Aus dem Institut für Tier- und Umwelthygiene
des Fachbereiches Veterinärmedizin
der Freien Universität Berlin

Empfindlichkeit von *Staphylococcus aureus* Stämmen isoliert von Kühen mit subklinischer Mastitis gegen verschiedene Arten von Desinfektionsmitteln und Antibiotika

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Susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different types of disinfectants and antibiotics

Thesis submitted
for the fulfilment of a doctor degree
in veterinary medicine (Dr. med. vet.)
at Free University of Berlin

Submitted by
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Dedication

I would like to dedicate my Doctoral dissertation to my beloved uncle Dr. Samir El Behiry who gave me unconditional love and support throughout his life

إهداء

إلى روح عمى الحبيب د. سمير البحيرى
الذى شملنى بعطفه ورعايته طوال حياته
تغمده الله برحمته وأسكنه فسيح جناته

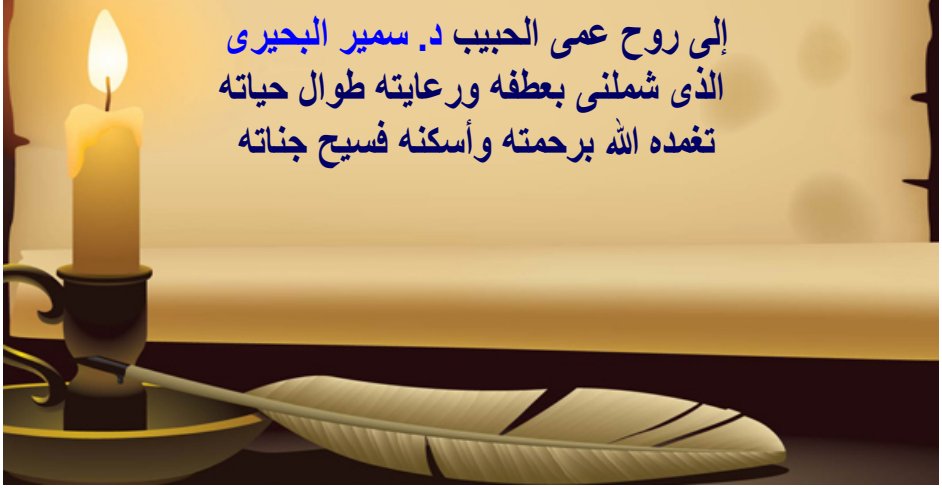

Table of contents

List of figures

List of tables

List of Abbreviations

ATCC	American Type Culture Collection
BAC	Benzalkonium chloride
Bp	base pair
C	Chloramphenicol
CLSI	Clinical Laboratory Standard Institute
CM	Cytoplasmic membrane
CMI	Cell-mediated immune system
CNS	Coagulase negative staphylococci
CPS	Coagulase positive staphylococci
Da	Daltons
°C	Degree Celsius
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
DVG	Deutsche Veterinärmedizinische Gesellschaft
E	Erythromycin
E. coli	*Escherichia coli*
FDA	Food and Drug Administration
GM	Gentamycin
H	hour
IgG	Immunoglobulin Gamma
IMI	Intramammary infection
I.U	International unit
LPS	Lipopolysaccharides
MALDI-TOF-MS	Matrix assisted laser desorption time of flight mass spectrometry
MIC	Minimum inhibitory concentration
µg	Microgram
µl	Microliter
Ml	Milliliter
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin sensitive *Staphylococcus aureus*
m/z	mass/charge ratio
N	Number
NaCl	Sodium chloride
NCCLS	National Committee for Clinical Laboratory Standard
-C	Negative control
OM	Outer membrane

OX	Oxacillin
+C	Positive control
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
%	Percent
P G	Penicillin G
PMF	Proton motive force
QACs	Quaternary ammonium compounds
Rpm	round per minute
SAS	Statistical analysis system
S. aureus	*Staphylococcus aureus*
SCC	Somatic Cell Count
T	Tetracycline
TSB	Tryptose Soya Broth
TBE	Tris/Borate/EDTA
TMP	Trimethoprim
TNase	Thermonuclease
V	Volt
WHO	World Health Organization
wt/vol	Weight/volume

ABSTRACT

The primary objective of the current study was to determine *in vitro* the efficacy of two types of commercial teat dips; Ujosan® dip (Nonoxinol-Iodine-Complex) and Eimü Chlorhexidin® dip (Chlorhexidine) against 56 *Staphylococcus* (*S.*) *aureus* strains isolated from quarter milk samples from various German dairy herds with different teat dipping schemes. 17 isolates stemmed from cows which were regularly dipped with the teat disinfectant Ujosan® dip; 29 isolates stemmed from cows regularly dipped with the disinfectant Eimü Chlorhexidin® dip and another 10 isolates isolated from a negative control group. The minimum inhibitory concentration (MIC) for all strains was determined using broth macrodilution method according to the guide lines for examination of chemical disinfectants in the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft, DVG). The mean MIC values of Ujosan® dip and Eimü Chlorhexidin® dip for the dipped and the control group were 45.70% ± 2.54%; 42.6% ± 1.64% and 97.51% ± 0.98%; 96.8 ± 0.78%, respectively, and showed no significant difference ($P<0.05$) between dipped and control groups for both Ujosan® dip and Eimü Chlorhexidin® dip.

A further main objective was an *in vitro* resistance induction (sensitivity reduction) against these two commercial teat dips with sub-lethal concentrations at ten different *S. aureus* strains. For each disinfectant, the 10 strains were repeatedly passed 10 times in growth media with sub-lethal concentrations of Ujosan® dip and Eimü Chlorhexidin® dip. The MIC values after the passages were determined and compared with the original MIC values before passages. 9 strains (90%) showed a strong susceptibility reduction to Ujosan® dip and only one strain (10%) to Eimü Chlorhexidin® dip. All isolates with increased MICs were passed every day for 10 days in tryptose soya broth (TSB) without disinfectant (active substance), to check whether the acquired resistance was stable or not. The stability of acquired resistance was noticed in all Ujosan® dip and Eimü Chlorhexidin® dip adapted *S. aureus* strains. In contrast, a co-induction of antibiotic resistances could not be observed at these ten investigated *S. aureus* strains.

Another objective was to check the sensitivity of 6 selected antimicrobial agents against 70 isolates of coagulase positive *S. aureus* and coagulase negative species (CNS) by using agar disk diffusion test. As can be seen, *S. aureus* isolates exhibited the highest degree of resistance to penicillin G (85.72%), whereas there has only been a limited occurrence of resistance to other antimicrobial agents.

From the present results, resistance of *S. aureus* to chemical disinfectants may be more likely to develop if they are used at concentrations lower than required for optimal biocidal effect. This reinforces the importance of always using disinfectants at the recommended concentrations and according to the label directions. Moreover, more research is needed to characterize the relationship between biocide nonsusceptibility and antibiotic resistance.

CHAPTER 1: INTRODUCTION

Bovine mastitis is an inflammatory reaction of the mammary gland and primarily caused by different pathogens that gain entry into the teat canal and mammary gland **(Bramley et al., 1996; Philpot and Nickerson, 1999)**. It represents one of the most costly diseases to the dairy industry all over the world, with losses estimated at about 2 billion dollars per year in the United States alone. These highly economic losses are due to several causes as rejected milk, reduced milk quality, drug costs, veterinary expenses, early culling and increased laboratory costs **(Hoblet et al., 1991; Gruet et al., 2001)**. Most cases of bovine mastitis are caused by various types of bacteria, and bacteria of the genus *Staphylococcus* are one of the most frequent pathogens causing mastitis worldwide. Anciently, the genus *Staphylococcus* is divided by the coagulase test into coagulase-negative (CNS) and coagulase-positive (CPS) species. Historically, CNS has often been considered to be minor important pathogens that cause intramammary infections (IMI)**.** In contrast, recent studies on mastitis prevalence have investigated that CNS may be of major importance in some countries **(Pyörälä and Taponen, 2008)**.

