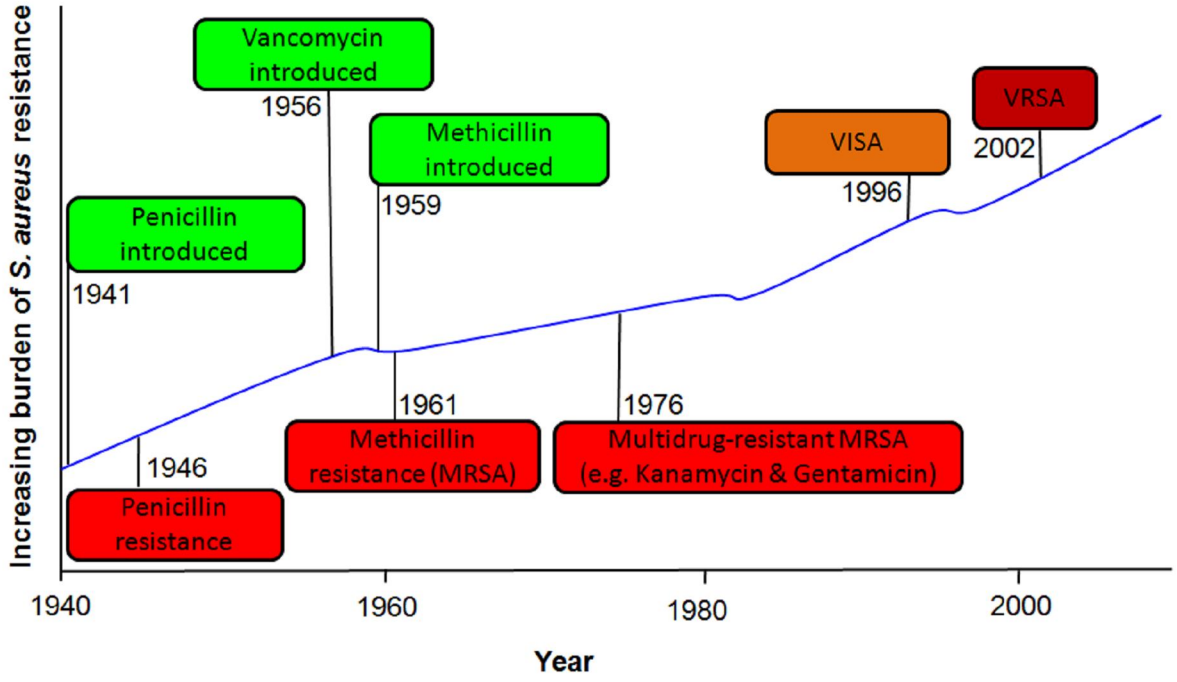


Figure 2.2: The time line of the emergence of antibiotic resistance in *S. aureus*. (VRSA = vancomycin-resistant *S. aureus*, VISA = vancomycin-intermediate *S. aureus*) (Chambers and Deleo, 2009).

molecules are trapped by high levels of free D-alanyl-D-alanine residues due to the much-reduced levels of cross-linking (Aires de Sousa and Lencaster, 2004).

In contrast, full vancomycin-resistance in *S. aureus* (VRSA) is encoded by three determinants, namely *van*A, *van*B and *van*D, normally associated with vancomycin-resistant enterococci (VRE). Resistance is brought about by the replacement of the native D-alanyl-D-alanine (D-Ala-D-Ala) residue of the cross-linking wall peptide with a D-alanyl-D-lactate (D-Ala-D-Lac) residue, which has a very low affinity to glycopeptides. It is noteworthy that the VanA induces

high level of resistance and also confers resistance to teicoplanin. The *vanA* gene cluster is carried on a large resistance plasmid (Berger-Bachi, 2002).

2.8 Emerging Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *S. aureus* were first discovered in the UK in 1961 by Jevons. In that study, three MRSA strains were reported among 5,440 isolates and MRSA infections were initially confined to hospital patients. The first nosocomial epidemic of MRSA was reported in 1963, when an MRSA strain was isolated from an infant who was treated unsuccessfully with penicillin. The same strain was isolated from 37 children in eight wards and from one nurse. Medical centres in several European countries (*e.g.* Denmark, France and Switzerland) described outbreaks of MRSA nosocomial infections in the 1960s. The first US MRSA outbreak did not occur until 1971 (Keane, 1992: Chambers, 1988). More recently, a number of MRSA infections have been shown to be community- rather than hospital-acquired (Chambers, 1988).

In the UK, the number of MRSA infections remained limited for several years. At the beginning of the 1971, MRSA represented 10% of all *S. aureus* isolates at the general hospital in Birmingham. Interestingly by the mid-1970s, the number of reported MRSA cases declined to virtually zero, a decrease which was thought to be due to a combination in the use of aminoglycoside antibiotics and better infection control procedures (Grundmann *et al.*, 2006: Johnson *et al.*, 2005: Griffiths *et al.*, 2004). However, in the early 1980s, concerns about MRSA were heightened with the emergence of gentamicin-resistant MRSA in the UK, Ireland and the USA.

In 1982, an epidemic strain of multi-resistant MRSA was reported in Australia, and the same strain was discovered in London in connection with a hospital outbreak. The staphylococcal reference laboratory of the UK Public Health Laboratory Service established a numerical prefix

for epidemic MRSA strains and, based on this system, 16 epidemic strains were identified in England and Wales up to 1995. Nevertheless, only three epidemic strains, UK EMRSA-3, UK EMRSA-15 and UK EMRSA-16, were still being recorded in the 1990s and EMRSA-15 and EMRSA-16 were behaviorally more dynamic. In the meantime, six epidemic MRSA strains have been recorded in some central Europe countries (Grundmann *et al.*, 2006). EMRSA15 and EMRSA16 strains have spread broadly and are associated with severe infections (Livermore, 2000). According to the Centers for Disease Control (CDC) and the National Nosocomial Infection Surveillance System (NNISS), the proportion of MRSA in US hospitals increased dramatically from 2.4% in 1975 to 29% in 1991 (Graffunder and Venezia, 2002). In the UK MRSA bacteraemias remained at 3% until 1992 and then rose considerably to reach 43% by 2002. Since then, the rate of isolation of MRSA strains has increased significantly every year worldwide (Grundmann *et al.*, 2006).

2.8.1 Mechanism of methicillin-resistance

Methicillin-resistance in staphylococci is due to the acquisition of a large mobile DNA element (20 to 100kb in size), the so-called "Staphylococcal cassette chromosome *mec"* (SCC*mec*) (Rohrer *et al.*, 2003). Currently, eight SCC*mec* types (I to VIII) have been described in detail.

β-lactam antibiotics destroy bacteria by inhibiting bacterial cell wall synthesis. The antibiotic, an analogue of D-alanyl-D-alanine, covalently attaches to penicillin-binding proteins (PBPs). These membrane-anchored proteins catalyse one or more of three reactions (transpeptidase, endopeptidase and carboxypeptidase) involved in cell wall synthesis. MRSA produces a modified PBP2a that can complete cell wall synthesis when the transpeptidation activities of the native PBPs are inactivated by the antibiotic (Chambers and Hackbarth, 1992). Resistance is due to the fact that PBP2a has low affinity to β-lactams antibiotics. The expression of the *mecA* gene

is controlled by the products of the *mecRI* and *mecI* genes. MecRI synthesis is induced upon exposure to β-lactam antibiotics. As a result, the MecI repressor is inactivated and PBP2a produced (Figure 2.3) (Lowy, 2003).

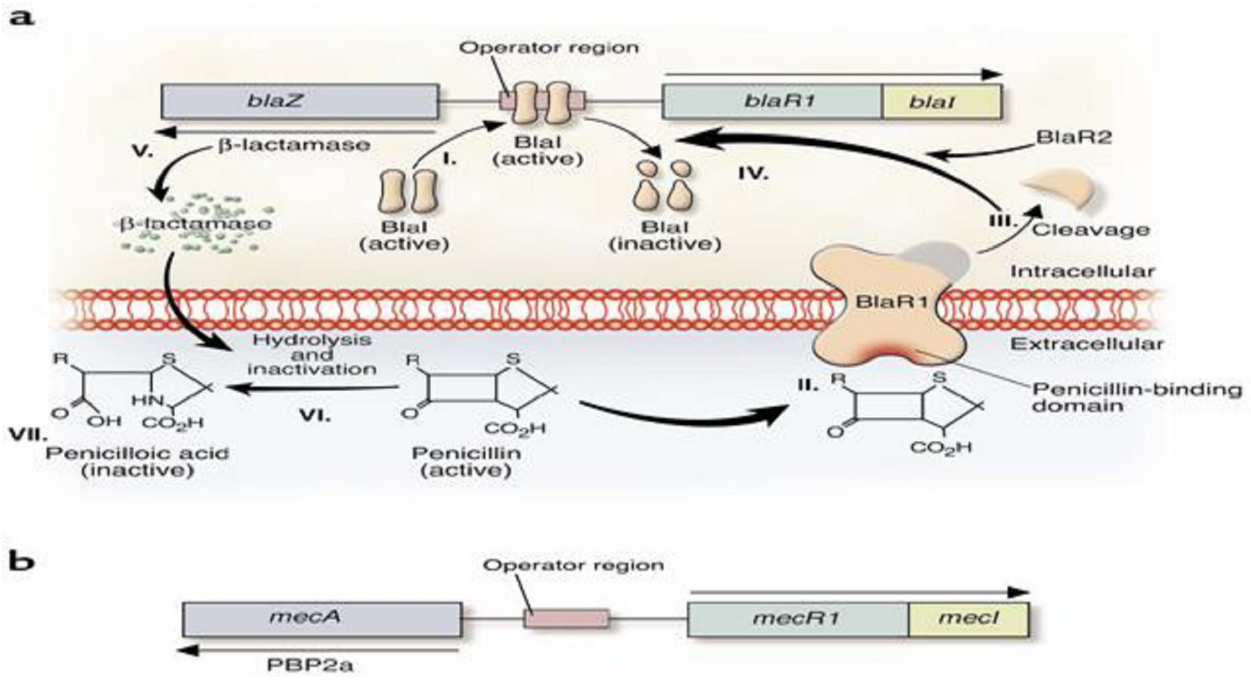


Figure 2.3: The mechanisms of β-lactam-resistance in *S. aureus*. (a) The production of β-lactamase by *blaZ* gene in the presence of penicillin, I. The operator region of *blaZ*, *blaR1* and *blaI* is bound by the DNA-binding protein Blal that represses the transcription of *blaZ*, *blaR1* and *blaI*. II- III. The presence of penicillin stimulates the transmembrane sensor-transducer BlaR1 to inactivate the Blal repressor. IV-V. This process results in the removal of Blal and allowing transcription of *blaZ* to produce β-lactamases that inactivate penicillin by cleaving the β-lactam ring, (b) The mechanism of methicillin-resistance by production of PBP2a encoded by *mecA* gene. PBP2a synthesis proceeds similarly to that shown for β-lactamases. MecR1 is induced by β-lactam antibiotics and MecR1 inactivates MecI repressor allowing PBP2a synthesis (Lowy, 2003).

2.8.2 Risk factors associated with nosocomial MRSA

A good knowledge of the epidemiological characteristics of MRSA is the cornerstone of establishing an effective infection control strategy. There are many risk factors associated with the emergence of MRSA nosocomial infections, most of which can be attributed to host factors, antibiotic pressure and infection control procedures. Those risk factors are older age, male gender, previous hospitalization and exposure to antibiotics, length of hospitalization, length of stay in ICU, and underlying disease. In addition, pneumonia infections are associated with ventilator therapy, and immunosuppressive therapy in ICUs. Invasive devices play role in increasing vulnerability to nosocomial infection. Intravascular (IV) catheters are, for example, the main risk factor for MRSA bloodstream infection in ICUs (Graffunder and Venezia, 2002: Washio *et al.*, 1997). There are other factors that are linked to spread of MRSA in hospitals: transferring of patients within or between hospitals, poor communication between hospital units, difficulties in isolation of MRSA patients and new epidemic MRSA strains (Vuopio-Varkila, 2007). These factors contribute not only to therapeutic failure and morbidity but also to increased mortality of MRSA among hospitalized patients. According to UK Government statistics, the number of deaths due to MRSA has increased dramatically from 51 in 1993 to 1,230 in 2008, although, there has been a noticeable decrease in MRSA deaths in 2008, compared with 2005, 2006 and 2007. It is noteworthy that colonized and infected patients and healthcare workers are the main reservoir for MRSA in hospitals. Moreover, the main route for transmission of MRSA is through the hands of health care workers (HCWs) in hospitals (Haddadin *et al.*, 2002).

2.8.3 Epidemiology of MRSA in the community

Although MRSA is mainly associated with nosocomial infections, it is becoming an increasing problem in the community. In 1993, community-acquired MRSA (CA-MRSA) was first reported

in Western Australia and by the end of 1999; four deaths were reported among children due to CA-MRSA infections in USA (Aires de Sousa and de Lencastre, 2004). CA-MRSA strains differ from nosocomial strains in a number of respects. Firstly, CA-MRSA strains are sensitive to non-β-lactams antibiotics, for example clindamycin, trimethoprim/sulfamethoxazole and doxycycline. Secondly, these strains have genotypes that are distinct from those of nosocomial strains. Finally, they have different methicillin-resistance cassettes and often encode PVL (Boyle-Vavra and Daum, 2007: Grundmann *et al.*, 2006). There is no standard definition for CA-MRSA and approximately eight classification systems have been applied to categorize community-acquired infections (Aires de Sousa and de Lencastre, 2004). The CDC defines CA-MRSA as an infection with MRSA that lacks the risk factors of HA-MRSA including: the isolation of MRSA more than 48 hours after admission, hospitalization history, recent surgery, previous isolation of MRSA and presence of an indwelling catheter or a percutaneous device at the time of culture (Brasel and Weigelt, 2008: Fridkin *et al.*, 2005). Most common CA-MRSA infections are bacteraemia, skin and soft tissue infections, septic arthritis, toxic shock syndrome, necrotizing fasciitis and necrotizing pneumonia (Grundmann *et al.*, 2006). CA-MRSA is also associated with the production of PVL that is encoded by phage-mediated *lukS-PV* and *lukF-PV* genes. The production of this toxin is associated with severe skin and soft tissue infections (Boyle-Vavra and Daum, 2007). The CA-MRSA infections have been documented among homeless people, homosexuals, military recruits, competitive athletes, residents of community-based health-care institutions and children in day-care centres (Grundmann *et al.*, 2006). There are several risk factors attributed to the acquisition of CA-MRSA such as overcrowding, high rates of skin infections, frequent use of broad-spectrum antibiotics and close contact with a person with these risk factors. It is assumed that health-care institutions are the most likely source for CA-MRSA

strains since some similarities have been found between sporadic nosocomial-MRSA and CA-MRSA infections (Aires de Sousa and de Lencastre, 2004).

2.8.4 Treatment and control of MRSA

MRSA strains are resistant to most β-lactam antibiotics and several other antimicrobial agents including aminoglycosides, clindamycin, chloramphenicol, fluoroquinolones, and macrolides (Schmitz and Jones, 1997). The glycopeptides have emerged as the most effective agents against MRSA. However, the emergence of MRSA strains with low sensitivity to vancomycin (VISA), first reported in 1996 and thereafter VRSA in 2002, has led to an increasing concern about the use of vancomycin as the drug of first choice for the treatment of MRSA infections (Cui and Hiramatsu, 2003).

The treatment of MRSA infections depends on the site of infection. In some cases, such as infected devices and abscess, the removal of these devices and draining of abscess are more important than antimicrobial therapy (Cunha, 2005). Recently, new guidelines for the prophylaxis and treatment of MRSA infections in the UK have been published. These guidelines recommend the use of glycopepides or linezolid as a first choice to treat MRSA pneumonia and severe skin and soft tissue infections where the risk of bacteraemia is high (Gemmell *et al.*, 2006). Linezolid is recommended for treatment of pneumonia since it has shown excellent penetration into lungs (Conte *et al.*, 2002). The same choice of glycopepides or linezolid is recommended but with longer treatment for uncomplicated bacteraemia. In bone and joint infections, glycopepides can be used particularly with multiresistant MRSA and/or in combination with rifampicin or fusidic acid. Glycopeptide prophylaxis is recommended for patients who require surgery and have a history with MRSA colonization (Gemmell *et al.*, 2006).