Among CPS isolated from bovine mastitis is *Staphylococcus (S.) aureus* and it is considered one of the most common causes of bovine mastitis in different areas of the world **(Ericsson Unnerstad et al., 2008)** and responsible for 25-30% of all IMI **(Sutra and Poutrel, 1994)**. Mastitis caused by *S. aureus* is most frequently subclinical; however, a major incidence rate of clinical mastitis is associated with this pathogen. *S. aureus* is regarded as a contagious mastitis pathogen because it is commonly spread from infected to non-infected cows at milking **(Sears and McCarthy, 2003)**. Despite *S. aureus* not being difficult to cultivate and easy to identify, there is still need for a rapid and sensitive DNA-based assay which is specific for *S. aureus* **(Saei et al., 2010)**. Most recent studies used polymerase chain reaction (PCR) for the identification of *S. aureus* and, in some cases, for its genotyping **(Ghoranpoor et al., 2007)**. In general more rapid identification of bacteria using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) can be an important method in the diagnosis of infections **(Bernardo et al., 2002; Sauer et al., 2008).**

Bovine *S. aureus* mastitis can be prevented and controlled to a manageable extent by the use of effective postmilking teat germicides, antibiotic therapy of all quarters at drying off, culling of animals with chronic infections, treatment of clinical mastitis during lactation and proper use of functioning milking machines **(Philpot and Nickerson, 1992)**. Postmilking teat disinfection is considered as one of the most effective procedures for reducing the rate of subclinical and clinical mastitis during lactation. More than 10 different active substances

have been used in teat disinfectants throughout the world in the last 20 years. In the United States, the National Mastitis Council reviewed and summarized nearly all the scientific literatures on teat disinfectants since 1980 and found that iodine and chlorhexidine were the major germicide classes used in teat dips **(National Mastitis Council, 2001)**. Despite universal acceptance of teat dipping as a method of mastitis control, restrictions are associated with most teat dips currently available. The most significant restriction is that teat dips do not provide equal protection against the huge amount of bacteria that cause bovine mastitis **(Oliver et al., 1990)**. Furthermore, prolonged in vitro exposure to germicidal teat dips has enhanced resistance of some bacteria to chemical disinfectants. Several passages of isolates through sub-lethal concentration of disinfectants either induced resistance or selected for resistant variants **(Szumala and Pemak, 1986)**. Germicides have multiple target sites against bacterial cells. This multiple target effect is thought to participate to their bactericidal activity and dictates against the development of resistance. Nevertheless, recent studies suggest that mutation or overexpression of triclosan and chlorhexidine target sites produces nonsusceptible microorganisms **(McMurry et al., 1998; Tattawasart et al., 1999; Levy, 2002)**.

In addition, resistance of bovine *S. aureus* mastitis to antimicrobial agents is a well-documented challenge in dairy cows **(Erskine et al., 2002; Makovec and Ruegg, 2003; Pitkala et al., 2004, Tenhagen et al., 2006)**. In fact, *S. aureus* pathogens have many features that make them difficult targets for antimicrobial therapy **(Sol et al., 2000)**. Results of susceptibility patterns for commonly used antibiotics indicate that the prevalence of β-lactamase producing *S. aureus* which are resistant to penicillin seems to have remained at a fairly constant level (40–60%) for the last twenty years **(Bennedsgaard et al., 2006)**. After the discovery and clinical application of antimicrobial agents, the morbidity and mortality caused by microbial infections were considerably reduced.

Recently, public health is facing a new challenge due to the most increase in bacterial resistance to most of the existing antibacterial agents as well as the emerging link between the resistance policies employed by bacteria toward antibiotics and biocides **(Braoudaki and Hilton, 2004)**. Microorganisms are limitlessly adaptable and have already demonstrated different mechanisms of resistance to these biocides; the concern is that these mechanisms may give cross-resistance to clinically important antibiotics. Several numbers of studies have been achieved to assess whether environmental and/or clinical strains that show decreased susceptibility to different types of biocides also display resistance to various types of antibiotics. Some laboratory studies suggest that the development of biocide and antibiotic resistance can be linked; other studies indicate no such link **(Russell et al., 1998; McDonnell and Russell, 1999)**.

Based on the previously mentioned facts, the current study was delineated to:

1. Determine *in vitro* the efficacy of two types of commercial teat dips; Ujosan® dip (Nonoxinol-Iodine-Complex) and Eimü Chlorhexidin® dip against *S. aureus* strains isolated from quarter milk samples from different German dairy herds with different teat dipping schemes.
2. *In vitro* induction of *S. aureus* resistance to commercial teat dips with sub-lethal concentration.
3. Check the antibiotic resistance patterns of bovine mastitis isolates of *S. aureus* and coagulase negative staphylococci (CNS).
4. Find a possible link (cross-resistance) between reduced susceptibility to teat disinfectants and antibiotic resistance commonly used in the treatment of bovine *S. aureus* mastitis.

CHAPTER 2: REVIEW OF LITERATURE

2.1 Overview of bovine mastitis

Mastitis is considered as one of the most predominant and most costly infectious disease of the dairy cattle industry worldwide **(Seegers et al., 2003; Petrovski et al., 2006)**. In Europe, the problem of mastitis is a highly relevant issue not only for the economic losses to producers, but also for the hygienic production of milk and the safety of dairy products for human consumption **(Moroni et al., 2005)**. The prevalence of mastitis in dairy cattle is relatively high. Subclinical mastitis is the main form of mastitis in dairy herds, exceeding 20 to 50% of dairy cows in given herds **(Wilson et al., 1997; Pitkala et al., 2004)**. It is very difficult to quantify the cost of subclinical bovine mastitis, however most experts accept that subclinical mastitis costs the average dairy farmer more than does clinical mastitis. Presuming a 45% prevalence of subclinical mastitis, the cost has been calculated at an average of $ 180 to $ 320 per case **(Wilson et al., 1997; Zhao and Lacasse, 2008)**. Around 70% of this cost is associated with a reduction in milk production.

Bovine mammary glands are exposed to different types of bacteria during lactation and in nonlactating periods. Pathogens commonly isolated from mastitic milk can be classified as noncontagious (are mainly environmental) and contagious pathogens. The environmental pathogens include *Streptococcus* (*Strept.*) *dysgalactiae*, *Strept. uberis, Escherichia* (*E.*) *coli*, and coagulase negative staphylococci (CNS) species, while the contagious pathogens include *Staphylococcus* (*S.*) *aureus* and *Strept. agalactiae* **(Zhao and Lacasse, 2008)**. The teat and streak canal are considered the initial line of the defense mechanism of the bovine mammary gland. **Capuco et al. (1992)** found that the keratin lining in the streak canal supports a physical and chemical barrier against bacterial penetration and a lot of bacteria may escape from the natural defense mechanisms by multiplication along the streak canal (especially after milking).

Sordillo and Streicher (2002) mentioned that after escaping of bacteria from the anatomical defense, they must attack the cellular and humoral defense mechanisms of the mammary tissue to establish disease. If the infection is not eliminated, bacterial levels in the mammary gland will rise to a level at which they begin to destroy the mammary tissue. As infection persists, the number of somatic cells in milk continues to increase and, concomitantly, tissue damage is deteriorated. The alveoli inside the gland start to lose structural integrity and the blood-milk barrier is breached. This permits extra-cellular fluid to enter the gland and mix with the milk. Moreover, visible changes in the milk and udder of the animals start to occur and clinical signs begin to appear.

2.2 Microorganisms most frequently associated with mastitis

Bacteria are the most common cause of bovine mastitis. Several reports clarified that more than 137 microbes are considered as etiological agents of mastitis **(Watts, 1988)**. The microbial causes of mastitis include a wide variety of microorganisms (aerobic and anaerobic bacteria, mycoplasmas, yeasts and fungi). The most common and important microorganisms of bovine mastitis are *Streptococci*, *Staphylococci*, *E. coli* and other Coliforms **(Giesecke et al., 1994; Quinn et al., 1994; Radostitis et al., 2000)**. The degree of importance of a specific agent, as a cause of mastitis in dairy cows, is mostly dependent on the nature of the organism, the pathogenicity of the agent, the challenge dose required to cause infection, and is influenced by management practices. Because most pathogens involved in mastitis are ever-present, mastitis can be managed but not eradicated **(Petzer, 2009)**. From an epidemiological point of view the main etiological agents responsible for mastitis can be divided into different groups of mastitogenic pathogens depending on the source of the organism involved. These include contagious, environmental and opportunistic pathogens **(Philpot and Nickerson, 1999)**.

2.2.1 Contagious pathogens

Contagious pathogens are usually found on the udder or teat surface of infected cows. Spreading occurs from diseased quarters to healthy quarters usually during milking. Programs for the control of contagious mastitis involve the improvement in hygiene and disinfection aimed at disrupting the cow-to-cow mode of transmission. Contagious mastitis found during the dry period of a dairy cow is mainly due to persistent infections not cured during lactation **(Petzer, 2009)**. Major contagious pathogens mainly cause clinical and subclinical mastitis include microorganisms such as *S. aureus*, *Strept. agalactiae and Mycoplasma bovis* **(Philpot and Nickerson, 1999; Quinn et al., 1999)**.

2.2.2 Environmental pathogens

Environmental mastitis is caused by bacteria that are transferred from the immediate surroundings of the cow, such as the sawdust, bedding of housed cows, the manure of cattle and the soil. Bacteria include streptococcal strains other than *Strept. agalactiae* such as *Strept. dysagalactiae*, *Strept. uberis*, *Strept. Bovis*, *Enterococccus faecium* and *Enterococcus faecalis* and Coliforms **(Quinn et al., 1999)**. These organisms are usually not well controlled by preventive measures such as teat dipping, because they are able to survive outside the udder, and cause infection only when given the opportunity such as low immunity, unhygienic conditions, etc. **(Radostitis et al., 2000)**.

2.2.3 Opportunistic pathogens

Opportunistic pathogens are responsible for the mild forms of mastitis and include CNS**.** The genus *Staphylococcus* is divided by the coagulase test into CNS and CPS species and all coagulase-negative isolates are generally regarded as non-pathogenic **(Quinn et al., 1999)**. They include *S. epidermidis*, *S. saprophyticus*, *S. simulans* **(Dos Santos Nacimento et al., 2005)**, *S. chromogenes* **(De Vliegher et al., 2003)**, *S. xylosus* **(Da Silva Santos et al., 2008)**.

2.3 Bovine *S. aureus* mastitis

Historically, infections caused by *S. aureus* were reported firstly by Sir Alexander Ogston, a Scottish surgeon, more than one hundred years ago. At the end of the eighteenth century *S. aureus* was reported to cause mastitis in cattle **(Haveri, 2008)**. It belongs to the family of Micrococcaceae and the group of staphylococci. Moreover, it is a gram-positive, catalase-positive, usually oxidase-negative, facultative anaerobic coccus; *S. aureus* can be differentiated from other staphylococcal species on the basis of gold colony pigmentation, their productivity to coagulase, fermentation of mannitol and trehalose, and production of heat stable thermonuclease. Most of *S. aureus* strains are surrounded by a polysaccharide capsule. Under the capsule there is a cell wall with a thick and a highly cross-linked peptidoglycan layer and teichoic acid, which is ideal of gram-positive bacteria **(Van Wely et al., 2001)**.

2.3.1 Pathology and virulence factors

S. aureus is a highly pathogenic bacterium persists in the mammary gland for several years. *S. aureus* have several virulence factors which are partially responsible for the subclinical and chronic type of mastitis that cause damage to secretory cells of the mammary gland **(Matthews et al., 1994)**. *S. aureus* has the ability to produce more than 30 virulence factors that participate to establishing and maintaining the infection in the mammary gland. These factors can be divided into two groups, including degradative enzymes and surface associated factors, together with exotoxins (Figure 1) **(Haveri et al., 2008)**. One of the most virulent factors produced by *S. aureus* is hyaluronidase enzyme which enables it to penetrate and adhere to the mammary tissue. Consequently, microabcesses form and eventually develop scar tissue which is impermeable to many types of antibiotics. *S. aureus* can be released if the microabcesses or scar tissue breaks down. This contributes to clinical flare-ups, chronicity and the ability of the infection to spread further within the gland. *S. aureus* also possesses another enzyme, coagulase, which is used to differentiate *S. aureus* from

other *S.* species. Coagulase reacts with the inflammatory products, forming fibrin-like clots. These clots prevent leukocyte movement and embarrass the action of the host's immune system phagocytes. These clots may also prevent drainage of milk from ducts of the mammary gland and lead to stasis or damage of secretory cells **(Bramley et al., 1996)**.

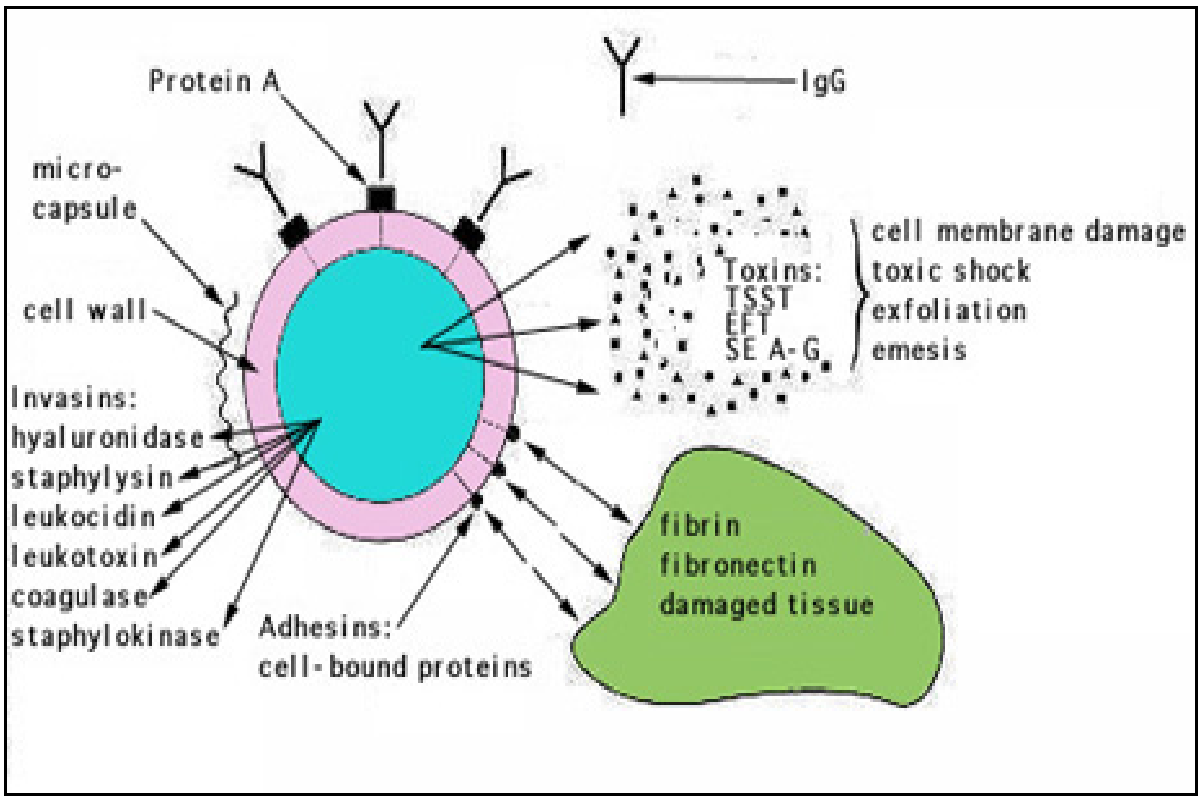

Figure 1: Virulence determinants of *S. aureus* (TSST = Toxic Shock Syndrome toxin, EFT = Exfoliative toxins SE A-G).