These recommendations take in their account not only the efficacy of the antibiotics but also their toxicity, selection of resistant bacteria and cost (Ben-David and Rubinstein, 2003)

The majority of studies have reported that the screening of infected and colonized patients, the early detection of MRSA, improved hand hygiene and the prudent use of antibiotics, are effective control methods for the MRSA infections (Wang and Barrett, 2007: Grundmann *et al.*, 2006: Hardy *et al.*, 2004: Frank, 2003). Furthermore, the most important step in controlling MRSA outbreaks is the typing of strains to distinguish between epidemic and sporadic strains (Frank, 2003)

2.8.5 The typing of MRSA

The ability to type isolates of MRSA is an important tool for understanding the epidemiology of MRSA and for developing effective control measures. In the last ten years, a variety of typing methodologies have been developed for use in both clinical and epidemiological studies (Wilailuckana *et al*., 2006). In recent years traditional typing methods have been augmented with a range of molecular typing methods. These include pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting (PF), arbitrarily primed polymerase chain reaction (AP-PCR), multiple locus variable number tandem repeat (VNTR) analysis (MLVA), multilocus sequence typing (MLST) and whole genome sequencing. For any typing method to be valuable it should have high discriminatory power, be reproducible, inexpensive, and easy to use and interpret (Sabat *et al*., 2003: Weller, 2000: Tenover *et al*., 1997).

2.9 Mechanism of Genetic Variability of Bacterial Genome

The concept behind all microbiological typing methods is that epidemiologically related strains are descended from a single ancestor. Consequently, the species share the similar, but not necessarily identical epidemiological characteristics that are distinct from those of unrelated strains. Consequently, the analysis of such characteristics can be useful for the study of population structures and the evolution of bacterial species. The stability of such characteristics within a strain and the general diversity of species are vital factors for epidemiological typing (van Leeuwen, 2003).

The genetic diversity among strains is attributed to several types of mutational process including the accumulation of spontaneous or induced point mutations, genetic recombination and the acquisition or loss of mobile genetics elements. The latter elements, which can confer virulence factors and antibiotic resistance determinants, include plasmids, bacteriophages and gene islands (van Leeuwen, 2003: Fitzgerald *et al*., 2001). The spontaneous mutations can be caused by DNA polymerases by mismatched base insertion or slippage errors during DNA replication resulting in changes in the base sequence (Atlas, 1997). Three types of replication errors have been characterized: (1) Single-base mis-pairs leading to a base substitution, (2) single-base bulges leading to a single-base frame shift and (3) multiple-base mismatches leading to a sequence substitution. These changes during the DNA replication play significant role in bacterial evolution and therefore in bacterial genetic diversities (Schumann, 2006).

Although, *S. aureus* clones arise more frequently (about 15-fold) by point mutation than by recombination, Robinson and Enright (2004) suggested that large chromosomal replacements could influence the long-term evolution of this species (Robinson and Enright, 2004: Feil *et al*., 2003). The SCC*mec* cassette, for example, is a large group of mobile genetic elements whose diversity is the result of extensive recombination. The SCC*mec* contains the *mec* gene

(methicillin-resistance gene) and other mobile genetic elements (*e.g.* transposons, plasmids and insertion sequences). SCC*mec* is horizontally transferred within *Staphylococcus* species, playing an important role in microbial evolution by promoting the genetic diversity (van Leeuwen, 2003).

Typing methods vary in their ability to monitor and define genetic changes, not only between species, but also between strains within a single species. This is reflected in differences in the resolving power of the various typing methods. For example, MLST is less discriminatory than PFGE for the analysis of *S. aureus* in hospital outbreaks, reflecting the fact that the evolution of diversity over short time periods is more likely to be due the positive selection pressure for virulence or antibiotic resistance genes based on mobile genetics elements genes, than for spontaneous mutations in housekeeping genes (Singh *et al*., 2006)

2.10 Phenotyping Methods

Phenotyping methods are used to characterize the products of gene expression in order to identify a species or to discriminate between strains. Examples of phenotyping methods include biochemical profiles, antibiogram profiles, bacteriophage susceptibilities (phage typing), the presence of surface antigens and protein analysis. However, there are limitations in using these methods extensively for typing MRSA strains because they are influenced by growth conditions and epigenetic modifications (van Leeuwen, 2003: Tenover *et al*., 1997)

2.11 Genotyping Methods

A variety of typing methods have been developed for the molecular typing of *S. aureus*. For a typing method to be effective it should be inexpensive, have high discriminatory power, good reproducibility, and be easy to perform and interpret. In the last decade, several molecular techniques have been developed for the typing of bacterial strains, including arbitrarily primed

polymerase chain reaction (AP-PCR), pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting (PF), variable number tandem repeat analysis (VNTR) typing, multiple-locus variable-number tandem repeat analysis (MLVA), multilocus sequence typing (MLST) and, in the case of MRSA, SCC*mec* typing. These techniques have been used primarily in reference laboratories but, with passage of time, are being used in an increasing number of clinical laboratories (Sabat *et al.*, 2003: Trindade *et al.*, 2003). Molecular methods have been increasingly applied to the epidemiology of nosocomial pathogens such as MRSA (van Leeuwen, 2003). The aim of any strategy for controlling the incidence of MRSA is to develop a thorough knowledge of its epidemiology and molecular typing techniques are ideally suited for this purpose because they are not phenotype-dependent (Deurenberg *et al*., 2007).

2.11.1 Pulsed-field gel electrophoresis (PFGE)

Bacterial chromosomes are generally between 2000-5000kb in length. The resulting patterns of digestion of the chromosomes can most easily be interpreted when using restriction endonucleases which generate a relatively few number of large fragments *i.e.* 10-30 fragments of between 10-800kb in length. In conventional electrophoresis the DNA fragments migrate in a straight line from the cathode to the anode. Because large molecules adopt a hairpin-like configuration, increasing the size of a fragment has little influence on its mobility. PFGE is highly discriminatory for identifying the relatedness of isolates from short time studies (e.g. outbreak within a hospital), but it is not suitable for long-term studies of global epidemiology, which require highly discriminatory techniques that are able to monitor genetic changes that accumulate slowly (Enright *et al*., 2002).

2.11.2 Multilocus sequence typing (MLST)

MLST is a relatively new approach to identifying and monitoring the spread of antibiotic resistant pathogens (Enright and Spratt, 1999). This technique, which is derived from multilocus enzyme electrophoresis (MLEE), was first introduced by Maiden *et al.* (1998) to resolve certain limitations of MLEE analysis. MLST is an excellent technique for investigating MRSA clonality. MLST is based on the analysis of seven ~500-bp sequences derived from housekeeping genes. The sequences represent distinct alleles and contain sufficient information to define the isolate using an online data search tool. The MLST website, http://www.mlst.net, contains information on 21 bacterial species, including *S. aureus*. The distinct sequences of each locus are given allelic numbers; for example, an Iberian clone of MRSA has the MLST profile "3-3-1-12-4-4-16" which categorizes it as sequence type ST247. Recently the nomenclature system has been expanded to include the SCC*mec* type (*e.g* MRSA-I) and the Iberian clone is now categorized as (ST247-MRSA-I) (Deurenberg *et al.*, 2007: Robinson and Enright, 2004). MLST can also be used to establish the evolutionary relationship of MRSA strains using the Electronic based upon related sequence types (eBURST) program that groups closely related STs into so-called clonal complexes (CC).

The first application of MLST to *S. aureus* was published in 2000 by Enright *et al*. who analyzed the target DNA sequences of 155 strains. The housekeeping genes used in this study were: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephoshate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*). In their study, Enright and colleagues clearly demonstrated the value of MLST with respect to the analysis of MRSA and MSSA, generating results that were comparable to PFGE (Enright *et al.*, 2000). These seven housekeeping genes are thought to

change slowly over time. They are also widely distributed around the genome, which decreases the impact of recent recombination events (Short and Enright, 2007). Therefore, MLST is a very useful technique for the study of evolutionary and population biology of bacterial pathogens but, is less discriminatory for the analysis of outbreaks (Cooper and Feil, 2004). Indeed, MLST provides unambiguous information and is very useful for studying the population structures of MRSA (Enright *et al.*, 2002). However, MLST technology is costly and requires specialist equipment. It is therefore not used extensively for routine epidemiological studies (Trindade *et al.*, 2003).

2.11.3 Staphylococcal protein A (*spa*) sequence typing

Protein A is an important virulence factor of *S. aureus*. It has five repeating analogous α-helical domains (A, B, C, D, and E) which involve in binding the Fc portion of immunoglobulin IgG. The gene encoding Protein A (*spa*) has a hyper-variable region (polymorphic X region or short sequences repeats (SSR)) that consists of 24bp repeats (variations from 21bp and 27bp have been identified) and located upstream of the region encoding the cell wall attachment sequences. This high degree of repeats is attributed to duplications, deletions and, rarely, to point mutations. The sequence of these tandem repeats in the X region has been used as alternative typing method to those currently in use. The *spa* typing approach was first introduced in 1996 as a single-locus sequence typing method by Frenay and his colleagues. Its discriminatory power lies between MLST and PFGE and has the advantages of being rapid, easy to use and interpret and has an established database (Deurenberg *et al.*, 2007: Moodley *et al.*, 2006: Shopsin *et al.*, 1999: Frenay *et al*., 1996).

Another advantage is the existence of a freely available software package for interpreting the resulting sequences. Although, the Ridom StaphType software (Ridom GmbH, Wurzburg,

Germany) is commonly used to analyze the *spa* sequences, many laboratories use the central online *spa* server (http://www.spaserver.ridom.de). This database has contributed in the widespread use of *spa* typing. To date, the *spa* database includes about 6167 *spa* types that are composed of a combination of 355 repeats from 108309 strains. Harmsen *et al.* (2003) and Koreen *et al.* (2004) have both introduced *spa* nomenclature systems. However, differences between these nomenclatures make comparisons between published *spa* data difficult (Deurenberg *et al.*, 2007). Although, *spa* typing has many advantages and is widely used as a single-locus typing technique, it is susceptible to chromosomal replacements that can falsely cluster genetically unrelated strains. Moreover, the cost of sequencing is still considered as a limitation of using *spa* typing extensively in routine clinical laboratories (Karynski *et al.*, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Kaduna state, Nigeria. Kaduna state is located in north western Nigeria on latitude 7º 26' E and longitude 10º 31' N, 1995ft above sea level (Google Earth, 2011). It has distinct wet and dry seasons within the guinea and part of the Sudan savannah zone of Nigeria.

Kaduna was the former regional capital of the defunct northern regional government. It is home to the traditional institutions of Zazzau and Birnin Gwari emirates with the Zazzau emirate being one of the foremost emirates of northern Nigeria. Zaria accommodates one of the largest populations of horses courtesy of this emirate, from the lowest income earners to the high class aristocrats keeping horses as symbols of tradition, pride and prestige.

Kaduna has also been host to the Nigerian army with several installations including the cavalry unit of the army and Mounted Troops of the police both keeping horses for their respective forces and providing stables and veterinary care for other horses.

Kaduna has played host to the oldest polo club in Nigeria; Kaduna Polo Club, which has been host of the oldest and most prestigious polo cup in Africa: The Georgian cup. All these reasons make Kaduna a suitable place to study MRSA in horses and horse handlers

3.2 Study Design

This was a cross-sectional study involving a one-stage cluster sampling of clinically normal horses selected from the study area. Horses selected were in three categories; horses used for

sports (mostly exotic/imported horses), horses kept locally for recreational, cultural or durbar, and agricultural activities, and institutional horses i.e., cavalry horses kept by the army and police. Animals were sampled only after owner's consent was given. All samples were collected by a Veterinarian.

3.3 Sample Size Determination

This was determined using the formula by Thrusfield, 1997

$N= Z^2pq/d^2$ using a confidence interval of 95%

Where N= sample size

p= prevalence (5% Weese *et al.,* 2006)

q= 1-p

d= 0.05

Z= 1.96

$N= 1.96^2 \times (0.05 \times 0.95)/0.05^2$

= 72.99

73 horses approximately.

A total of 240 horses and a convenience sample of 65 persons were taken.

Minimum number of clusters sampled; this was determined using the formula by Thrusfield, (1997) for the minimum number of clusters:

$g= 1.96^2(nVc+Pexp(1-Pexp))/nd^2$

Where;

g= number of clusters to be sampled

n= predicted average number of animals per cluster

Pexp= expected prevalence

d= desired absolute precision

Vc= between cluster variation

$g = 1.96^2(10\times0.0345+0.05(1-0.05))/10\times0.05$

$= 3.8416\times0.3925/0.5$

$= 3.0$ clusters

3.4 Sample Collection

Each horse sampled was properly restrained and its bio-data taken. A single nasal swab was collected from each horse. A moistened cotton-tipped culture swab was inserted approximately 10 cm into one nasal passage and withdrawn after 3 to 4 rotations with the swab in contact with the nasal mucosa. Swabs were promptly placed into appropriately labelled sample bottles containing liquid Stuart's medium (Oxoid Basingstoke, UK) and kept at 4ºC in a cool box and immediately transported to the bacteriology laboratory of the Department Of Veterinary Public Health and Preventive Medicine, A.B.U, Zaria for processing.

All consenting individuals within a farm collected a single nasal swab themselves according to instructions that were provided. A moistened cotton-tipped swab was inserted approximately 1cm into each anterior nasal cavity and withdrawn with the swab in contact with the nasal mucosa. Each of the samples collected were identified by appropriate labeling. Inclusion criteria for being sampled included individuals of any age, gender, or profession who reside with 1 or more horse of any age, gender, or breed. (Hanselman *et. al.*, 2006)

3.5 Materials Used

3.5.1 Bacteriological culture media

All the media used in this study were obtained from Oxoid (Basingstoke, UK), and these included; Brain Heart Infusion Broth (CM1135), Columbia agar base (CM0331B), Baird Parker

agar (CM1127B), and Egg Yolk tellurite emulsion (SR0054C), Nutrient agar (CM3), Mueller Hinton agar (CM0337), and Andrade peptone water (CM0061).

3.5.2 Sugars

The sugars used for the confirmation of *Staphylococcus aureus* are mannitol, glucose, sucrose, maltose, and inositol. They were obtained from Fluka, USA. These sugars were prepared and reconstituted with Andrade's peptone water according to label instructions (Oxoid, Basingstoke, UK).

3.5.3 Antibiotic impregnated discs and their concentrations.

All antibiotic discs were obtained from Oxoid, Basingstoke, UK. These antimicrobial agents were selected based on the class differences in their modes of action and mechanisms of resistance. The details of the antibiotics used and their concentrations are given in Table 3.1.

3.5.4 Other materials used

Other materials used include; distilled water (Juhel®), Pasteur loop, cotton wool, sterile swab sticks (Tyconpacey®), normal saline (Juhel®), syringes and needles (Agar-Jec®), permanent marker (Laries®), aluminium foil, masking tape, test tubes, sample bottles (Sterile-CEO®), EDTA bottles (Sterile-CEO®), test tube racks, bijoux bottles, disposable petri dishes, conical flasks, measuring cylinder, disposable pipettes (Cosar®), micropipette (Per-Fect®), refrigerator, incubator (Gallenhamp®), weighing balance (Gallenhamp®), hot air oven (Gallenhamp®), autoclave (Gallenhamp®), Bunsen burner, mini-columns (Bioneer®), PCR water (Bioneer®), micro centrifuge tubes (Bioneer®).

3.6 Isolation of *S. aureus*

3.6.1 Brain heart infusion (BHI) broth

Brain Heart Infusion broth was used for enrichment. This was prepared according to the manufacturer's instructions. Swabs from liquid Stuarts transport medium were transferred into the sterile BHI broth (3ml) and incubated aerobically at 37°C for 24hours. Enrichment was manifested by increased turbidity of the medium after the incubation period.

3.6.2 Columbia media with 5% sheep blood.

Columbia Agar (Oxoid, Basingstoke, United Kingdom) was prepared according to the manufacturer's instructions. The media were streaked for isolation with inoculating loop. Cultures were incubated aerobically at 37°C and examined for growth after 24hours. *Staphylococcus aureus* was identified as duff colored, smooth, round and raised colonies that exhibited an area of clearance around the colonies (β-hemolysis).

3.6.3 Baird parker-egg yolk+tellurite media.

Baird parker-egg yolk agar (Oxoid, Basingstoke, United Kingdom) was prepared according to the manufacturer's instructions and inoculated directly on the surface of the prepared medium with a single pure colony of suspected *Staphylococcus aureus* from the Colombia media using an inoculating loop. Cultures were incubated at 37°C and examined for growth at 24 hours. *Staphylococcus aureus* was identified as small (1-2mm in diameter), discrete, shiny, black colonies that had clear zone around the colonies.

3.7 Biochemical characterization

3.7.1 Catalase test

This test was used to detect the enzyme catalase. Catalase breaks H_2O_2 down into water and oxygen. Colonies from the Baird Parker media were used to perform this test and a drop of 3%

hydrogen peroxide was added to the growth directly. A positive test was manifested by bubbles becoming visible (these would be the O_2 bubbling up). A lack of bubbles was regarded as absence of catalase and a negative test.

3.7.2 Coagulase test

Coagulase test is a method for differentiating between pathogenic and non-pathogenic strains of *Staphylococcus*. Bacteria that produce coagulase use it as a defense mechanism by clotting the areas of plasma around them thereby enabling them resist phagocytosis by the host's immune system. The tube test is more definitive than the slide test because it can detect both bound and free plasminogen, and was carried out using rabbit plasma. Growth/organisms conforming to staphylococcus morphology after Gram staining and microscopy were inoculated into sterile BHI (1ml) broth overnight. After incubation, the broth was inoculated into 5ml of diluted rabbit plasma and incubated anaerobically at 37°C for 24hours. Coagulase positive organisms cause fibrin threads to form between the cells, causing them to agglutinate. If agglutination occurred within 4hours post inoculation, it was graded +4, if it occurred within 4-24hours post inoculation, it was graded +1 to +3. A negative test did not show clumping within 24hours post inoculation.

3.7.3 Sugar fermentation

Five sugars were used; mannitol, maltose, glucose, sucrose, and inositol. 1% concentration of each sugar was prepared using Andrade peptone as basal medium, in bijoux bottles. These sugar solutions were then inoculated with the organisms from pure culture with gentle agitation for proper mixing and incubated anaerobically (tightening the caps) at 37°C for 24-72hours. A positive sugar fermentation test was manifested by colour change from bright red to pink.

3.8 Antibiotic sensitivity testing

The disk diffusion method was used to determine the antibiotic susceptibility profile of the *Staphylococcus aureus* isolates following the Clinical and Laboratory Standards Institute (CLSI) methods (2010). Mueller Hinton agar plates were inoculated with a colony suspension of 0.5 McFarland units prepared through dilution of a 1ml overnight culture of the test isolate with 7ml sterile water to make a final volume of 8ml, 200µl of the suspension was spread on Mueller Hinton medium using a sterile cotton-wool swab stick. After few minutes to allow for pre diffusion, antibiotic discs were placed on the dried plate inoculated with test organism and incubated at 35-37ºC for 16-20h. After 16hours incubation the plates were examined and diameter of the zones of growth inhibition measured in millimeters. The results of sensitivity or resistance were interpreted according to the Clinical and Laboratory Standards Institute Guidelines (2010). Eight classes of antibiotics were tested.

Table 3.1. Antibiotics discs used and break points for each based on CLSI, 2010.

Antibiotics	Concentration	Resistant	Intermediate	Susceptible
Oxacillin	5µg	≤10mm	11-12mm	≥13mm
Penicillin G	10 units	≤28mm		≥29mm
Gentamicin	10µg	≤12mm	13-14mm	≥15mm
Amikacin	30µg	≤14mm	15-16mm	≥17mm
Erythromycin	5µg	≤13mm	14-22mm	≥23mm
Sulphamethoxazole+Trimethoprim	25µg	≤12mm	13-16mm	≥17mm
Tetracycline	30µg	≤14mm	15-18mm	≥19mm
Ciprofloxacin	5µg	≤15mm	16-20mm	≥21mm
Chloramphenicol	30µg	≤12mm	13-17mm	≥18mm
Vancomycin	30µg			≥15mm

3.9 PBP2a latex agglutination test

This test was carried out using the PBP2a latex agglutination test kit obtained from Oxoid, Basingstoke, UK. The PBP2a extraction procedure was carried out by first adding 4 drops of extraction reagent 1 into a microcentrifuge tube, a loopful of the test culture was then suspended in the microcentrifuge tube with the reagent and placed in boiling water for 3minutes. The tube was then removed and allowed to cool to room temperature, followed by addition of a drop of extraction reagent 2 and mixed thoroughly. This was followed by centrifugation at 1500g for 5minutes. The supernatant was then used for the latex agglutination test. Each circle on the test card was labelled test circle and control circle with one drop of the test latex and control latex added to the labelled circles respectively. 50µl of supernatant was then added to each circle and mixed thoroughly with a mixing stick. The card was gently rocked for up to three minutes and results interpreted thus: Agglutination was seen with test latex but not control latex within 3minutes; PBP2a positive (MRSA), no agglutination in either test latex nor control latex within 3minutes; PBP2a negative (MSSA), and agglutination was seen with the control latex within 3minutes; Indeterminate.

3.10 Genotyping

3.10.1 DNA extraction; Extraction of the DNA by lysostaphin treatment using the Wizard® Genomic DNA purification Kit.

Six millilitres of overnight culture of the test organism in BHI broth was transferred to four 1.5ml micro centrifuge tubes and centrifuged for 3 min at 13000*g*. The supernatants were discarded and the pellets suspended in 180µl lysis buffer lacking lysozyme but with 10µl lysostaphin (1mg/ml). The suspension was incubated at 37ºC for 30 min and then 25µl of proteinase K and 200µl AL buffer were added and the mixture incubated at 56ºC for 30 min.

200µl of ethanol (100%) was added and mixed thoroughly. The mixture was transferred into a Wizard® Mini spin column placed in a 2ml collection tube and centrifuged for 1 min at 8000*g*. The collection tube with flow-through was removed and replaced with a new collection tube. 500µl of AW1buffer was added and the tube centrifuged for 1 min at 8000*g* and the flow-through discarded. 500µl of AW2 buffer was added and centrifuged for 2 min at 13,000*g* and the flow-through again discarded. Finally, the Wizard® Mini spin column was placed in a 1.5ml micro centrifuge tube and 200µl of AE buffer was added onto Wizard® membrane, incubated for 1 min at room temperature and centrifuged for 1 min at 8,000*g*. This step was repeated in a new 1.5ml micro centrifuge tube and the DNA stored at -20ºC.

3.10.2 Multiplex polymerase chain reaction (M-PCR)

The staphylococcal cassette chromosome *mec* (SCC*mec*) types were determined by multiplex PCR for the *mecA-IS431* gene, IS1272 gene, *ccr*C gene, and *ccr*A2-B gene, to confirm the identification and methicillin resistance of *S. aureus*. This was carried out as described by Boye *et al.,* (2006). Four primer sets were used to ensure amplification of two DNA targets from SCC*mec* type IV and two targets from SCC*mec* type V. The targets were chosen so that one target would be amplified from each of SCC*mec* type's I–V (Table 3.2). PCR was performed in a total volume of 50µL containing 1*AmpliTaq PCR buffer, 1.5mM MgCl, 200µM each dNTP and 1U of AmpliTaq DNA polymerase. Based on optimisation experiments, primer concentrations were as follows: primers b and a3, 0.2µM each; *ccr*CF and *ccr*CR, 0.25µM each; 1272F1 and 1272R1, 0.08µM each; and 5RmecA and 5R431, 0.1µM each. Amplification comprised 4 min at 94°C, followed by 30 cycles of denaturation 30 s at 94°C, 30 s at 55°C and 60 s at 72°C, with a final extension for 4 min at 72°C. PCR products (10µL) were analyzed by electrophoresis on agarose 1.5% W/V gels, with ethidium bromide added to the gel. The SCC*mec*

type was determined on the basis of the band pattern obtained. Isolates with no visible bands, or with a band pattern that was not in agreement with one of the five predicted band patterns, were classified as not-typed (NT).

Table 3.2. Primers used in the multiplex SCC*mec* PCR and the resulting amplicon sizes for SCC*mec* type I–V.

Name	Primer sequence (5'---3')	Amplicon length expected	Target gene
β	ATTGCCTTGATAATAGCCYTCT[a]	937 bp	*ccrA2-B*
α3	TAAAGGCATCAATGCACAAACACT[a]		
***ccrC*F**	CGTCTATTACAAGATGTTAAGGATAAT[b]	518 bp	*ccrC*
***ccrC*R**	CCTTTATAGACTGGATTATTCAAAATAT[b]		
1272F1	GCCACTCATAACATATGGAA[c]	415 bp	IS1272
1272R1	CATCCGAGTGAAACCCAAA[c]		
5R*mecA*	TATACCAAACCCGACAACTAC[c]	359 bp	*mecA*–IS431
5R431	CGGCTACAGTGATAACATCC[c]		

a. Ito *et al.,* 2001

b. Ito *et al.,* 2004

c. Boye *et al.,* 2006

3.11 Identification of Risk factors associated with MRSA in horses and horse handlers.

A structured questionnaire pretested on a subset of the sample population was administered to all horse handlers whose horses were sampled. Written consent was sought for prior to administration. The questionnaire was administered through interview for non-literate individuals while for literate horse handlers they were given the questionnaire to fill themselves. A total of 305 samples were collected from horses and horse handlers (persons with occupational contact) during the period from 19th of April 2012 to 25th September 2012. Clinical information about the horses and handlers was recorded in a database – samples were coded to facilitate cross-reference between horses and handlers, but no person's name were used. Results were transferred to excel spread sheet, filtered for consistency and finally put in SPSS version 16.0 for analysis.

3.12 Data Analysis

Variables were grouped into two categories based on expected outcomes/responses; binomial outcomes and multinomial outcomes/responses. Variables with binomial outcomes were placed in 2x2 contingency tables and assessed for risk of association with MRSA colonisation using Odds Ratio at 95% confidence interval, Chi square and p-values were calculated to check for statistical significance. Variables with multinomial outcomes/ responses were assessed using Chi Square with p-values determined at 95% confidence interval to check for statistical significance, risk was assessed for these variables in ordered tables using concordances and discordances ratio. Knowledge was assessed through scoring of responses and subsequent grading of such scores as poor, fair, and good knowledge.

CHAPTER FOUR

RESULTS

4.1 Isolation and Identification of MRSA from horses and horse handlers.

Of the 240 samples collected from the horses during the period, 15.4% (37/240) of these samples were confirmed as *Staphylococcus aureus* using the standard microbiological approach. While of the 65 samples collected from the horse handlers during the same period, 10.8% (7/65) were *S. aureus*. Of these, 24 from horses and 4 from horse handlers were identified as MRSA through antibiotic susceptibility testing using the disk diffusion method.

A prevalence of 10% (24/240, $P \leq 0.05$) was recorded for MRSA in horses while the MSSA had a prevalence of 8.3% (20/240, $P \leq 0.05$). Amongst the three horse categories, highest prevalence was recorded for the institutional and performance horses with 11.3% (9/80, $P \leq 0.05$) each. Traditional horses had a relatively lower prevalence of 7.5% (6/80, $P \leq 0.05$). (Table 4.1)

An overall prevalence of 6.2% (4/65, $P \leq 0.05$) was found in horse handlers, with highest prevalence of 6.7% (1/15, $P \leq 0.05$) each amongst horse handlers within the traditional and performance horses' categories. Horse handlers of institutional horses had a lower prevalence of 5.7% (2/35). (Table 4.2)

Table 4.1. Prevalence of MRSA isolated by category of horses

Category	**No. MRSA positive/No. sampled**	**Prevalence**
Institutional	9/80	11.3%
Performance	9/80	11.3%
Traditional	6/80	7.5%
Total	**24/240**	**10%**

$\chi^2 = 41.0$ df= 1 p= 0.000

Table 4.2. Prevalence of MRSA in horse handlers by category of horses.

Category	No. MRSA positive/No. sampled	Prevalence
Institutional	**2/35**	**5.7%**
Performance	**1/15**	**6.7%**
Traditional	**1/15**	**6.7%**
Total	**4/65**	**6.2%**

$\chi^2 = 46.0$ df= 1 p= 0.000

4.2. Antibiotic Susceptibilities of the MRSA Isolates from the Horses and Horse Handlers.

There was 100% resistance to penicillin and oxacillin by the MRSA isolates from both horses and horse handlers. High resistance of 62.5% and 50% to erythromycin was encountered for the horses and horse handlers respectively. This was followed by resistance of the horse isolates to tetracycline with 41.7% and amikacin with 33.3%. Resistance level recorded for sulphamehoxazole+trimethoprim was 12.5%. Gentamicin, ciprofloxacin, and chloramphenicol recorded resistance levels of 4.2% each. (Table 4.3)

Nineteen resistance profiles were obtained ranging from no resistance (4.5%) to resistance to six antibiotics (6.8%). Resistance to four antibiotics had highest rate of occurrence of 22.7% with the profile: Pen:Oxa:Ery:Van being most common (11.3%). Resistance to three antibiotics recorded a rate of 20.5% with the profile: Pen:Oxa:Ery being most common (9.1%). Resistance to five antibiotics recorded 13.6% occurrence rate, while 18.1% and 15.9% were recorded for resistance to two and one antibiotic respectively. (Table 4.4)

There was marked expression of antibiotic resistance amongst the MRSA isolates, with 74.8% of the isolates exhibiting multidrug resistance (MDR).

Table 4.3. Antimicrobial Resistance of MRSA Isolates from Horses and Horse Handlers.

Antibiotic	MRSA from horses, N=24	MRSA from horse handlers, N=4
β-lactams		
Penicillin (5units)	24 (100)	4 (100)
Oxacillin (5µg)	24 (100)	4 (100)
Aminoglycosides		
Amikacin (30µg)	8/24 (33.3)	0 (00)
Gentamicin (10µg)	1/24 (4.2)	0 (00)
Macrolide		
Erythromycin (5µg)	15/24 (62.5)	2/4 (50)
Tetracycline		
Oxytetracycline (30µg)	10/24 (41.7)	0 (00)
Sulphonamide		
Sulphamethoxazole+trimethoprim (25µg)	3/24 (12.5)	0 (00)
Fluoroquinolone		
Ciprofloxacin (5µg)	1/24 (4.2)	0 (00)
Phenicols		
Chloramphenicol (30µg)	1/24 (4.2)	0 (00)
Glycopeptide		
*Vancomycin (30µg)	10/24 (41.7)	1/4 (25)

Key; N=number of MRSA, percentage of resistance in parenthesis.
*vancomycin resistance is only confirmed after MIC test.

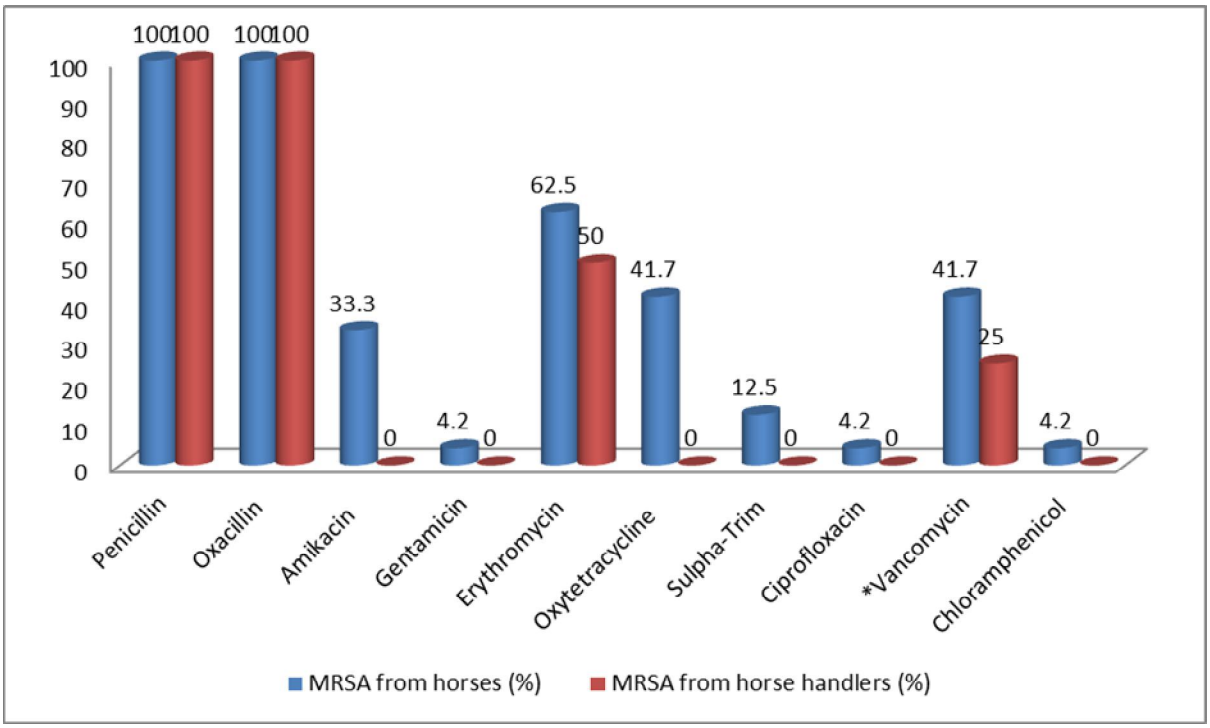


Figure 4.1. Resistance levels to various antibiotics for the MRSA isolates from horses and horse handlers.