Additionally*, S. aureus* releases toxins, including alpha, beta, gamma, and delta toxins. Of these, alpha toxin appears to be the most toxic. It is particularly harmful to mammary tissue causing vasoconstriction, which leads to localized ischemia and cell necrosis **(Guidry, 1985)**. In times of rapid *S. aureus* growth, the effects of alpha toxin may lead to gangrenous mastitis **(Bramley et al., 1996)**. Moreover, **Foster et al. (1990)** observed a lack of phagocytic cells (macrophages and neutrophils) in areas where alpha toxin-producing *S. aureus* were growing in vitro mouse mastitis models. The authors theorized that this was due to decreased chemotaxis of macrophages and neutrophils into regions where alpha toxin-positive bacteria were growing. **Guidry (1985)** also noticed that beta and gamma toxins were mostly tissue irritants, with beta toxin being the most predominant toxin of *S. aureus* isolated from animals. However, beta toxin has also been found to increase bacterial growth in vitro mouse mastitis experiments **(Foster et al., 1990)**.

Components of the cell wall of *S. aureus* can also contribute to virulence. The main component, peptidoglycan, causes delayed hypersensitivity which can lead to flare-ups in chronic cases of subclinical *S. aureus* in which additional tissue damage results **(Guidry, 1985)**. Teichoic acid is a second component of the cell wall and it can be converted in vivo to teichuronic acid. The cell-mediated immune system (CMI) and the humoral immune system may face difficulties in recognition of teichuronic acid after the conversion **(Guidry, 1985)**. Protein A is the third cell wall component that may participate to *S. aureus* virulence by binding to the Fc portion of IgG. By doing this, Protein A prevents opsonization of *S. aureus* by IgG **(Fox et al., 2000)**. However, there are two subtypes of IgG, IgG1 and IgG2. **Sutra and Poutrel (1994)** found that Protein A binds strongly to IgG2, but only weakly binds to IgG1. Moreover, they noticed that some strains of *S. aureus* may also form capsules or pseudocapsules (slime layer). These may cover cell wall antigens and inhibit opsonization by complement and antibodies to cell wall components. In concordance, **Nickerson (1999)** indicated that the *S. aureus* pseudocapsule/slime layer was sufficient to impede antibody and complement attachment, which would block phagocytosis. In fact, when cows were immunized with a vaccine designed to promote opsonization of the *S. aureus* capsule, phagocytic activity improved **(Guidry et al., 1994)**.

Last of the virulence factors are “superantigens”, which are skeptical in their existence in *S. aureus* IMI. It has been hypothesized that the alpha, beta, gamma, delta toxins and leukocidin may be superantigens. The best known example of a superantigen is the staphylococcal enterotoxin B **(Mallard and Barnum, 1993)**. Additional research needs to be done to completely understand the superantigens possessed by *S. aureus* that cause bovine mastitis **(Fox et al., 2000)**. *S. aureus* can continue to resist the effects of antibiotics, biocides and/or the immune system if the microabcesses and scar tissue present from an established infection are bypassed. Production of β-lactamase enzyme (penicillinase) and conversion to L-forms are two additional ways possessed by *S. aureus* to protect themselves from lysis in the mammary environment. Penicillinase is an enzyme found in some strains of *S. aureus* that causes hydrolysis of the β-lactam ring. The β-lactamase enzyme of *S. aureus* has shown variability between herds. This may be due to antibiotic treatment habits and cow individuality. **Owens and Watts (1988)** indicated that resistance of *S. aureus* to penicillin was flactuating from 0 to 60% between herds. In addition, *S. aureus* can be converted to L-forms. It is thought that L-forms of *S. aureus* act as a temporary stage to survive conditions such as disruption of bacterial cell wall synthesis by antibiotics that are deleterious to cellular integrity. Cell survival is possible due to the lack of an organized cell wall in these *S. aureus* L-forms. L-forms provide *S. aureus* with benefits that include the ability to withstand antibiotic therapy, persist in the mammary gland, and re-emerge (flare-up) when conditions improve **(Owens, 1987)**.

2.3.2 Prevalence and significance

S. aureus is considered as one of the most commonly isolated pathogens in bovine mastitis all over the world **(Chaves et al., 2001; Gianneechini et al., 2002; Barrett et al., 2005; Tenhagen et al., 2006)** and the most frequent contagious mastitis pathogen isolated from raw milk **(Piccinini et al., 2003; Olde Riekerink et al., 2006)**. In the Nordic countries, more than 95% of sub-clinical and 60% of clinical cases of mastitis were caused by gram-positive cocci **(Sanholm et al., 1995)**. Among these, the most frequent pathogen was *S. aureus* which was responsible for 30-40% of sub-clinical and 20-30% of clinical cases of bovine mastitis. A survey carried out on Danish herds found that 21-70% of all dairy cows and 5-35% of all quarters were infected with *S. aureus* **(Aarestrup et al., 1995)**. Prolonged surveys propose that the importance of *S. aureus* in the dairy industry has remained unchanged **(Sol, 2002; Swinkels et al., 2005)**. IMI caused by *S. aureus* lead to high economic losses such as: decrease in milk production, reduced milk quality brought about by bacterial contamination and increased number of somatic cell count (SCC) in the milk of infected animal, veterinary and treatment costs, premature culling and loss of genetic potential. Economic losses due to *S. aureus* mastitis may be higher than for an average case of mastitis, especially in primiparous cows **(Gröhn et al., 2004)**. Multiparous cows are generally more often infected with *S. aureus* as compared with heifers **(McDougall et al., 2007)**. However, a high prevalence of *S. aureus* IMI has occasionally been reported for heifers.

2.3.3 Reservoirs and transmission

The infected mammary gland is considered the primary and most significant reservoir of *S. aureus*. From all sites where *S. aureus* has been isolated from cows, the infected mammary gland is considered the primary source for IMI **(Davidson, 1961)**. In concurrence, many strains of *S. aureus* were eliminated from extramammary body sites once the udder was treated, that are why Davidson theorized that the udder is the main reservoir that seeded other areas. Persistent colonization of *S. aureus* on the teat skin and several others body sites, initially mucosal external orifices, have been observed in heifers **(Roberson et al., 1994)** suggesting persistent colonization. However, although *S. aureus* has been found to survive in the barn environment (bedding material, on the floor, in dust, and in feed), it cannot be considered an environmental bacterium **(Kloos, 1997)**. Mastitis caused by *S. aureus* is mainly contagious, as the variety of mastitis causing strains is low, suggest a common source of infection and control programs planned for contagious mastitis have reduced occurrence of *S. aureus* **(Wilson et al., 1995; Buzzola et al., 2001)**. Traumatized sites such as injuries on teats, legs, bends and navel, typically infected by *S. aureus*, are considered as secondary sources of *S. aureus* causing bovine mastitis. Transmission occurs mainly at the

time of milking through contaminated clothes, milking machines and milker's hand or machine operators **(Radostitis et al., 1994)**. In herds that do not practice back flushing, the dairy advisor can look inside the teat cups and see residual milk. If the last cow milked with that contaminated unit with *S. aureus* IMI, then the next cow milked, with the same unit, will be directly exposed to *S. aureus* -laden milk. If employed, common clothes or sponges can be a major means of spreading *S. aureus*, as nearly every cow in the herd would be exposed on a daily basis **(Roberson, 1999)**.

2.3.4 Clinical manifestation and outcome

Barkema et al. (2006) noticed that the clinical signs of bovine mastitis caused mainly by *S. aureus* following IMI were changed from a subclinical to a peracute, gangrenous form. Subclinical mastitis is the most famous and likely the most field-problematic. In general, the clinical signs include pain, heat and swelling of the affected quarter or half of the gland and abnormality of milk either as clots or flakes and wateriness of the liquid phase **(Miffin, 2004; Abera et al., 2010)**. Bovine mastitis can be clinical with local clinical signs and milk abnormalities or subclinical with production losses and lowered milk quality. Older cows are more frequently infected with *S. aureus* compared with primiparous cows **(Pyörälä and Pyörälä, 1997; McDougall et al., 2007)**.