Table 4.4. Antibiotic resistance patterns of *S. aureus* isolates (n=44).

Resistance Profiles	No. of *S aureus* (%)
No resistance	2 (4.5%)
Penicillin	7 (15.9%)
PEN;OXA	5 (11.3%)
PEN;TET	3 (6.8%)
PEN;OXA;AMI	2 (4.5%)
PEN;OXA;ERY	4 (9.1%)
PEN;OXA;TET	1 (2.3%)
PEN;ERY;TET	1 (2.3%)
PEN;TET;AMI	1 (2.3%)
PEN;OXA;ERY;VAN	5 (11.3%)
PEN;OXA;TET;AMI	3 (6.8%)
PEN;OXA;GEN;AMI	1 (2.3%)
PEN;TET;ERY;VAN	1 (2.3%)
PEN;OXA;TET;ERY;VAN	2 (4.5%)
PEN;OXA;TET;ERY;AMI	2 (4.5%)
PEN;OXA;ERY;AMI;VAN	1 (2.3%)
PEN;OXA;ERY;CHL;VAN	1 (2.3%)
PEN;OXA;TET;ERY;SUL;CIP	1 (2.3%)
PEN;OXA;TET;ERY;SUL;VAN	2 (4.5%)

4.3. PBP2a latex agglutination test to confirm methicillin resistance.

There was good correlation between resistance testing using disk diffusion and latex agglutination for identification of MRSA and MSSA. Twenty five of the isolates out of the twenty eight identified as MRSA through disk diffusion tested positive for the penicillin binding protein 2a (89%) whereas all the sixteen MSSA isolates identified using disk diffusion all tested negative (100%). (Table 4.5)

Table 4.5. PBP2a latex agglutination test using PBP2A latex agglutination test kit (Oxoid, United Kingdom).

Isolate	Disk diffusion	PBP2a (%)
MRSA	28	25/28 (89%)
MSSA	16	16/16 (100%)

4.4. SCC*mec* Typing

Staphylococcal cassette chromosome (SCC) *mec* typing results showed high occurrence of SCC*mec*III with 13 isolates (46%) from eleven horses and two horse handlers. This was followed by 5 isolates (18%) having SCC*mec*I from one horse handler and four horses. Four isolates (14%) belonged to SCC*mec*IV wih four horses also and one horse handler while only one (4%) isolate was SCC*mec*V isolated from a horse. Five isolates (17.9%) were non-typeable and SCC*mec*II types were not identified in this study (Plates 5 and 6)

Table 4.6. SCC*mec* typing result based on method described by Boye *et al,* (2009).

Isolate	SCC*mec* type	Source
ATCC 33591	Type III	Food
HMT 05	Type III	Human
HRT 10	Type IV	Horse
HRT 19	NT	Horse
HRT 35	Type III	Horse
HRT 57	Type III	Horse
HRT 64	Type III	Horse
HRT 80	Type III	Horse
HMIA 13	Type I	Human
HRIA 16	NT	Horse
HRIA 22	Type III	Horse
HRIA 24	Type III	Horse
HMIP 05	Type IV	Human
HRIP 01	Type III	Horse
HRIP 07	Type IV	Horse
HRIP 18	Type V	Horse
HRIP 21	NT	Horse
HRIP 26	Type III	Horse
HMS5 14	Type III	Human
HRS5 24	NT	Horse
HRS5 41	Type I	Horse
HRS5 52	Type III	Horse
HRS5 57	Type III	Horse
HRS5 67	Type I	Horse
HRS5 76	Type I	Horse
HRS5 82	Type III	Horse
HRS5 84	Type IV	Horse

HRS5 87	Type I	Horse

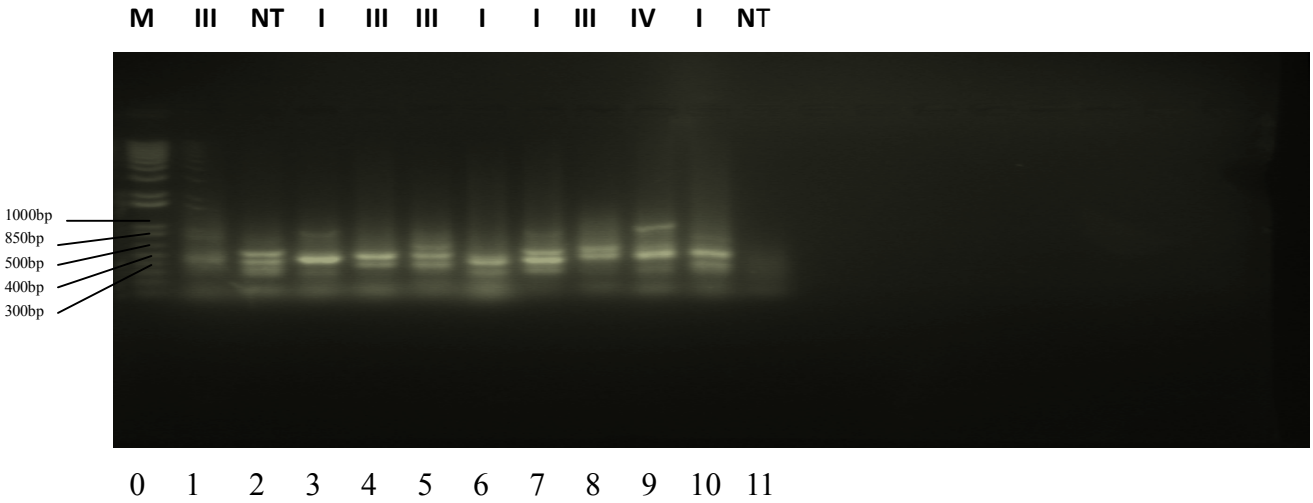


Plate 1**.** Gel electrophoresis of multiplex PCR analyses of 11 MRSA isolates, to identify their SCC*mec* type. Control strain with known SCC*mec* was also analysed (lane 2). 100bp marker was used (lane M). Lanes 2 and 11 (SCC*mec* Non-Typeable), lanes 3, 6, 7, 10 (SCC*mec* I), lanes 1, 4, 5, 8 (SCC*mec* III), lane 9 (SCC*mec* IV).

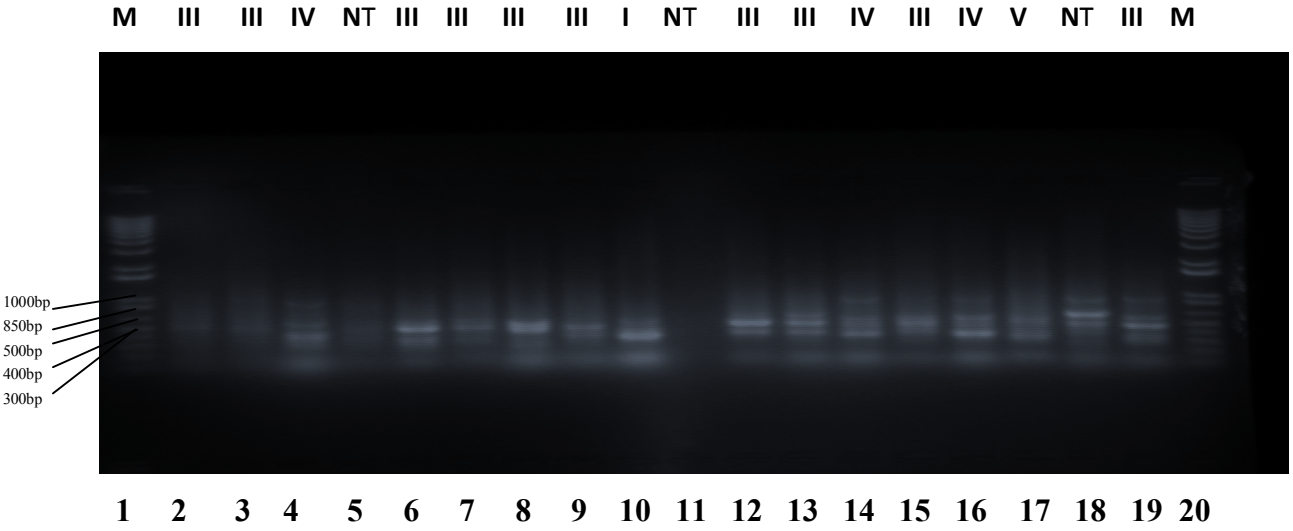


Plate 2**.** Gel electrophoresis of multiplex PCR analyses of 18 MRSA isolates, to identify their SCC*mec* type. Control strain with known *SCCmec* was also analysed (SCC*mec* III; lane 2). 100bp marker (lanes 1 and 20) was used. Lanes 5, 11, 18 (SCC*mec* Non-Typeable), lane10 (SCC*mec* I) lanes 3, 6, 7**,** 8, 9, 12, 13, 15, and 19 (SCC*mec* III), lanes 4, 14, 16 (SCC*mec* IV) and lane 17 (SCC*mec* V)

4.5. Risk Factor Analysis

Risk factors identified amongst variables with binary outcomes in this study included being a veterinarian (OR=9.0), and using protective clothing such as face mask (OR=10.3) and hand gloves (OR=19.0). (Table 4.6)

For variables with multinomial outcomes, risk factors identified include daily contact during restraint of the animal (OR=3.9) and visiting a hospital for medical reasons or to see a patient (OR=2.09). (Table 4.7)

Sex (male or female), feeding and watering activity, washing hands with water only after handling, previous antimicrobial therapy 4months and beyond, hospital visitation 4months and beyond, and tertiary education were not found to be risk factors for MRSA carriage in horse handlers (OR≤1).

CHAPTER FIVE

DISCUSSION

In this study, MRSA prevalence rate of 10% in horses screened in three horse categories was relatively higher than the prevalence rates recorded in previous studies (Weese *et al.,* 2007: Vengust *et al.,* 2006) . However, care must be taken when interpreting these data because screening was performed only once. Similar limitations are present with the prevalence data regarding MRSA colonization for traditional/recreational horses, which was 7.5% but was lower in comparison with the prevalence rates for the institutional and performance horses which was 11.3% each. This may not be unrelated to the population setup in these categories of horses; traditional horses are tethered to trees or poles in front or at the back of the household sparsely located from other neighbouring horses at relatively safe distances to reduce the chances of transmission between the horses, unlike for the latter two where the horses are housed in stables side by side closely clustered in their multitudes thus increasing the likelihood of transmission between the horses. The prevalence of 6.2% for the horse handlers was different from previous studies where 4.7% samples were MRSA positive (Weese *et al.,* 2006). Conversely the horse handlers of traditional and performance horse categories had a prevalence rate of 6.7% slightly higher when compared with horse handlers of institutional horses at 5.7% prevalence rate; this may be explained by the fact that horse handlers from the institutional horses do not reside with or in close proximity with their horses nor engage in any other activity other than feeding and watering of the horses and sometimes cleaning the stables all of which are done when the horses are out of the stable. This is unlike in the case of the traditional and performance horses where the handlers live in close proximity with the horses and engage the horses frequently from the activities mentioned above to grooming, hoof care and shoeing, exercising and restraint, thus

increasing the chances of transmission. Horse handlers interact with both hospital and community population as part of their job and thus have the potential for exposure to MRSA from both sources. Why MRSA has emerged in the equine population is unclear, but it may reflect increased exposure of horses to MRSA-infected persons, a unique ability of strains to colonize horses, the increasing use of certain antimicrobial drugs in veterinary medicine, or a combination of these factors.

The non-expression of the *mecA* gene in 2 out of the 27 MRSA isolates could not be readily explained in this study, however other researchers have reported such phenomenon and referred to such isolates as pre-MRSA (Lewis and Dyke, 2000: Brown *et al.,* 2005).

Antibiotic resistance appeared to be more pronounced among the *S. aureus* isolated from horses compared to the *S. aureus* isolated from horse handlers. This enhanced antibiotic resistance of the horse *S. aureus* strains might be caused by horizontal gene transfer under the pressure of an enhanced antibiotic treatment of the host. These results also indicate that *S. aureus* of human origin, at least the strains investigated in the present study seemed to be of minor importance. As previous studies from South-West Nigeria had shown (Ghebremedhin *et al.,* 2009), it appears that there is a high proportion of *S. aureus* isolates resistant to erythromycin and tetracycline. This could be associated with the fact that tetracycline historically had wide clinical application, is inexpensive, orally administered and available from diverse sources where they are sold with or without prescription in Nigeria. In this study, all the trimethoprim resistant *S. aureus* isolates were MDR and carried SCC*mec* III element which is large and can carry a lot of resistance genes (Deurenberg *et al.,* 2007), but has also been suggested that mutation of the dihydrofolate reductase (DHFR) enzyme of the bacteria is responsible for such resistance (Chongtrakool *et al.,* 2006).

Minor differences between human and equine isolates were found in the SCC*mec* composition of MRSA from this study such as identification of the site specific recombinase gene *(ccrAB2)* in four equine isolates, the same recombinase system associated with a majority of human MRSA strains (SCC*mec* IV): of which only one human isolate was identified in this study. This may reflect independent acquisition of allelic variants from coagulase-negative staphylococci equine strains or from human MRSA (Vengust *et al.,* 2008). However, 12 MRSA isolates possessing the SCC*mec* type III element was alarming as this element is associated with HA-MRSA, thus reflecting the possible shift of MRSA from a nosocomial pathogen to a community associated pathogen and may even explain the high level of multidrug resistance encountered.

This study identified a variety of factors that were associated with MRSA colonization in horses. However, it was surprising that residence on a farm where one or more horses had previously been diagnosed with MRSA carriage/colonization was not associated with MRSA carriage in the handlers, as identification of multiple colonized horses on a farm is not uncommon (Weese *et al.,* 2006). In this study there was no association between MRSA and equipment sharing as reported in earlier studies (Weese, 2007). It was not particularly surprising that previous antimicrobial therapy within the last 4months was associated with MRSA colonization in this study as has been reported previously (Mamoon *et al.,* 2008). Previous antimicrobial therapy has also been reported as a risk factor in humans, yet there is increasing recognition of MRSA infection in humans without risk factors, such as antimicrobial use. Regardless, the association of antimicrobials with MRSA colonization highlights the need for prudent antimicrobial use in veterinary medicine. The finding that being a veterinarian or veterinary assistant was risk factor was not surprising and is without a doubt understandable. However, it was surprising that protective clothing such as face mask and hand gloves were risk factors. This perhaps may not be

unrelated to the fact that they can serve efficiently as fomites for the carriage and transmission/spread of MRSA. Interestingly, previous antimicrobial therapy was not a risk factor for horses in this study: in fact, the only identified risk factor for horses with statistical significance was allergy. In humans, contact with healthcare facilities is a commonly reported risk factor for CA-MRSA infection or colonization (Cuny *et al.,* 2006) as was also found in this study. Care must be taken in comparing human and equine data, because the nature of the equine population and equine healthcare is quite different from that of humans. In fact, some major subsets of the equine population, such as breeding farms, may mimic the human healthcare situation more closely than the human community situation because of the frequent contact of horses with veterinary personnel, frequent antimicrobial use, regular inter-farm movement of horses, and care by a constant group of farm personnel that may concurrently be managing sick horses. Also identified in this study as a risk factor was contact during restraint; this may be explained by the fact that restraint in horses has to do mainly with head or leg restraint (prevent kicking) thus enhancing the likelihood of transmission of MRSA from the nares, facial area, and hoof of the horse. Surprisingly, protective factors identified from this study include exercise contact, handling 4-5horses daily, and being a groom; these are without a real explanation. Unsurprisingly perhaps were protective factors such as feeding and watering contact which is explainable; since the horses are usually out in the paddocks and stables cleaned when this activity is taking place thus minimizing contact between the handler and horses. Also unsurprising is washing hands with water only following contact with the horse, a tertiary education and a fair knowledge of MRSA. Another interesting protective factor was sex (being female); this may however be as a result of sampling bias since only two females, both of them

veterinarians were available for this study. This finding emphasizes the need to consider putative risk factors in concert with others when investigating relationships with any outcome.