2.3.5 Prevention and control strategies

The five point plan for mastitis control has been the corner stone of control strategies for many years worldwide **(Giesecke et al., 1994)**. The main aim of the control program was to eradicate *S. aureus* and *Strept. agalactiae* from dairy herds. The elements were post-milking teat disinfection, dry cow therapy, treatment of clinical cases during lactation, proper maintenance of the milking machinery and culling of chronically infected cows. The five point plan, or some of its components, has considerably reduced *Strept. agalactiae* mastitis, but for *S. aureus* mastitis the effect has been less satisfactory. Separation of infected cows alone has not be shown sufficient **(Fox et al., 1991)**; cure rates for dry-cow therapy have been low and ranged from 40 to 70% **(Leslie and Dingwell, 2003)** and there is no scientific evidence to suggest that culling alone is of economic importance. Epidemiological studies of *S. aureus* in the environment of dairy cows have increased knowledge on the dynamics of *S. aureus* intramammary infections. Current strategies for control and prevention of *S. aureus* mastitis have been expanded to include isolation or elimination of the reservoir by segregation, therapy, and/or culling, isolation or removal of the fomites by applying improved milking hygiene, evaluation of teat skin condition, teat disinfection and back flush. In some countries, host resistance has been enhanced by improving management of the cows and vaccinating

against mastitis **(Talbot and Lacasse, 2005)**. In spite of the introduction of large-scale mastitis control programs, *S. aureus* remains a major mastitis pathogen. It causes mastitis epidemics even in well-managed dairy herds **(Smith et al., 1998)** and can persist for long periods in the mammary glands **(Anderson and Lyman, 2006)**. The current control practices may fail to prevent the spread of particularly virulent strains.

2.4 Teat dips and control of bovine *S. aureus* mastitis

2.4.1 Definitions

Biocide: is a chemical substance capable of killing living organisms **(Block, 2001)**. Because it varies in antimicrobial activity, other terms may be more specific, including "-static," referring to agents which inhibit growth (e.g., bacteriostatic, fungistatic, and sporistatic) and "-cidal," referring to agents which kill the target organism (e.g., sporicidal, virucidal, and bactericidal).

Antiseptics: are biocides or products that destroy or inhibit the growth of microorganisms in or on living tissue (e.g. health care personnel handwashes and surgical scrubs) **(Joklik, 1992)**.

Disinfectants: are products or biocides that are applied directly to an inanimate object to destroy or irreversibly inactivate most pathogenic microorganisms, some viruses, but not usually spores **(Quinn and Markey, 2001)**.

Teat dips: are biocides that are applied to the teats of lactating animals immediately after milking to control the spread of contagious bovine mastitis.

2.4.2 Biocides as teat dips

The udders of animals used for milk production to be free from microbes, may be contaminated with faecal and other dirty materials. That's why, before milking, udders are cleaned with water that may contain teat dips, although this is less common. More frequently, after the milking process, so-called teat dips are applied to protect the teat skin from different contagious pathogens. Control of mastitis in dairy cows is important for the production of high quality milk. Teat dipping is proposed as one of the most common investments applied in prevention and control of contagious bovine mastitis, and is an essential part of the five point plan **(Kingwill et al., 1970)**.

The prevention of bovine mastitis is the chief significant part of a mastitis control program, and both pre- and postmilking teat disinfectants are considered as the most effective procedures for preventing new IMI in different dairy herds. These procedures involve dipping teats of dairy cows immediately before and after milking with an effective germicidal preparation to reduce teat skin colonization and contamination with mastitis-causing bacteria and minimize penetration of bacteria into the teat canal **(Nickerson, 2001)**. The concept of teat disinfection after milking dates back to **Moak (1916)** when diluted pine oil was used to reduce the spread of *Strept. agalactiae*. However, the practice was not adopted widely for several decades because supporting research data were not available on existing teat dip products. In the end of the 1950's, **Newbould and Barnum (1960)** indicated that use of the germicidal teat dips after milking reduced the staphylococcal populations on milking machine. Afterwards, milking hygiene programs including teat dipping were evaluated in two field trials in England **(Neave et al., 1966; Neave et al., 1969)**. The hygiene programs in the farm reduced infection rates, and teat dipping was shown to be a highly effective component of the prevention and control programs.

Furthermore, the efficacy of teat dipping was established in field trials in England **(Kingwill, 1973)** and New York **(Natzke et al., 1972)** in which a mastitis control program, including post milking teat dips in combination with dry cow therapy of all cows, proved effective and prevented new IMI. Consequently, in Canada, where researchers at the University of Ontario, Guelf, observed that the practice of teat dipping in a chemical disinfectant after milking led to reductions in mastitis-causing bacterial populations on teat cup liners. Subsequent studies at the National Institute for Research in Dairying in England confirmed the Canadian observations in large field trials and led to extensive investigations at Cornell University, where postmilking teat dipping was included as a component of bovine mastitis control program **(Nickerson, 2001)**.

It is widely accepted that most postmilking teat dip products will reduce the new IMI rate by at least 50 to 90% **(Farnsworth, 1980)**. Only products shown by research to be effective and safe must be used. This involves using a product registered with the Food and Drug Administration (FDA). The label for such products will provide a lot of information on each active ingredient, steps for use, the manufacturer, a production lot number, and an expiration date. Responsibility for generating conclusive evidence of effectiveness belongs to the manufacturer. Dairy farmers should require evidence that a product meets FDA regulations and is effective in preventing new udder infections **(Nickerson, 2001)**. In the last twenty-five years, teat dipping with a post milking teat disinfectant has been proven to be an effective milking management practice to reduce the rate of new IMI. Therefore, postmilking teat antisepsis is regarded as the single most effective mastitis control practice in lactating dairy

cows and the reason for this is that teat dipping is a simple and economical way to decrease the colonization of bacteria on the teat skin **(Radostitis et al., 1994)**. Not all types of IMI are reduced equally by germicidal teat dips. Infections by contagious pathogens, those spread primarily from quarter to quarter and from cow to cow during the milking process, are reduced markedly by germicidal teat dips **(Østerås et al., 2008).** In contrast, some studies investigated the effect of teat dipping on IMI and observed that there was no significant difference between treated and control group of animals and this was due to many of mastitis causing bacteria having already established in the quarter before using teat dips **(Edinger et al., 2000)**. It is not expected that post milking teat dips would have any effect on already established infections **(Whist et al., 2007)**.

In the last 20 years, more than 10 active substances have been used in post milking teat disinfectants worldwide. In the United States, the National Mastitis Council has reviewed all the scientific literature on teat dips published since 1980 and noticed that iodine and chlorhexidine were the most frequent germicide classes used in teat dips **(National Mastitis Council, 2001)**. Iodophor and chlorhexidine teat dips decreased new IMI caused by *S. aureus* and *Streptococcus agalactiae* under experimental conditions. New IMI caused by *S. aureus* were reduced by the chlorhexidine and the iodophor products by 73.2 and 75.6%, respectively. Characterization of the condition of the teat skin and teat ends before and after each trial indicated that both products had no effects on the parameters measured **(Boddie et al., 1997)**.

2.4.2.1 Active ingredients commonly used in teat dips

2.4.2.1.1 Iodine

Iodine as a potent bactericide agent was first used in the remedy of bronchocele, a dilatation of the air passages in the lungs **(Gottardi, 1991)**. Recently, iodine is used as a topical antiseptic, germicidal handwash, surgical scrub, disinfectant of hard surfaces, and teat dip as Nonoxinol (9)-Iodine for dairy cows as an aid in the prevention of mastitis (**Flachowsky et al., 2007)**. It is a broad spectrum germicide, which is rapidly acting and effective against most mastitis-causing bacteria as well as fungi, viruses, and bacterial spores. Iodine has been widely used worldwide as the active ingredient in the majority of mastitis control teat dips, with concentrations ranging from 0.10% to 1.0 **(Boddie et al., 2000; Leslie et al., 2005)**. Iodine, in the form of a tincture or an iodophor, has long been known as an effective antiseptic and disinfectant. It has a broad spectrum of antimicrobial activity against vegetative bacteria, fungi, viruses, and even bacterial spores **(King et al., 1981)**. Iodine has several properties that make it difficult to use alone, such as being poorly soluble in water;

irritating in alcoholic solutions; it stains and has unpleasant acrid odor **(Windholz, 1976)**. These problems were reduced significantly by combining iodine with a solubilizing agent or carrier molecule to form iodophor compounds. **Nickerson (2001)** reported that all of the available iodine in the iodophor was found in the complexed but unbound form, and so it is not antimicrobial. The uncomplexed form is pointed to as free iodine and are provides the antimicrobial activity by oxidation of the microorganisms. The free and the complexed iodine components of the iodophor represent the available iodine, and present in a state of chemical equilibrium. Upon reacting with organic matter, milk and bacteria, the free iodine is used up, but is immediately replaced from the complexed iodine. Therefore, free iodine is usually available until the total amount of available iodine in the iodophor is consumed.