CHAPTER SIX

CONCLUSION

This study has shown that MRSA infection may be an emerging disease in horses, which agrees with earlier reports (Weese, 2007). Emergence of MRSA as an equine pathogen is of concern because horses may be a community reservoir of MRSA and source of infection or reinfection for persons. In view of the size of the Nigerian horse population and the frequent close contact between many persons and horses, this concern must not be dismissed. However, the results of this study are important as they indicate high levels of multidrug resistance and possible interspecies transferability of certain MRSA genotypes. This may contribute to clarifying the question of why some genotypes seem to be easily transferable between different hosts. Also shown are factors associated with horses being colonized and the associated risk for the horse handlers that work with such horses or horse environment, something that is important to know for the design and implementation of infection control programs.

CHAPTER SEVEN

RECOMMENDATIONS

Based on the findings of this study, the following recommendations are advocated;

1. There is need for detailed surveillance for MRSA in horses and horse handlers

2. Prudent use of antimicrobial agents accompanied with reporting of suspected or confirmed cases of resistance for *S. aureus* and MRSA by veterinarians and other professionals

3. Improvement/implementation of strict infection control measures on farms/stables and in hygiene (especially hand hygiene) for persons working with horses or animals generally with isolation of infected animals and use of barrier precautions

4. Public enlightenment on effects of indiscriminate use of antibiotics as well as enactment of effective drug control policies

5. Further studies should be undertaken to design farm- and hospital-based screening programs to lessen the dissemination of this potentially important veterinary and zoonotic pathogen.

REFERENCES

Aarestrup F. M., Agerso Y., Ahrens P., Jorgensen J. C. O., Madsen M., and Jensen L. B. (2000). Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. *Veterinary Microbiology*. 74:353–364

Aarestrup F. M., Cavaco L., Hasman H. (2010). Decreased susceptibility to zinc chloride is associated with methicillin resistant *Staphylococcus aureus* CC398 in Danish swine. *Veterinary Microbiology*; 142(3-4):455-7.

Abbott Y., Leggett B., Rossney A. S., (2010). Isolation rates of methicillin-resistant *Staphylococcus aureus* in dogs, cats and horses in Ireland. *Veterinary Record.* 166:451–455.

Aires de Sousa M., and de Lencastre H. (2004). Bridges from hospitals to the laboratory: genetic portraits of methicillin-resistant *Staphylococcus aureus* clones. *FEMS Immunology and Medical Microbiology*. 40: 101-11.

Ako-Nai A. K., Ogunniyi A. D., Lamikanra A., and Torimiro S. E. (1991). The characterisation of clinical isolates of *Staphylococcus aureus* in Ile-Ife, Nigeria. *Journal of Medical Microbiology.* 34109-112.

Al Zahrani I. A., (2011). A novel multiplex PCR-based tool of typing strains of *Staphylococcus aureus*: Chapter 1, page 4; PhD thesis.

Anderson M. E. C., Lefebvre S. L., Weese J. S. (2007). Evaluation of prevalence and risk factors for Methicillin-resistant *Staphylococcus aureus* colonization in veterinary personnel attending an international equine veterinary conference. *Veterinary Microbiology*, 129, 410-417.

Anzai T., Kamada M., Kanemaru T., Sugita S., Shimizu A., Higuchi T. (1996). Isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) from mares with metritis and its zooepidemiology. *Journal of Equine Science.* 7, 7–11.

Archer G. L. (1998). *Staphylococcus aureus*: a well-armed pathogen. *Clinical Infectious Disease*. 26, 1179–1181.

Atlas R. M. (1997). Genetic Mutation, Recombination, and Mapping. pp. 296-349 Principles of Microbiology, 2nd Edition. Wm. C. Brown Publishers, Dubuque, Iowa.

Baptiste K. E., Williams K., Willams N. J., Wattret A., Clegg P. D., Dawson S., Corkill J. E., O'Neill T., and Hart C. A. (2005). Methicillin-resistant staphylococci in companion animals. *Emerging Infectious Diseases*. 11:1942–1944.

Bartley J. (2002) First case of VRSA identified in Michigan. *Infection Control and Hospital Epidemiology*. 23:480.

Ben-David D., and Rubinstein E. (2003). Treatment of MRSA infections. pp. 275-316. In, MRSA Current Perspectives (Ad C. Fluit and Franz-Josef Schmitz, Eds) Caister Academic Press, Norfolk, England.

Berger-Bachi B. (2002). Resistance mechanisms of gram-positive bacteria. *International Journal of Medical Microbiology*. 292: 27-35.

Binh A. D. (2006). Roles of 34 Virulence Genes in the Evolution of Hospital- and Community-Associated Strains of Methicillin-Resistant Staphylococcus aureus. *Journal of Infectious Disease*. 193:1495-1503.

Bokarewa M. I., Jin T., (2006). "Pathogens in focus *Staphylococcus aureus* staphylokinase." *The international Journal of Biochemistry and Cell biology.* 38: 504-509.

Bottone, E.J., Girolami R., Stamm .J.M., (1984). Schneierson's Atlas of Diagnostic Microbiology 9th edition Abbott Park IL: Abbott Laboratories; *Staphylococcus*; p 10-13.

Bowersox John (1999). "Experimental Staph Vaccine Broadly Protective in Animal Studies"National Institutes of Health. Archived from the original on 5 May 2007. Retrieved 28 July 2007.

Boye K., Bartels M. D., Andersen I. S., Møller J. A., and Westh H. (2007) A new multiplex PCR for easy screening of methicillin-resistant Staphylococcus aureus SCCmec types I-V. *Clinical Microbiology and Infection.* 13: 725–727.

Boyle-Vavra S., and Daum R. S. (2007). Community-acquired methicillin-resistant *Staphylococcus aureus*: the role of Panton-Valentine leukocidin. Laboratory Investigation. 87: 3-9.

Brasel K. J., and Weigelt J. A. (2008). Community-Acquired MRSA as a Pathogen. pp. 43-54. In, MRSA (J.A. Weigelt, Ed). Informa Healthcare USA, Inc. New York.

Brown D. F. J., Edwards D. I., Hawkey P. M., Morrison D., Ridgway G. L., Towner K. J., and Wren M. W. D. (2005)**.** Guidelines for the laboratory diagnosis and susceptibility testing of methicillin-resistant *Staphylococcus aureus* (MRSA). *Journal of Antimicrobial Chemotherapy*. 56; 1000–1018

Catry B., Van Duijkeren E., Pomba M. C., Greko C., Moreno M. A., Pyörälä S., Ruzauskas M., Sanders P., Threlfall E. J., Ungemach F., Törneke K., Munoz-Madero C., Torren-Edo J. (2010). Reflection paper on MRSA in food-producing and companion animals: epidemiology and control options for human and animal health. *Epidemiology of Infection*. 138(5):626-644.

Centers for Disease Control and Prevention (2002). *Staphylococcus aureus* Resistant to Vancomycin - United States. *Morbidity Mortality Weekly Report*. 51:565-567

Centers for Disease Control and Prevention (1999). Four Pediatric Deaths from Community-Acquired Methicillin-Resistant Staphylococcus aureus-Minnesota and North Dakota, 1997-1999. *Morbidity and Mortality Weekly Report*. 48(32); 707-710.

Chambers H. F., and Deleo F. R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*. 7(9):629-641.

Chambers H. F. (2001). The changing epidemiology of Staphylococcus aureus. *Emerging Infectious Diseases*. 7:178-82.

Chambers H. F., and Hackbarth C. J. (1992). Methicillin-Resistant *Staphylococcus aureus*: Genetics and mechanisms of Resistance. pp. 21-35 In, Methicillin-Resistant *Staphylococcus aureus*: Clinical management and Laboratory Aspects (M.T. Cafferkey, Ed) Marcel Dekker, INC. New York, USA.

Chambers H. F. (1988). Methicillin-resistant *Staphylococci*. *Clinical Microbiology Review*. 1: 173-186.

Chang, S., Sievert D. M., Hageman J. C., Boulton M. L., Tenover F. C., Downes F. P., Shah S., Rudrik J. T., Pupp G. R. (2003). Infection with vancomycin-resistant *Staphylococcus aureus* containing the *van*A resistance gene. *New England Journal of Medicine.* 348: 1342–1347.

Chickering H. T., Park J. H. (1919). *Staphylococccus* pneumonia. *Journal of the American Medical Association*, 72:617-626.

Chongtrakool P, Ito T., Ma X. X., Kondo Y., Trakulsomboon S., Tiensasitorn C., Jamklang M., Chavalit T., Song J. H., and Hiramatsu K., (2006). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus. Antimicrobial Agents and Chemotherapy.* 50: 1001.

Cockfield J. D., Pathak S., Edgeworth J. D., and Lindsay J.A. (2007). Rapid determination of hospital-acquired meticillin-resistant *Staphylococcus aureus* lineages. *Journal of Medical Microbiology*. 56; 614-619.

Conte J. E., Golden J. A., Kipps J., and Zurlinden E. (2002). Intrapulmonary pharmacokinetics of linezolid. *Antimicrobial Agents and Chemotherapy.* 46: 1475-1480.

Cookson B. D., and Phillips I. (1988). Epidemic methicillin-resistant *Staphylococcus aureus. Journal of Antimicrobial Chemotherapy.* 21; 57-65.

Cooper J. E., and Feil E. J. (2004). Multilocus sequence typing--what is resolved? *Trends in Microbiology.* 12; 373-7.

Craig E. Z., Byron C., Mark A. M., James E. L., Adam A., Bruce K. B. (2004). Community-acquired methicillin-resistant *Staphylococcus aureus* among military recruits. *Emerging Infectious Disease*. 10 (5); 1941-1944.

Cui L., and Hiramatsu K. (2003). Vancomycin-resistant *Staphylococcus aureus*, p. *187*-212. In A. C. Fluit and F. J. Schmitz (ed.), *MRSA: current perspectives*. Caister Academic Press, Norfolk, England.

Cunha B. A. (2005). Methicillin-resistant *Staphylococcus aureus*: clinical manifestations and antimicrobial therapy. *Clinical Microbiology and Infection*. 11(4): 33-42.

Cuny C., Kuemmerle J., Stanek C., Willey B., Strommenger B., and Witte W. (2006). Emergence of MRSA infections in horses in a veterinary hospital: strain characterisation and comparison with MRSA from humans. *Eurosurveillance*. 11:44–47

Cuny C., Friedrich A., Kozytska S., Layer F., Nübel U., Ohlsen K., Strommenger B., Walther B., Wieler L., Witte W. (2010) Emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different animal species. *International Journal of Medical Microbiology*. 300(2-3):109-17.

De Lencastre, H., Oliveira D., and Tomasz A. (2007). Antibiotic resistant *Staphylococcus aureus*: a paradigm of adaptive power. *Current Opinion in Microbiology*. 10:428-435.

Devriese L. A., Van Damme L. R., Fameree L. (1972). Methicillin (cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases. *Zentralblatt für Veterinärmedizin Reihe B. 19:* 598-605.

Deurenberg R. H., Vink C., Kalenic S., Friedrich A. W., Bruggeman C. A., and Stobberingh, (2007). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus. Clinical Microbiology and Infection.* 13: 222.

Duquette R. A., and Nuttal T. J. (2009). Methicillin–resistant *Staphylococcus aureus* in Dogs and Cats: an emerging Problem? *Journal of Small Animal Practice*. 45; 591-597.

Enright M. C., Robinson D. A., Randle G., Feil E. J., Grundmann H., and Spratt B. G. (2002). The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academic Science,* U S A. 99: 7687-92.

Farzana K., Shah S. N., Jabeen F., (2004). Antibiotic resistance pattern against various isolates of Staphylococcus aureus from raw milk samples. *Journal of Research Science*. (Bahauddin Zakariya University, Multan, Pakistan) 15, 145–151.

Feil, E. J., Cooper J. E., Grundmann H., Robinson D. A., Enright M. C., Berendt T., Peacock

S. J., Smith J. M., Murphy M., Spratt B. G., Moore C. E., and Day N. P. (2003). How clonal is *Staphylococcus aureus*? *Journal of Bacteriology.* 185:3307-16.

Fitzgerald J. R., Sturdevant D. E., Mackie S. M., Gill S. R. and Musser J. M. (2001). Evolutionary genomics of Staphylococcus aureus: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. *Proceedings of the National Academy of Science,* U S A. 98; 8821–8826.

Foster T. J. (2005). Immune evasion by *Staphylococci. Nature Reviews Microbiology.* 3:948-58.

Francis J. S., (2005). Severe Community-Onset Pneumonia in Healthy Adults Caused by Methicillin-Resistant Staphylococcus aureus carrying the Panton-Valentine Leukocidin Genes. *Clinical Infectious Diseases.* 40:1378-9.

Frank U. (2003). Prevention and Control of Methicillin-resistant *Staphylococcus aureus* (MRSA). pp. 317- 336. In, MRSA Current Perspectives (A. C. Fluit and Franz-Josef Schmitz, Eds). Caister Academic Press, Norfolk, England.

Fred C. Tenover, Emily A. Gay, Stacie Frye, Samantha J. Eells, Mimi Healy, and John E. McGowan, Jr. (2009). Comparison of Typing Results Obtained for Methicillin-Resistant *Staphylococcus aureus* Isolates with the DiversiLab System and Pulsed-Field Gel Electrophoresis. *Journal of Clinical Microbiology*. 47 (8); 2452–2457.

Frenay H. M., Bunschoten A. E., Schouls L. M., van Leeuwen W. J., Vandenbroucke-Grauls C. M., Verhoef J., and Mooi F. R. (1996). Molecular typing of methicillin-resistant *Staphylococcus aureus* on the basis of protein A gene polymorphism. *European Journal of Clinical Microbiology and Infectious Disease.* 15:60-4.

Fridkin S. K., Hageman J. C., Morrison M., Sanza L. T., Como-Sabetti K., Jernigan J. A., Harriman K., Harrison L. H., Lynfield R., and Farley M. M. (2005). Methicillin-resistant *Staphylococcus aureus* disease in three communities. *New England Journal of Medicine*. 352: 1436-1444.

Fridkin S. K. (2001). Vancomycin-intermediate and Resistant Staphylococcus aureus: What the Infectious Disease Specialist Needs to Know. *Clinical Infectious Diseases*. 32:108-15.

Gemmell C. G., Edwards D. I., Fraise A. P., Gould F. K., Ridgway G. L., and Warren R. E. (2006). Guidelines for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the UK. *Journal of Antimicrobial Chemotherapy.* 57: 589-608.

Ghebremedhin B., Olugbosi M. O., Raji A. M., Layer F., Bakare R. A., König B., and König W. (2009). Emergence of a Community-Associated Methicillin-Resistant *Staphylococcus aureus* Strain with a Unique Resistance Profile in Southwest Nigeria. *Journal of Clinical Microbiology*. 47(9): 2975–2980.

Golding G. R., Campbell J. L., Spreitzer D. J., Veyhl J., Sury-nicz K., Simor A., Mulvey M. R. and Program C.N.I.S. (2008). A preliminary guideline for the assignment of methicillin-resistant *Staphylococcus aureus* to a Canadian pulsed-field gel electrophoresis epidemic type using spa typing. *Canadian Journal of Infectious Diseases and Medical Microbiolog*y. 19; 273–281.

Gordon J. Rachel and Franklin D. Lowy (2008). Pathogenesis of Methicillin-Resistant *Staphylococcus aureus* Infection. *Clinical Infectious Disease.* 46: 350-359.

Graffunder E. M., and Venezia R. A. (2002). Risk factors associated with nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) infection including previous use of antimicrobials. *Journal of Antimicrobial Chemotherapy*. 49: 999-1005.

Griffiths C., Lamagni T. L., Crowcroft N. S., Duckworth G., and Rooney C. (2004). Trends in MRSA in England and Wales: analysis of morbidity and mortality data for 1993-2002. *Health Stat*. Q: 15-22.

Grundmann H., Aires-de-Sousa M., Boyce J., Tiemersma E., (2006). Emergence and resurgence of meticillin-resistant *Staphylococcus aureus* as a public-health threat. *The Lancet*. 368: 874–885.

Guardabassi L., Stegger M., and Skov R. (2007). Retrospective detection of methicillin resistant and susceptible *Staphylococcus aureus* ST398 in Danish slaughter pigs. *Veterinary Microbiology*. 122:384–386.

Guardabassi L., O'Donoghue M., Moodley A., Ho J., Boost M. (2009). Novel lineage of methicillin-resistant *Staphylococcus aureus*, Hong Kong. *Emerging Infectious Diseases*; 15(12):1998-2000.

Haddadin A. S., Fappiano S. A., and Lipsett P. A. (2002). Methicillin resistant *Staphylococcus aureus* (MRSA) in the intensive care unit. *Postgraduate Medical Journal*. 78: 385-92.

Hanselman B. A., Kruth S. A., Rousseau J., Low D. E., Willey B. M., McGeer A., and Weese J. S. (2006). Methicillin-resistant *Staphylococcus aureus* colonization in veterinary personnel. *Emerging Infectious Diseases*. 12:1933–1938.

Hanssen A. M., and Ericson Sollid J. U. (2006). SCC*mec* in *staphylococci*: genes on the move. *FEMS Immunology and Medical Microbiology*. 46; 8–20.

Hardy K. J., Hawkey P. M., Gao F., and Oppenheim B. A. (2004). Methicillin resistant *Staphylococcus aureus* in the critically ill. *British Journal of Anaesthesia*. 92:121-30.

Harmsen D., Claus H., Witte W., Rothganger J., Claus H., Turnwald D., and Vogel U. (2003). Typing of methicillin-resistant *Staphylococcus aureus* in a university hospital setting by using novel software for spa repeat determination and database management. *Journal of Clinical Microbiology*. 41:5442-8.

Hartmann F. A., Trostle S. S., and Klohnen A. A. (1997). Isolation of methicillin-resistant *Staphylococcus aureus* from a postoperative wound infection in a horse. *Journal American Veterinary Medical Association*. 211:590–592.

Hiramatsu K., Aritaka N., Hanaki H. (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *Lancet.* 350: 1670-1673.

Holden M. T., Feil E. J., Lindsay J. A., Peacock S. J., Day N. P., Enright M. C., Foster T. J., Moore C. E., Hurst L. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proceedings of the National Academy of Science,* U S A. 101; 9786–9791.

Huijsdens X. W., van Dijke B. J., Spalburg E., M. van Santen-Verheuvel G., Heck M. E., Pluister G. N., Voss A., Wannet W. J., and de Neeling A. J. (2006). Community acquired MRSA and pig farming. *Annals of clinical microbiology and antimicrobials.* 5:26

Humphreys H., Glynn G., Rossney A., McDonald P., Johnson H., McDonnell R., Doyle D., Mitchell E., Burd M. (2002). Methicillin-resistant *Staphylococcus aureus*: laboratory detection methods in use in the Republic of Ireland and Northern Ireland. *British Journal of Biomedical Science.* 2002; 59(1):7-10.

International Working Group on the Classification of Staphylococcal Cassette Chromosome (IWG-SCC) Elements (2009). 'SCC*mec* up to date'.

Ito T., Katayama Y., Asada K. (2001). Structural comparison of three types of Staphylococcal Cassette Chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*; 45: 1323–1336.

Ito T., Ma X. X., Takeuchi F., Okuma K., Yuzawa H., Hiramatsu K. (2004). Novel type V Staphylococcal Cassette Chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC*. *Antimicrobial Agents and Chemotherpy*; 48: 2637–2651.

Johnson A. P., Pearson A., and Duckworth G. (2005). Surveillance and epidemiology of MRSA bacteraemia in the UK. *Journal of Antimicrobial Chemotherapy.* 56: 455-62.

Johnson AP, James D, Livermore DM. (1999). Increasing prevalence of methicillin resistance amongst *Staphylococcus aureus* blood culture isolates. *Journal of Antimicrobial Chemotherapy*. 43: 160.

Johnson L. B., Bhan A., Pawlak J., Manzor O., Saravolatz L. D. (2003). Changing epidemiology of community-onset Methicillin-resistant *Staphylococcus aureus* bacteremia. *Infection Control and Hospital Epidemiology,* 24, 431-435.

Johnson M. D., and Decker C. F. (2008). Antimicrobial agents in treatment of MRSA infections. *Disease-a-Month*. 54: 793-800.

Karynski M., Sabat A. J., Empel J., and Hryniewicz W. (2008). Molecular surveillance of methicillin-resistant *Staphylococcus aureus* by multiple-locus variable number tandem repeat fingerprinting (formerly multiple-locus variable number tandem repeat analysis) and spa typing in a hierarchic approach. *Diagnostic Microbiology and Infectious Disease.* 62:255-62.

Kazakova S. V., Hageman J. C., Matava M. (2005). A clone of methicillin-resistant *Staphylococcus aureus* among professional football players. *New England Journal of Medicine*. 352:468–75.

Keane C. T. (1992). MRSA: an overview. pp. 1-9 In, Methicillin-Resistant *Staphylococcus aureus*: Clinical management and Laboratory Aspects (M.T. Cafferkey, Ed) Marcel Dekker, INC. New York, USA.

Kesah, C., Ben Redjeb S., Odugbemi T. O., Boye C. S., Dosso M., Ndinya Achola J. O., Koulla-Shiro S., Benbachir M., Rahal K., and Borg M. (2003). Prevalence of methicillin-resistant *Staphylococcus aureus* in eight African hospitals and Malta. *Clinical Microbiology and Infection*. 9153-156.

Kerr S., Kerr G. E., Mackintosh C. A. (1990). A survey of methicillin-resistant *Staphylococcus aureus* affecting patients in England and Wales. *Journal of Hospital Infection*. 16: 35–48.

Khanna T., Friendship R., Dewey C., Weese J. S. (2008). Methicillin resistant *Staphylococcus aureus* colonization in pigs and pig farmers. *Veterinary Microbiology*. 128; 298–303.

Klevens R. M. (2007). Invasive Methicillin-resistant *Staphylococcus aureus* infections in the United States. *Journal of American Medical Association*, 298, 1763-1771.

Kluytmans J., van Belkum A., Verbrugh H. (1997). Nasal carriage of Staphylococcus aureus: Epidemiology, underlying mechanisms, and associated risks. *Clinical Microbiology Review.* 10 (3): 505–20.

Koreen, L., Ramaswamy S. V., Graviss E. A., Naidich S., Musser J. M., and Kreiswirth B. N.

(2004). Spa-typing method for discriminating among *Staphylococcus aureus* isolates: implications for use of a single marker to detect genetic micro- and macrovariation. *Journal of Clinical Microbiology*. 42:792-9.

Kottler S., Middleton J. R., Perry J., Weese J. S., and Cohn L. A. (2010). Prevalence of *Staphylococcus aureus* and Methicillin resistant *Staphylococcus aureus* carriage in three populations. *Journal of Veterinary Internal Medicine.* 24(1): 132-139.

Kwon N. H., Park K. T., Moon J. S., Jung W. K., Kim S. H., Kim J. M., Hong S. K., Koo H. C., Joo Y. S., and Park Y. H. (2005). Staphylococcal cassette chromosome *mec* (SCC*mec*) characterization and molecular analysis for methicillin resistant *Staphylococcus aureus* and novel SCC*mec* subtype IVg isolated from bovine milk in Korea. *Journal of Antimicrobial Chemotherapy*. 56:624–632.

Kwon N. H., Park K. T., Jung W. K., Youn H. Y., Lee Y., Kim S. H., Bae W., Lim J. Y., Kim J. Y., Kim J. M., Hong S. K., Park Y. H. (2006). Characteristics of methicillin resistant *Staphylococcus aureus* isolated from chicken meat and hospitalized dogs in Korea and their epidemiological relatedness. *Veterinary Microbiology*; 117(2-4):304-12.

Lee J. H. (2003) (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Applied Environmental Microbiology*; 69(11):6489-94.

Lee J. H., Jeong J. M., Park Y. H., Choi S. S., Kim Y. H., Chae J. S., Moon J. S., Park H., Kim S., Eo S. K. (2004). Evaluation of the methicillin-resistant *Staphylococcus aureus* (MRSA)- Screen latex agglutination test for detection of MRSA of animal origin. *Journal of Clinical Microbiology*; 42(6):2780-2.

Lewis R. A., and Dyke K. G. H. (2000). *MecI* represses synthesis from the β-lactamase operon of *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. 45; 139–144.

Lin Y., Barker E., Kislow J., Kaldhone P., Stemper M. E., Pantrangi M., Moore F. M., Hall M., Fritsche T. R., Novick T., Foley S. L., Shukla S. K. (2011). Evidence of multiple virulence subtypes in nosocomial and community-associated MRSA genotypes in companion animals from the upper midwestern and northeastern United States. *Clinical Medicine and Research.* 9(1):7-16.

Lindsay J. A., Holden M. T., Feil E. J., Peacock S. J., Day N. P., Enright M. C., Foster T. J., Moore C. E., and Hurst L. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. *Proceedings of the National Academy of Science,* U S A. 101; 9786–9791.

Loeffler A., Lloyd D. H. (2010). Companion animals: a reservoir for methicillin-resistant *Staphylococcus aureus* in the community? *Epidemiology and Infection.* 138, 595–605

Lowy F. D. (1998). *Staphylococcus aureus* infections. *New England Journal of Medicine*. 339: 520–32.

Maiden M. C., Bygraves J. A., Feil E., Morelli G., Russell J. E., Urwin R., Zhang Q., Zhou J., Zurth K., Caugant D. A., Feavers I. M., Achtman M., and Spratt B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of National of Academy of Science,* USA. 95:3140-5.

Malcolm B. (2011). Rise of MRSA in U.S correctional populations. *Journal of Correct Health Care*. 17 (3); 254-265

Mamoon A. A., Dominique L. M., Jose´ Marı´a Lo´pez-Lozano, Carmel M. H., Michael G. S., Mary P. K., Fidelma A. M., and James C. M. (2008). Modelling the impact of antibiotic use and infection control practices on the incidence of hospital-acquired methicillin-resistant Staphylococcus aureus: a time-series analysis. *Journal of Antimicrobial Chemotherapy*. 62, 593–600

Marshall J. H., Wilmoth G. J. (1981). Proposed pathway of triterpenoid carotenoid biosynthesis in *Staphylococcus aureus*: evidence from a study of mutants. *Journal of Bacteriology*. 147:914–919.

Mazmanian S. K., Liu G., Ton-That H., Schneewind O. (1999). *Staphylococcus aureus* sortase, an enzyme that anchors surface proteins to the cell wall. *Science.* 285:760–763.

Middleton J. R., Fales W. H., Luby C. D., Oaks J. L., Sanchez S., Kinyon J. M., Wu C. C., Maddox C. W., Welsh R. D., and Hartmann F. (2005). Surveillance of *Staphylococcus aureus* in veterinary teaching hospitals. *Journal of Clinical Microbiology*. 43:2916-2919.

Milheirico C., Oliveira D. C., Lencastre H. (2007). Update to the Multiplex PCR Strategy for assignment of the mec Element types in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 51, 3374-3377.

Moran G. J. (2006). Methicillin Resistant Staphylococcus aureus Infections among Patients in the Emergency Department. *New England Journal of Medicine*. 355:666-74.

Moodley A., Latronico F., Guardabassi L. (2010). Experimental colonization of pigs with methicillin-resistant *Staphylococcus aureus* (MRSA): insights into the colonization and transmission of livestock-associated MRSA. *Epidemiology of Infection*. 15:1-7.

Moodley A., Stegger M., Bagcigil A. F., Baptiste K. E., Loeffler A., Lloyd D. H., Williams N. J., Leonard N., Abbott Y., Skov R., and Guardabassi L. (2006). Spa typing of methicillin-resistant *Staphylococcus aureus* isolated from domestic animals and

veterinary staff in the UK and Ireland. *Journal of Antimicrobial Chemotherapy*. 58:1118-23.

Moon J. S., Lee A. R., Kang H. M., Lee E. S., Kim M. N., Paik Y. H., Park Y. H., Joo Y. S., Koo H. C. (2007). Phenotypic and genetic antibiogram of methicillin-resistant staphylococci isolated from bovine mastitis in Korea. *Journal of Dairy Science*. 90, 1176–1185.

Moore-Keir A. A., Stampfli H. R., Crawford J. (1999). Outbreak of acute colitis on a horse farm with tetracycline-contaminated sweet feed. *Canadian Veterinary Journal*, 40, 718-720

National Nosocomial Infections Surveillance (NNIS) System, (2004). Report, data summary from January 1992 through June 2004. *American Journal of Infection and Control.* 32:470-85

Nemati M., Hermans K., Lipinska U., Denis O., Deplano A., Struelens M., Devriese L. A., Pasmans F., Haesebrouck F. (2008). Antimicrobial resistance of old and recent *Staphylococcus aureus* isolates from poultry: first detection of livestock-associated methicillin-resistant strain ST398. *Antimicrobial Agents and Chemotherapy*; 52(10):3817-9.

Ochoa T. J. (2005).Community-associated Methicillin-resistant Staphylococcus in Pediatric Patients. *Emerging Infectious Disease.* 11: 966-968.

Oliveira, D. C., Tomasz A., and de Lencastre H. (2002). Secrets of success of a human pathogen: molecular evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*. *Lancet Infectious Disease*. 2:180-189.

O'Mahony R., Abbott Y., Leonard F. C., Markey B. K., Quinn P. J., Pollock P. J., Fanning S., Rossney A. S. (2005) Methicillinresistant *Staphylococcus aureus* (MRSA) isolated from animals and veterinary personnel in Ireland. *Veterinary Microbiology*; 109(3-4):285-96.

O'Rourke K. (2003). Methicillin-resistant *Staphylococcus aureus*: an emerging problem in horses? *Journal of American Veterinary Medical Association*. 223(10):1399-1400.

O'Sullivan N. P., and Keane C. T., (2000). The prevalence of methicillin-resistant *Staphylococcus aureus* among residents of six nursing home for the elderly. *Journal of Hospital Infection*. 45:322-329

Pak S. I., Han H. R., and Shimizu A. (1999). Characterization of methicillin resistant *Staphylococcus aureus* isolated from dogs in Korea. *Journal of Veterinary Medical Science*. 61:1013–1018.

Perry N. S., Bollen C., Perry E. K., Ballard C. (2003). Salvia for dementia therapy: review of

Pharmacological activity and pilot tolerability clinical trial. *Pharmacology, Biochemistry and Behaviour*.75 (3): 651-659.

Persoons D., Van Hoorebeke S., Hermans K., Butaye P., de Kruif A., Haesebrouck F., Dewulf J. (2009). Methicillin-resistant Staphylococcus aureus in poultry. *Emerging Infectious Diseases*. 15, 452–453.

Plata K., Rosato A. E., Wegrzyn G. (2009). *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. *Acta Biochimica Polonica*. 56: 597–612.

Ramdani-Bouguessa N., Bes M., Meugnier H., Forey F., Reverdy M. E., Lina G., Vandenesch F., Tazir M., and Etienne J. (2006). Detection of methicillin-resistant *Staphylococcus aureus* strains resistant to multiple antibiotics and carrying the Panton-Valentine leukocidin genes in an Algiers hospital. *Antimicrobial Agents and Chemotherapy*. 50, 1083-1085.