Winicov (1982) noticed that as iodophors had enhanced bactericidal activity and reduced vapor pressure that decreased the problems of odor and staining, a wide range of stable dilutions in water became possible. Moreover, iodophors are relatively nonirritating to skin **(Gershenfeld, 1977)**. Color is one of the common features of iodophors for on-farm use because an iodophor teat dip is visible on teats. In addition, **Windholz (1976)** observed that iodophors are considered relatively non-toxic but should be used in accordance with label directions as some irritation can develop. However, atmospheric temperature plays a significant role in the efficacy of iodophors and other teat dip for example the killing time of all germicides at temperatures near freezing decrease the efficacy of teat dips. Because natural protective oils are removed from the teat skin as consequence of their use, detergents are used as compensatory agents in iodophor teat dips. Therefore, conditioners are often added to iodine teat dips. These include emollients such as propylene and glycerin, which are normally added to teat dips at concentrations ranging from 2 to 10%, as well as lanolin, which serves as an emollient to replace natural oils lost from the skin **(Nickerson, 2001)**.

2.4.2.1.2 Chlorhexidine

The biguanide chlorhexidine is a significant disinfectant, antiseptic, pharmaceutical preservative and antiplaque agent **(Walhauser, 1984)**. It exists as acetate (diacetate), gluconate and hydrochloride salts. It is a colorless, odorless organic compound which is soluble in water and used at 0.5% concentration and a dye is commonly added to commercial products to allow the solution to be seen on the teat skin. It has a wide spectrum of bactericidal and antiviral activity and is a common ingredient in various formulation ranging from skin disinfectants in healthcare products to antiplaque agents in dentistry **(Paulson, 1993; Albandar et al., 1994)**. At present, chlorhexidine is used in veterinary medicine for preventing the spread of bacteria associated with bovine IMI due to its wide range of antimicrobial activity. Different chlorhexidine preparations are marketed as topical

postmilking teat dips and udder washes for use in commercial dairy milking operations **(Oliver et al., 1990)**. Because chlorhexidine residues are unlikely to be transferred into milk, it is considered a nonfood antiseptic. Additionally, it has also been shown to be efficacious as a therapeutic agent for treating bovine mastitis **(Boddie and Nickerson, 1993)**. This treatment relies on direct intramammary infusion into the udder, so chlorhexidine residues may be transferred to the milk during the milking process and lead to a negative effect on human dietary exposure. For understanding the mechanism of action of chlorhexidine, **Gjermo (1974)** studied the chemical structure of chlorhexidine and found that it contains two symmetrically positioned basic chlorophenyl guanide groups attached to a lipophilic hexamethylene chain (Figure 2) to help in the rapid absorption through the outer bacterial cell wall, causing irreversible bacterial membrane injury, cytoplasmic leakage, and enzyme inhibition.

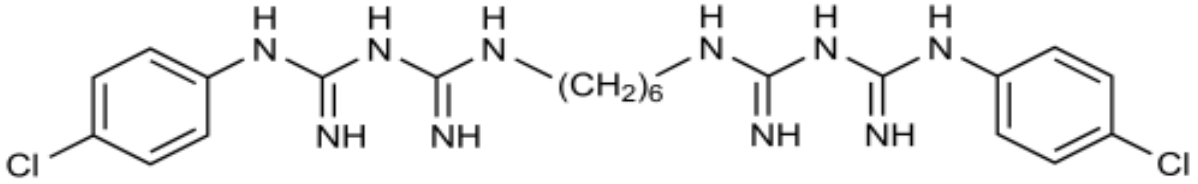

Figure 2: Chemical structure of chlorhexidine

Chlorhexidine exerts its bactericidal effect at an optimum pH range from 5.0-8.0. Thus, any deviation from this range leads to reduction in its action. Moreover, chlorhexidine is a cationic molecule that readily forms complexes with organic anions or other negatively charged agents, such as carbonate, phosphate, sulphate and chloride. When chlorhexidine is mixed with water that is 'hard', high in organic matter, which it has been treated with chlorine, insoluble salts are formed and its bactericidal effect is decreased. Reduction in bactericidal activity of chlorhexidine begins when water has a hardness of 20 parts per million. Chlorhexidine is entirely precipitated and inactive, when water hardness becomes above 200 parts per million **(Denton 2001)**. Emollients are often used in conjunction with chlorhexidine to enhance teat health.

2.4.2.2 Determining the germicidal activity and efficacy of teat dips

The efficacy of the teat dip can be determined by its ability to reduce the incidence of natural infection under field conditions, but it is a very expensive method and requires several efforts, such as studying of many cows for a long time. These limitations led to development of model systems in which efficacy could be evaluated more efficiently **(Pankey et al., 1984)**. The National Mastitis Council recommended three protocols. Protocol A, intended as a

screening test only, determined the germicidal activity of a teat dip formulation on teat skin. A teat was dipped in a bacterial suspension and then in the teat dip under test. Reduction of bacterial numbers was then calculated relative to number of bacteria recovered from control teats dipped in bacterial suspension only. Techniques of investigators, climatic conditions, and cow differences, however led to wide variations of results, thus this method was modified subsequently and performed on excised teats to minimize differences and generate more reproducible results **(Phlipot and Pankey, 1975; Phlipot et al., 1978)**.

Protocol B delineated steps to determine the ability of teat dips in the prevention of new IMI under experimental challenge conditions. This protocol evaluates the effectiveness of a product to reduce the incidence of new IMI compared with undipped control when teats are challenged experimentally with mastitis causing pathogens to increase the infection rate (**Nickerson, 2001)**. The last model recommended by the National Mastitis Council was Protocol C, which based on natural infection under field conditions. Attempts to evaluate teat dips using this method are usually performed by cooperating dairymen in commercial dairy herds. This model is similar to protocol B, evaluates the effectiveness of teat dip in reducing the incidence of new IMI compared with undipped controls; however, teats are not challenged with mastitis-causing bacteria, rather, the new IMI rate is dependent upon natural exposure to mastitis-causing pathogens on the farm. After milking, half the teats of cows are dipped in the teat dip under study and half are left as undipped controls. Quarter milk samples are collected every two weeks or month for approximately 1 year (to cover all seasons), and, at the end of the trial, the numbers of new infections in dipped and control quarters are compared and the efficacy is determined **(Phlipot et al., 1978; Nickerson, 2001)**.

2.4.2.3 Limitations and hazards of teat dips

Although general approval exists for teat dipping as one of the important component of a mastitis control program, the practice has several restrictions, and some risks may be associated with its use. Dipping of teats in a post milking teat dip will prevent many new infections, but duration of existing infections persists for a long time. Most IMI persist for months or years, and using teat dips alone requires several months before the infection in a herd is reduced substantially. **Dychdala (1968)** investigated that a 50% reduction of new IMI reduced the percentage of quarters infected by only 14% in the 12 month. Therefore, using of teat dips alone in a control program of bovine mastitis is not enough to obtain satisfactory results. The impact of teat dipping on mastitis is enhanced by simultaneous use of culling and dry cow therapy, measures designed to reduce the duration of existing infections. Antibiotic therapy of all dry cows is a practical and effective complementary to teat dipping.