Rich M., Roberts L., Kearns A.M. (2005). Methicillin-resistant Staphylococcus aureus isolates from companion animals. *Veterinary. Microbiology*. 105, 313–314.

Robinson D. A., and Enright M. C. (2004). Multilocus sequence typing and the evolution of methicillin-resistant *Staphylococcus aureus*. *Clinical Microbiology and Infection*. 10:92-7.

Rohrer S., Bischoff M., Rossi J., Berger-Bächi B. (2003). Mechanisms of methicillin resistance. In: A.C. Fluit and F. J. Schmitz, Editors, MRSA. Current Perspectives, Caister Academic Press, Wymondham, pp. 31– 53.

Sabat A., Krzyszton-Russjan J., Strzalka W., Filipek R., Kosowska K., Hryniewicz W., Travis J., and Potempa J. (2003). New method for typing *Staphylococcus aureus* strains: multiple-locus variable-number tandem repeat analysis of polymorphism and genetic relationships of clinical isolates. *Journal of Clinical Microbiology*. 41:1801-4.

Schumann W. (2006). Origin of Mutations. pp.124-146 Dynamics of the bacterial chromosome: structure and function. WILEY.VCH Verlag GmbH&Co. KGaA, Weinheim.

Schwartz D. C., and Cantor C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell*. 37:67-75.

Seaman P., Day M., Denver Russell A., and Ochs D. (2004). Susceptibility of capsular *Staphylococcus aureus* strains to some antibiotics, triclosan and cationic biocides. *Journal of Antimicrobial Chemotherapy*. 4; 696–698

Seguin J. C., Walker R. D., Caron J. P., Kloos W. E., George C. G., Hollis R. J., Jones R. N., and Pfaller M. A. (1999). Methicillin-resistant *Staphylococcus aureus* outbreak in a veterinary teaching hospital: potential human-to-animal transmission. *Journal of Clinical Microbiology*. 37:1459–1463.

Seybold U. (2006). Emergence of Community-Associated Methicillin-Resistant *Staphylococcus aureus* USA300 Genotype as a Major Cause of Health Care Associated Blood Stream Infections. *Clinical Infectious Disease*. 42:647-656

Shimizu A., Kawano J., Yamamoto C., Kakutani O., Anzai T., and Kamada M. (1997). Genetic analysis of equine methicillin-resistant *Staphylococcus aureus* by pulsed-field gel electrophoresis. *Journal of Veterinary Medical Science*. 59:935–937.

Shittu A., Johnson L., and Donald M. (2007). Molecular identification and characterization of mannitol-negative methicillin-resistant *Staphylococcus aureus. Diagnostic Microbiology and Infectious Disease*. 57; 93–95.

Shopsin B., Gomez M., Montgomery S. O., Smith D. H., Waddington M., Dodge D. E., Bost D. A., Riehman M., Naidich S., and Kreiswirth B. N. (1999). Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *Journal of Clinical Microbiology*. 37:3556-63.

Short B. R., and Enright M. C. (2007). The evolution of MRSA. pp. 21-28. In, MRSA in Practice (I. Gould, Ed). The Royal Society of Medicine Press Ltd. London, UK.

Singh A., Goering R. V., Simjee S., Foley S. L., and Zervos M. J. (2006). Application of molecular techniques to the study of hospital infection. *Clinical Microbiology Review*. 19:512-30.

Stapleton P. D. and Taylor P. W. (2002). Methicillin resistance in *Staphylococcus aureus*: mechanisms and modulation. *Science Progress*. 85, 57–72.

Stepan J., Pantucek R., and Doskar J. (2004). Molecular diagnostics of clinically important *Staphylococci*. *Folia Microbiology (Praha)*. 49:353-86.

Stranden A., Frei R., and Widmer A. F. (2003). Molecular typing of methicillin-resistant *Staphylococcus aureus*: can PCR replace pulsed-field gel electrophoresis? *Journal of Clinical Microbiology*. 41:3181-6.

Strommenger B., Kehrenberg C., Kettlitz C., Cuny C., Verspohl J., Witte W., Schwarz S. (2006). Molecular characterization of methicillin-resistant Staphylococcus aureus strains from pet animals and their relationship to human isolates. *Journal of Antimicrobial Chemotherapy*. 57, 461–465.

Taiwo S. S., Bamidele M., Omonigbehin E. A., Akinsinde K. A., Smith S. I., Onile B. A.,

and Olowe A. O. (2005). Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Ilorin, Nigeria. *West African Journal of Medicine*. 24, 100-106.

Tanner A. C. (2000). Antimicrobial drug use in poultry, p. 637–655. *In* J. F. Prescott, J. D. Baggot, and R. D. Walker (ed.), *Antimicrobial Therapy in Veterinary Medicine*. Iowa State University Press, Ames, IA.

Tenover F. C., Vaughn R. R., McDougal L. K., Fosheim G. E., and McGowan J. E. (2007). Multiple-locus variable-number tandem-repeat assay analysis of methicillin-resistant *Staphylococcus aureus* strains. *Journal Clinical Microbiology*. 45:2215-9.

Tenover F. C., Arbeit R. D., and Goering R. V. (1997). How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. *Molecular Typing Working Group of the Society for Healthcare Epidemiology of America; Infection Control and Hospital Epidemiology*. 18:426-39.

Thomas W. D., and Archer G. L. (1989). Mobility of gentamicin resistance genes from *Staphylococci* isolated in the United States: identification of Tn4031, a gentamicin resistance transposon from *Staphylococcus epidermidis*. *Antimicrobial Agents and Chemotherapy*. 33(8):1335–1341.

Tokateloff N., Manning S. T., Weese J. S., Campbell J., Rothenburger J., Stephen C., Bastura V., Gow S. P., and Reid-Smith R. (2009). Prevalence of methicillin-resistant *Staphylococcus aureus* colonization in horses in Saskatchewan, Alberta, and British Columbia. *The Canadian Veterinary Journal*. 50(11), 1177-80.

Trindade P. A., McCulloch J. A., Oliveira G. A., and Mamizuka E. M. (2003). Molecular techniques for MRSA typing: current issues and perspectives. *Brazilian Journal Infection Diseases*. 7:32-43.

van Belkum Alex, (2007). Methicillin-Resistant and -Susceptible *Staphylococcus aureus* Sequence Type 398 in Pigs and Humans. *Emerging Infecious Diseases*. 14(3); 479-483

van den Broek I. V., van Cleef B. A., Haenen A., Broens E. M., van der Wolf P. J., van den Broek M. J., Huijsdens X. W., Kluytmans J. A., van de Giessen A. W., Tiemersma E. W. (2009). Methicillin-resistant *Staphylococcus aureus* in people living and working in pig farms. *Epidemiology and Infection*. 137(5):700-8.

van Duijkeren E., Wolfhagen M., Box A. T., Heck M., Wannet W. J. B., Fluit A. C., (2004). Human-to-dog transmission of methicillin-resistant *Staphylococcus aureus. Emerging Infectious Disease*. 10: 2235–2237.

van Leeuwen W. (2003). Molecular Approaches for Epidemiological Characterization of *Staphylococcus aureus*. pp 95-136. *In*, MRSA Current Perspectives (A. C. Fluit and Franz-Josef Schmitz, Eds). Caister Academic Press, Norfolk, England.

Vengust M., Anderson M. E., Rousseau J., Weese J. S. (2006). Methicillin-resistant staphylococcal colonization in clinically normal dogs and horses in the community. *Letters of Applied Microbiology*; 43(6):602-6.

Vitale C. B., Gross T. L., Weese J. S. (2006). Methicillin-resistant *Staphylococcus aureus* in cat and owner. *Emerging Infectious Diseases*; 12(12):1998-2000.

Voss A., Loeffen F., Bakker J., Klaassen C., Wulf M. (2005) Methicillin-resistant *Staphylococcus aureus* in pig farming. *Emerging Infectious Diseases*: 11; 1965–6.

Vuopio-Varkila J. (2007). Epidemiology of MRSA. pp. 29-35. In, MRSA in Practice (I. Gould, Ed). The Royal Society of Medicine Press Ltd. London, UK.

Walther B., Monecke S., Ruscher C., Friedrich A. W., Ehricht R., Slickers P., Soba A., Wleklinski C. G., Wieler L. H., Lübke-Becker A. (2009). Comparative molecular analysis substantiates zoonotic potential of equine methicillin resistant *Staphylococcus aureus*. *Journal of Clinical Microbiology*; 47(3):704-10.

Wang L., and Barrett J. F. (2007). Control and Prevention of MRSA Infections. pp. 209-225. *In,* Methicillin-Resistant *Staphylococcus aureus* (MRSA) Protocols (Y. Ji, Ed). Humana Press Inc. Totowa, New Jersey, USA.

Washio M., Mizoue T., Kajioka T., Yoshimitsu T., Okayama M., Hamada T., Yoshimura T., and Fujishima M. (1997). Risk factors for methicillin-resistant *Staphylococcus aureus* (MRSA) infection in a Japanese geriatric hospital. *Public Health*. 111: 187-190.

Weese J. S. (2004). Methicillin-resistant *Staphylococcus aureus* in horses and horse personnel. *Veterinary Clinics of North American Equine Practice*. 20:601–613.

Weese J. S., Rousseau J., Traub-Dargatz J. L., Willey B. M., McGeer A. J., and Low D. E. (2005). Community-associated methicillin-resistant *Staphylococcus aureus* in horses and humans who work with horses. *Journal of American Veterinary Medical Association*. 226:580–583.

Weese J. S., Archambault M., Willey B. M. (2005). Methicillin resistant *Staphylococcus aureus* in horses and horse personnel,2000–2002. *Emerging Infectious Diseases*; 11: 430–435.

Weese, J. S., Dick H., Willey B. M., McGeer A. (2006). Suspected transmission of Methicillin resistant *Staphylococcus aureus* between domestic pets and humans in veterinary clinics and in the household. *Veterinary Microbiology*, 115, 148-155.

Weese J. S., Caldwell F., Willey B. M., Kreiswirth B. N., McGeer A., Rousseau J., Low D. E. (2006). An outbreak of Methicillin-resistant *Staphylococcus aureus* skin infections resulting from horse to human transmission in a veterinary hospital. *Veterinary Microbiology*, 114, (1-2), 160-164.

Weese J. S. (2007). MRSA infection in horses. *Veterinary Record*. 161:359–360.

Weller T. M. (2000). Methicillin-resistant *Staphylococcus aureus* typing methods: which should be the international standard? *Journal of Hospital Infection*. 44:160-72.

Wenzel M. D., and Richard P. (2004). The Antibiotic Pipeline; Challenges, Costs, and Values. *New England Journal of Medicine*. 351; 6.

White D. G., Ayers S., Maurer J. J., Thayer S. G., and Hofacre C. (2003). Antimicrobial susceptibilities of *Staphylococcus aureus* isolated from commercial broilers in Northeastern Georgia. *Avian Disease*. 47:203–210.

Wieland B., Feil C., Gloria-Maercker E., Thumm G., Lechner M. (1994). Genetic and biochemical analyses of the biosynthesis of the yellow carotenoid 4,4'-diaponeurosporene of *Staphylococcus aureus*. *Journal of Bacteriology.* 176: 7719-7726.

Wilailuckana C., Tribuddharat C., Tiensasitorn C., Pongpech P., Naenna P., Rugdeekha S., Dhiraputra C., and Danchaivijitr S. (2006). Discriminatory powers of molecular typing techniques for methicillin-resistant *Staphylococcus aureus* in a university hospital, Thailand. *Southeast Asian Journal of Tropical Medical Public Health*. 37:327-34.

Wulf M., Nes A. V., Eikelenboom-Boskamp A., (2006). Methicillin-resistant *Staphylococcus aureus* in veterinary doctors and students, the Netherlands. *Emerging Infectious Diseases*, 12, 1939-1941.

Wulf M. W., Markestein A., van der Linden F. T., Voss A., Klaassen C., Verduin C. M. (2008). First outbreak of methicillin resistant *Staphylococcus aureus* ST398 in a Dutch Hospital, June 2007. *Eurosurveillance*; 28:13(9).

Wulf M. W., Tiemersma E., Kluytmans J., Bogaers D., Leenders A. C., Jansen M. W., Berkhout J., Ruijters E., Haverkate D., Isken M., Voss A. (2008). MRSA carriage in healthcare personnel in contact with farm animals. *Hospital Infection*; 70(2):186-90.

Zhang, K., McClure J. A., Elsayed S., Louie T., and Conly J. M. (2005). Methicillin resistance in *Staphylococci*: molecular and biochemical basis and clinical implications. *Journal of Clinical Microbiology*. 43:5026-5033.

APPENDICES

Appendix I: Composition of media

Brain hear infusion broth (CM1135; Oxoid, Basingstoke, UK)

Typical formula (g/l) ----------pH 7.4±0.2 at 25°C

Brain infusion solids ----------12.5

Beef hear infusion solids ----------5.0

Proteose peptone ----------10.0

Glucose ----------2.0

Sodium chloride ----------5.0

Di-sodium phosphate ----------2.5

Suspend 37g of agar base into 1000ml of water. Mix well to dissolve and distribute into final containers. Sterilize by autoclaving a 121°C for 15minutes.

Columbia blood agar (CM0331; Oxoid, Basingstoke, UK)

Typical formula (g/l) ----------pH 7.3±0.2 at 25°C

Special peptone ----------23.0

Starch ----------1.0

Sodium chloride ----------5.0

Agar ----------10.0

Suspend 39g in 1000ml of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121°C for 15minutes. Cool to 50°C and add 5% sterile defebrinated sheep blood.

Baird Parker Agar-Egg Yolk Tellurite emulsion (CM0275 and SR0054C)

Typical formula (g/l) ----------pH 6.8±0.2 at 25°C

Tryptone ----------10.0

Lab-lemco powder ----------5.0

Yeast extract ----------1.0

Sodium pyruvate ----------10.0

Glycine ----------12.0

Lithium chloride ----------5.0

Agar ----------20.0

Suspend 63g in 1000ml of distilled water and bring to the boil to dissolve the medium completely. Sterilize by autoclaving at 121°C for 15minutes. Cool to 50°C and aseptically add 50ml of egg yolk-tellurite emulsion.

Nutrient agar (CM003; Oxoid, Basingstoke, UK)

Typical formula (g/l) ----------pH 7.4±0.2 at 25°C

Lab-lemco beef extract ----------1.0

Yeast extract ----------2.0

Peptone ----------5.0

NaCl ----------5.0

Agar ----------15.0

Suspend 28g of the agar into 1000ml of distilled water. Heat gently until dissolved completely. Sterilize b autoclaving at 121°C for 15minutes.

Mueller Hinton agar (CM0337; Oxoid, Basingstoke, UK)

Typical formula (g/l) ----------pH 7.3±0.1 at 25°C

Beef dehydrated infusion ----------300.0

Casein hydrolysate ----------17.5

Starch ----------1.5

Agar ----------17.0

Suspend 38g of the agar base into 1000ml of distilled water. Heat gently until dissolved completely and sterilize by autoclaving a 121°C for 15minues.

Andrade Peptone water (CM0061; Oxoid, Basingstoke, UK)

Typical formula (g/l) ----------pH 7.4±0.2 at 25°C

Peptone ----------10.0

Sodium chloride ----------5.0

Andrade's indicator ----------0.1

Suspend 15g of the agar base into 1000ml distilled water. Mix well to dissolve completely and distribute into containers fitted with Durham's tubes. Sterilize by autoclaving a 121°C for 15minutes.

Appendix II; Flow chart outline of isolation and characterization of MRSA from horses and horse handlers

Swab sample collection and transportation in transport media (liquid Stuart's medium 4°C)

⇩

Enrichment in Brain Heart Infusion broth for 24hours at 37°C

⇩

Culture on Columbia Blood agar and incubated for 24hours at 37°C

⇩

Subculture growth from above onto Baird Parker Egg Yolk-tellurite media and incubated for 24-48hours at 37°C

⇩

Biochemical characterization; Gram staining, catalase test, coagulase test, sugar fermentation (mannitol, inositol, glucose, sucrose, maltose)

⇩

Antibiotic sensitivity test on Mueller Hinton agar using disc diffusion method

⇩

Confirm methicillin resistance (through *mec* presence) using PBP2a latex agglutination test kit following manufacturers guide

⇩

Multiplex polymerase chain reaction (M-PCR) for SCC*mec* typing of confirmed MRSA according to Boye *et., al.* (2006)

Appendix III; Plates



Plate 1; Colonies of *Staphylococcus aureus* on Baird Parker Egg Yolk with tellurite agar.