A second restriction is that teat dips do not afford equal protection against all types of bacterial that cause bovine mastitis or IMI. The effectiveness of teat dips in reducing new IMI caused by contagious mastitis pathogens like *S. aureus* and *Strept. agalactiae* is well documented, but infections by other species of streptococci and coliform bacteria are not reduced as equalles markedly **(Bramley, 1981)** and in several studies coliform infections were not reduced at all by using teat dips **(Wesen and Schultz, 1970)**. This variation of efficacy is probably not due to the inability of germicides to destroy some species of bacteria but is more likely due to differences in the epidemiology of the various mastitis pathogens. Infections with *S. aureus* and *Strept. agalactiae* are contagious and are transmitted from infected to uninfected quarters and cows primarily during the milking process. An effective teat dip, applied to teats after each milking, often destroys these bacteria prior to teat skin colonization or penetration of the teat canal **(Pankey et al., 1984)**.

Irritation of teats is considered a third restriction usually associated with teat dipping. Some germicides incorporated in teat dips are mildly irritating. This problem may result from low or high pH, high acidity or alkalinity, of the product. Such problems may originate from manufacturing errors, deterioration of products from freezing or overheating, or from stratification through long storage without mixing. Severe problems may occur when highly acidic utensil sanitizers or udder washes are used as teat dips. Use of such products, for even a few milkings, can cause severe teat end lesions that may predispose to a serious outbreak of mastitis within a herd **(DeWitte et al., 1980)**. Sometimes irritation appears to be caused by interaction between teat dip and management or environmental factors in a herd. Products used safely in most herds appear to cause irritation in individual herds. Under extremely cold weather, it may be advisable not to dip teats. If teats are dipped, only the lower end should be dipped and should be dried before exposure to extremely cold weather. To inhibit irritation of teats and to improve skin conditioning, teat dip manufacturers often add emollients, such as glycerin or lanolin, into formulations. The germicidal activity of teat dips may be reduced if concentrations of emollients are added by more than 10 to 12% **(Pankey et al., 1984)**.

2.5 Mechanism of action of biocides

Impressive progress has been made in understanding how different types of biocides exert action and became an essential issue with the emergence of bacterial resistance to biocides and the suggestion that biocides and antibiotic resistance in bacteria might be associated. There is still a lack of understanding of the mode of action of biocides, especially when used at sub-lethal concentrations. Although such data might not be required for highly reactive biocides (e.g. alkylating and oxidizing agents) and biocides used at high concentrations, the

use of biocides as preservatives or in products at sub-lethal concentrations, in which a bacteriostatic rather than a bactericidal activity is achieved, is driving the need to better understand microbial target sites **(Maillard, 2002)**. The same methods used for evaluation the mechanism of action of antibiotics are used for the biocides. These methods include an evaluation of the effects on intracellular components such as interactions with macromolecules and their biosynthetic processes, inhibition of oxidative phosphorylation, and interference with enzymes and electron transport. They also include effects upon membranes such as a microscopic examination of cells exposed to biocides by effects on model membranes and examination of uptake, lysis, and leakage of intracellular components **(McDonnell and Russell, 1999)**. Biocides mechanisms of action depend on their chemical nature, the pathogens used in the evaluation (e.g., gram-positive bacteria, gram-negative bacteria, yeasts, and viruses), and on test conditions (e.g., concentration, pH, duration of exposure, and temperature). The cell wall of gram-positive bacteria is composed of a cytoplasmic membrane (CM), which overlies the cytoplasm and a thick peptidoglycan (PG) outer layer. Gram-negative bacteria add an outer membrane (OM), consisting of a lipopolysaccharides (LPS) layer, lipoproteins, and proteins (Figure 3).

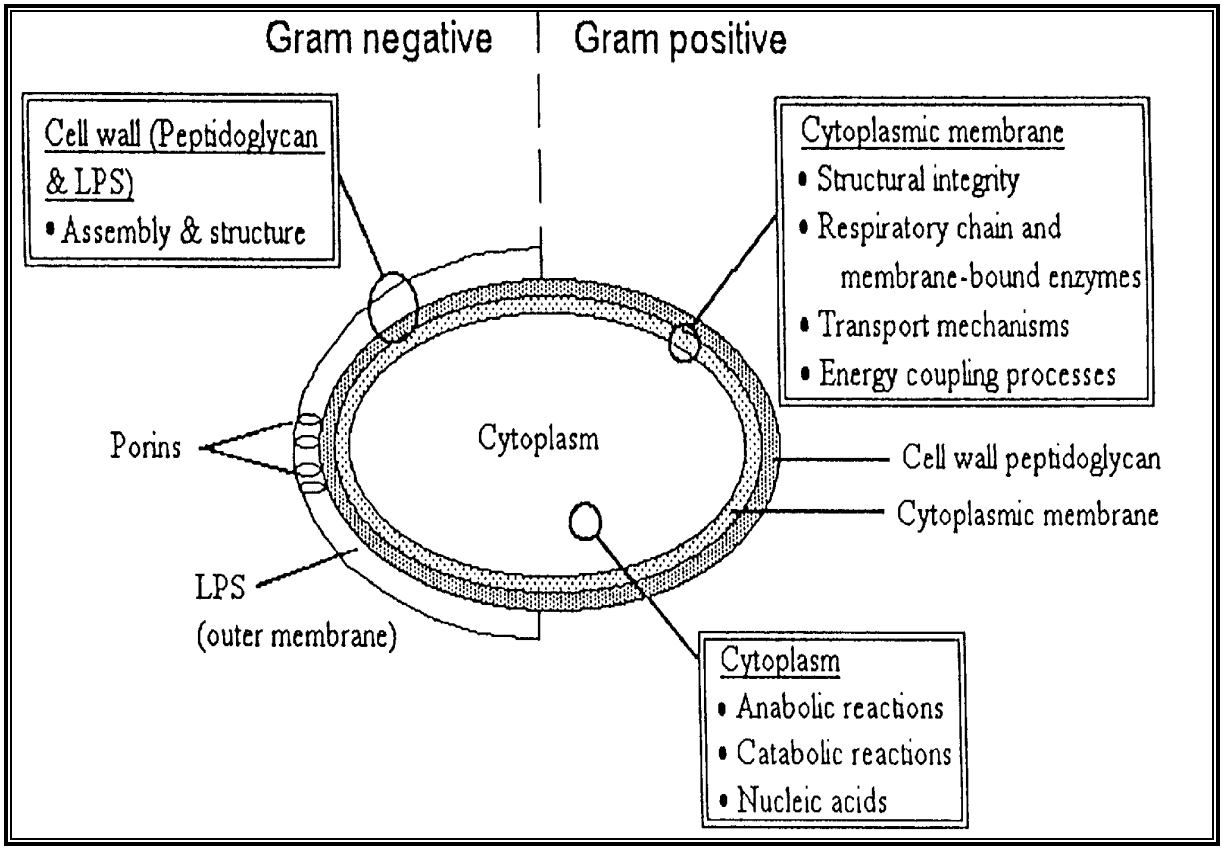

Figure 3: Potential targets for biocides reproduced by permission from **Denyer (1995)**

The OM in gram-negative bacteria is skeptical in maintaining the cell wall's integrity as a permeability barrier. Gram-negative bacteria are less sensitive to biocides than gram-positive bacteria because of the LPS layer. The core region of the LPS is negatively charged, impeding permeability and reducing susceptibility to negatively charged antiseptics. Anionic biocides, such as chlorhexidine, neutralize the negative charge and mediate changes in hydrophobicity of the OM, thereby promoting uptake **(McDonnell and Russell, 1999)**. Biocides also disrupt the CM by dissipating the proton motive force (PMF) of efflux pumps, and by interacting with CM enzymes **(Maillard, 2002)**. The PMF is a proton gradient across the CM that develops when the extracellular concentration of protons (H+) is greater than the intracellular concentration. Efflux pumps use the PMF by coupling biocide efflux to the counterflow of protons **(Maillard, 2002)**. Generally, the biocide initially binds to targets within the cell wall to disrupt the latter's integrity and then penetrates the cell wall and interacts with cytoplasmic constituents **(Cole et al., 2003)**. Biocides, unlike antibiotics, have multiple targets within the microbial cell (Figure 4). This multiple target effect is thought to participate to their bactericidal activity and dictates against the emergence of resistance. However, recent studies suggest that mutation or overexpression of triclosan and chlorhexidine target sites produce nonsusceptible microorganisms **(McMurry et al., 1998; Tattawasart et al., 1999; Levy, 2002)**.