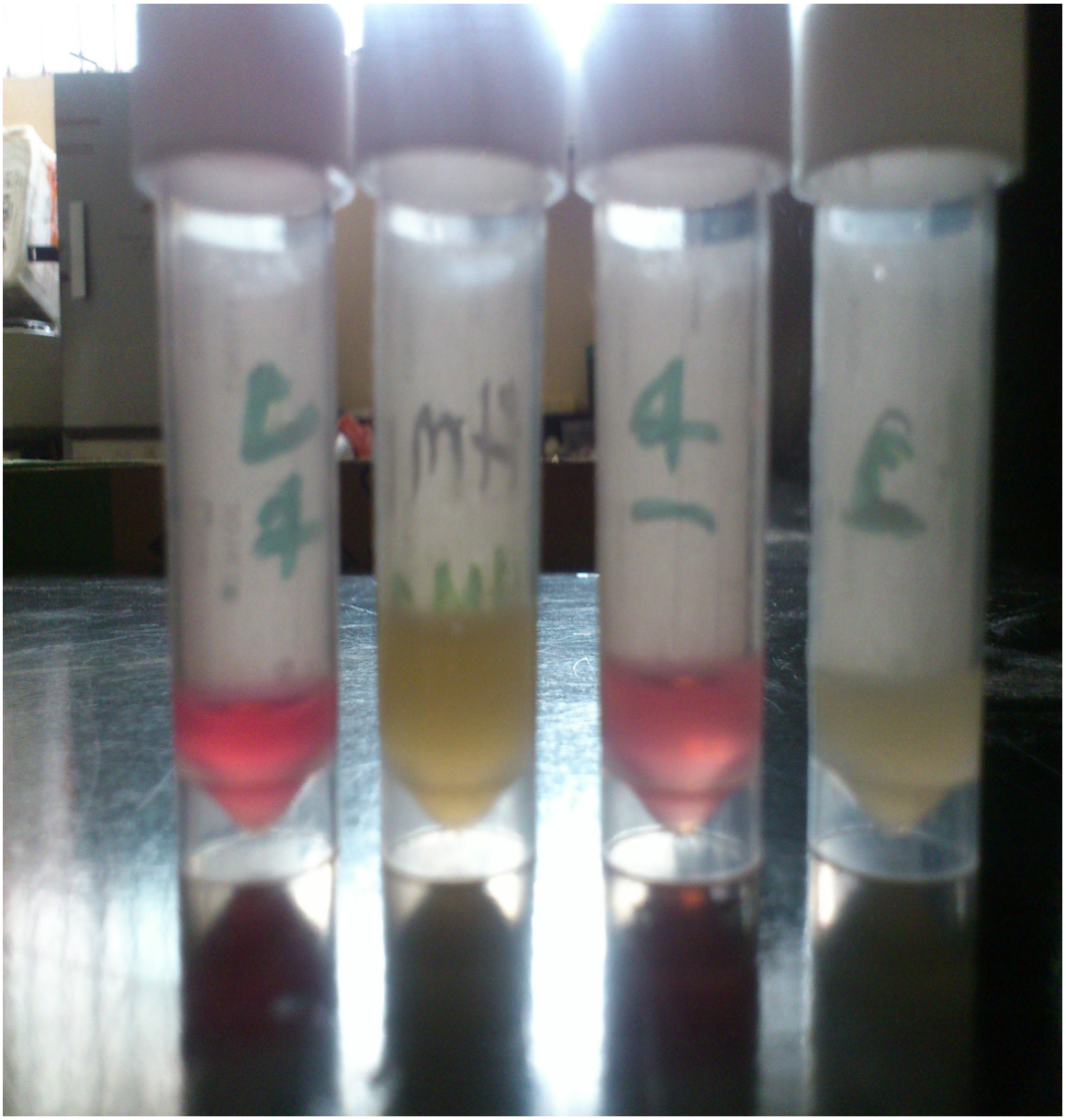


Plate 2; Sugar fermentation after 24hours incubation showing color changes with pink being positive while the cloudy yellow being negative.

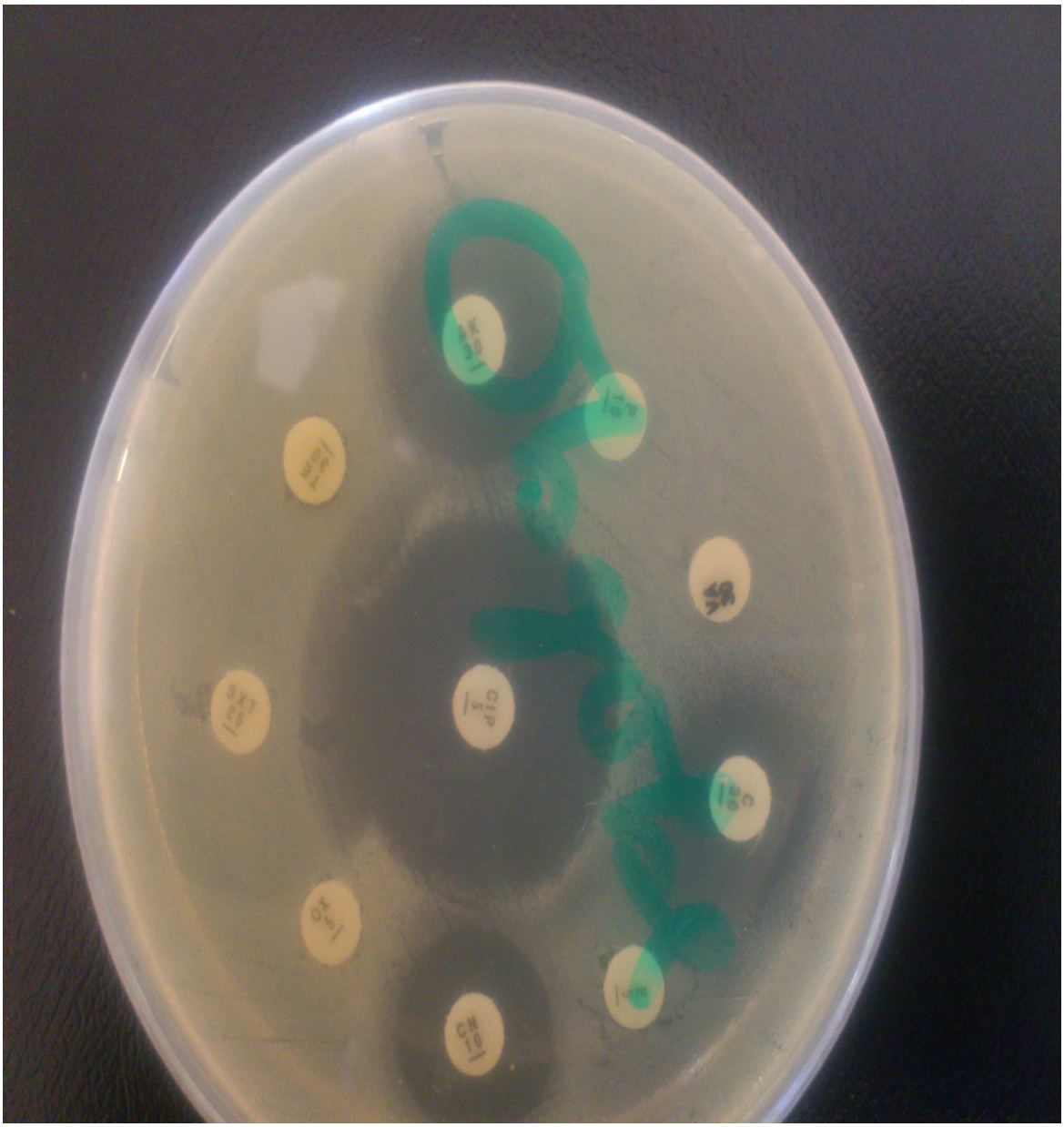


Plate 3; Antibiotic sensitivity testing on Mueller Hinton agar showing zone of inhibition

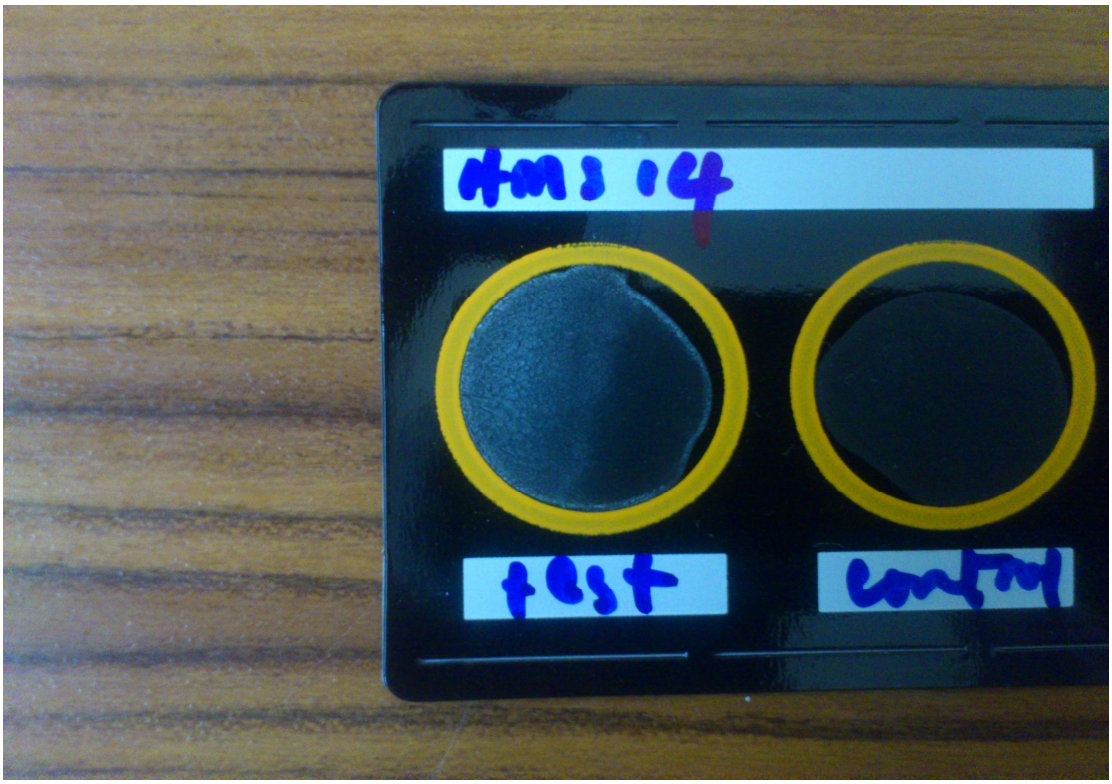


Plate 4. PBP2a latex agglutination test.

Appendix IV; Results of the isolates based on biochemical characterization

SAMPLES	BHI 7.5% SALT	COLUMBIA	BAIRD PARKER	CATALASE	COAGULASE	GLUCOSE	SUCROSE	MANNITOL	MALTOSE	INOSITOL
HMIA 12	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMIA 13	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIA 03	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIA 06	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIA 16	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIA 22	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIA 24	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMIP 05	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 01	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 07	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 18	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 21	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 24	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRIP 26	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMS5 08	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMS5 14	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMS5 15	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 01	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 16	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 24	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 41	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 48	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 52	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 55	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 57	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 64	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 67	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 76	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 79	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 82	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 84	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 85	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRS5 87	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HMT 05	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 06	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 10	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 19	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	4+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 28	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 35	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 40	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 57	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 64	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 72	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE
HRT 80	POSITIVE	BETA HAEMOLY	POSITIVE	POSITIVE	3+	POSITIVE	POSITIVE	POSITIVE	POSITIVE	POSITIVE

ANTIBIOGRAM

Isolate	Resistance profile
HMIA 12	PENICILLIN
HMIA 13	PEN;OXA
HRIA 03	AMI;PEN;ERY;TET;OXA
HRIA 06	AMI;PEN;OXA
HRIA 16	VAN;AMI;PEN;ERY;OXA
HRIA 22	PEN;TET;OXA
HRIA 24	AMI;PEN;TET;OXA
HMIP 05	PEN;OXA
HRIP 01	VAN;SUL;PEN;ERY;TET;OXA
HRIP 07	VAN;SUL;PEN;ERY;TET;OXA
HRIP 18	VAN;PEN;ERY;TET;OXA
HRIP 21	PEN;OXA
HRIP 24	PEN;ERY;TET
HRIP 26	PEN;OXA
HMS5 08	PEN;TET
HMS5 14	VAN;PEN;ERY;OXA
HMS5 15	PENICILLIN
HRS5 01	PENICILLIN
HRS5 16	AMI;PEN;TET
HRS5 24	AMI;PEN;OXA
HRS5 41	VAN;PEN;ERY;CHL;OXA
HRS5 48	PENICILLIN
HRS5 52	PEN;OXA

HRS5 55	PEN;TET
HRS5 57	VAN;PEN;ERY;OXA
HRS5 64	NR
HRS5 67	VAN;PEN;ERY;TET;OXA
HRS5 76	AMI;PEN;ERY;TET;OXA
HRS5 79	PEN;TET
HRS5 82	PEN;ERY;OXA
HRS5 84	PEN;ERY;OXA
HRS5 85	NR
HRS5 87	VAN;PEN;ERY;OXA
HMT 05	PEN;ERY;OXA
HRT 06	PENICILLIN
HRT 10	AMI;PEN;TET;OXA
HRT 19	AMI;PEN;GEN;OXA
HRT 28	PENICILLIN
HRT 35	CIP;SUL;PEN;ERY;TET;OXA
HRT 40	VAN;PEN;ERY;TET
HRT 57	VAN;PEN;ERY;OXA
HRT 64	PEN;ERY
HRT 72	PENICILLIN
HRT 80	VAN;PEN;ERY;OXA

NR; no resistance

DEFINITIONS OF VARIABLES

- Sex; male or female
- Years of Practice; duration of work/being around horses for work
- Educational Qualification; level of certified education
- Occupation; specific work with/around the horses
- Horses No., Handled; number in counts of horses cared for routinely
- Other Animals Kept; animals other than horses taken care in association with the horses
- Contact Activity/Contact Frequency; specific work and duration of such work routinely with the horses
- Knowledge;

Awareness to Occupational Hazard; knowledge of any health hazard associated with working with horses

Predisposition to Infectious Disease; knowledge of specific aspect of work that predisposes to any health hazard

Horse Zoonosis Known; knowledge of any disease of horse that can be transmitted to humans

MRSA Knowledge; any knowledge of MRSA

- Veterinary Care; which category of professional provides veterinary care
- Previous Antimicrobial Therapy; any therapy self-prescribed or otherwise of any antimicrobial agent with the past (period specified)
- Hospital Visitation; any visit recent or past to the hospital (period specified)
- Reason for Visit; any reason for visiting the hospital such as medical or visit a patient etc
- Hand Washing Habit; specific hand washing routine such as beginning of days' work only, irregular etc
- What Used For Washing; specific element(s) used for washing hands such as water only, water and soap etc
- Protective Clothing Use; any item/equipment/material used for protection physically against any health hazard during work with horses
- Residency; place of resident with respect to distance to the horses such close to the stable (within 1km radius from stable), within stable (within the same perimeter fencing with the stable), and away from stable (>1km radius from the stable)

Risk factor association with MRSA colonization for variables with binary outcomes in 2*2 contingency table.

Variable	**Chi square**	**P<0.05**	**Odds ratio**
Sex	15.488	0.000	0.047
Veterinarian	5.486	0.019	9.000
Feeding & watering	54.069	0.000	0.088
Face mask	6.744	0.009	10.333
Hand gloves	10.544	0.001	19.000

Water wash only	15.238	0.000	0.048

Risk factor association with MRSA for variables with multinomial outcomes.

Variable	**Chi-square**	**P<0.05**	**Estimated Risk**
Restraint contact	65.877	0.000	3.93
Previous antimicrobial therapy	56.937	0.000	0.689

Last hospital visitation	33.969	0.000	0.600
Reason for visitation	33.875	0.000	2.090
Educational qualification	33.187	0.000	0.150