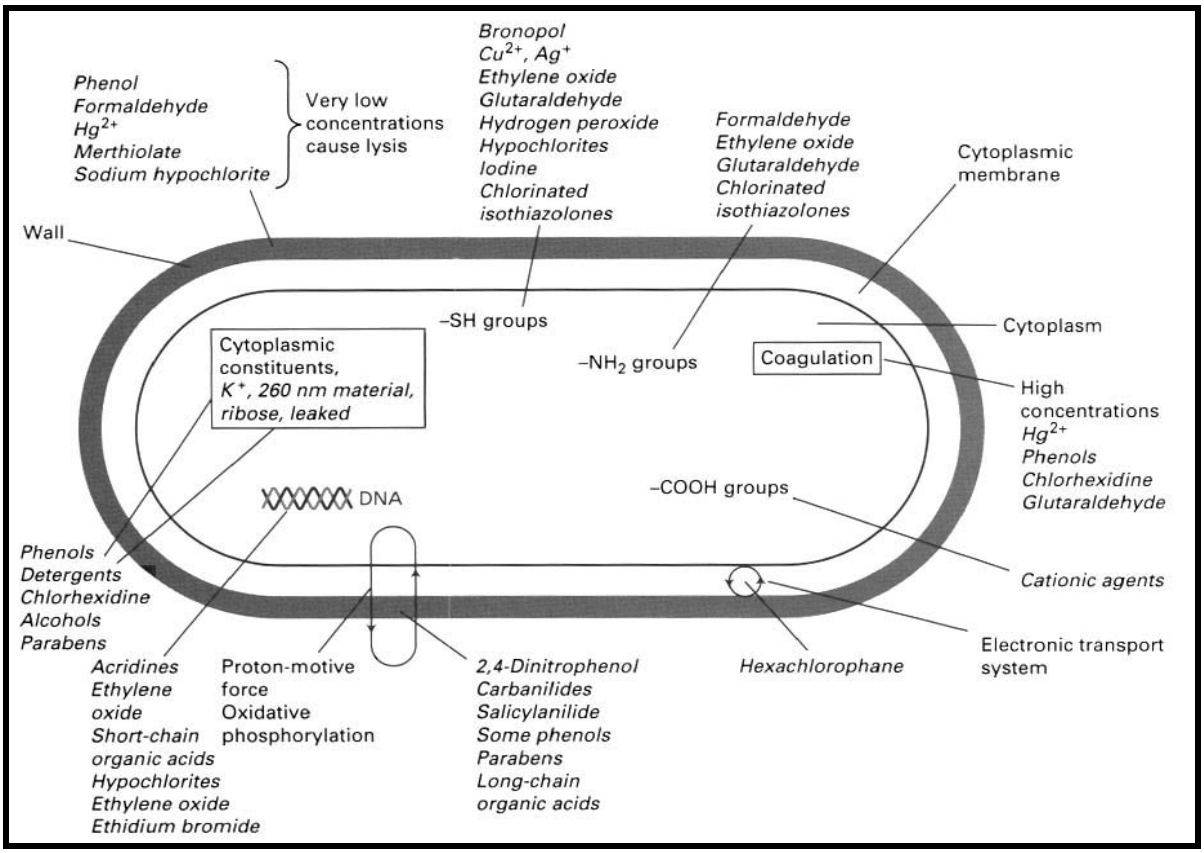

Figure 4: Different sites of action of biocides **(Maillard, 2002)**

2.5.1 Iodine and Iodophors mechanism of action

The antimicrobial action produced by iodine is very rapid and exerts its action even at low concentrations; however, the exact mode of action is unknown. Iodine can penetrate into microorganisms rapidly **(Chang, 1971)** and raids key groups of protein particularly the free sulfur amino acids cysteine and methionine **(Kruse, 1970; Gottardi, 1991)**, fatty acids and nucleotides, which lead to cell death. Despite of iodine and iodophors being less reactive than chlorine, iodine is a quickly bactericidal, fungicidal, virucidal, and sporicidal agent **(Gottardi, 1991)**. Although iodine solutions have been used as antiseptics from about 150 years ago, they are associated with several problems such as irritation and excessive staining; moreover, aqueous solutions of iodine are commonly unstable **(Anderson et al., 1990)**. These problems were solved by the development of iodophors, which are defined as iodine-releasing agents or iodine carriers and the most widely used are povidone-iodine and poloxamer-iodine in both disinfectants and antiseptics. Iodophors act as a reservoir of the active “free” iodine **(Gottardi, 1991)**. To ensure that lethal action is obtained, most disinfectants are used in a high concentration, substantially more than the MIC. At this level cell death is likely to be caused by non-specific disruptive effects such as membrane damage or protein coagulation rather than by subtle, selective inhibition of individual enzymes **(Maillard, 2002)**.

2.5.2 Chlorhexidine mechanism of action

Chlorhexidine is a bactericidal agent **(Denyer, 1995)**. Its interaction and uptake by bacteria were studied primarily by **Hugo and Longworth (1964)**, who found that the absorption of chlorhexidine was very rapid and depended on its concentration and pH. More recently, by using chlorhexidine gluconate, the absorption by bacteria was shown to be extremely rapid, with a maximum effect occurring within 20 second. Damage to the outer cell layers takes place **(El Moug et al., 1985)** but is not enough to induce lysis or cell death. The agent then crosses the cell wall or outer membrane, probably by passive diffusion, and subsequently attacks the bacterial cytoplasmic or inner membrane and leads to intracellular leakage. Thus, chlorhexidine at low concentrations is a strong membrane-active agent against both gram-positive and gram-negative bacteria, including the release of K+, 260 nm-absorbing material and pentoses and lead to intracellular leakage (Figure 4). The biguanide is also an inhibitor of adenosine triphosphatase (ATPase) activity. At higher bactericidal concentrations, chlorhexidine induces precipitation of cytoplasmic protein and nucleic acids and causes coagulation of intracellular constituents. As a result, the cytoplasm becomes congealed, with a consequent reduction in leakage **(Longworth, 1971)**, so that there is a biphasic effect on membrane permeability. As the concentration of chlorhexidine increases, the initial high rate

of leakage rises but leakage is reduced at higher biocide concentrations because of the coagulation of the cytosol.

2.6 Bacterial resistance to biocides

SCENIHR (2009) reported that biocidal products need to be approved before they are released on the market in the different areas of Europe. Their active substances must be safe for humans, animals and the environment. Nevertheless, the products being safe, the fact that they are used in huge amounts should have safety implications. If biocides kill all bacteria that are reasonably easy to eradicate, the only bacteria left are resistant strains and these are free to grow with no competition from other bacterial populations. It is probable that the large amount of biocides released into the environment alone may already exert a biological danger by applying a selective pressure on bacterial populations, leading to the selection and spreading of resistant bacteria. Recently, bacterial resistance to different types of biocides was not recognised as a problem. However, there is experimental evidence that particular bacteria do have the ability to develop resistance to some biocides, including chlorhexidine diacetate **(Tattawasart et al., 1999)** and iodophor **(O'Rourke et al. 2003)**. Resistance to biocides may be more likely to develop if they are used at concentrations lower than required for optimal biocidal effect. This reinforces the importance of always using biocides at the recommended concentrations and according to the label directions.

Bacterial resistance to biocides, like antibiotic resistance, can be either intrinsic or acquired. Antimicrobial resistance can occur through mutation or amplification of a chromosomal gene, or by acquiring resistance determinants on extra-chromosomal pieces of DNA (e.g., plasmids) **(Poole, 2002)**. Other mechanisms of biocide nonsusceptibility include a decrease in membrane permeability, active efflux, changes in bacterial target sites, or growth in biofilms. Biocides have several target sites against microbial cells. Thus, the incidence of general bacterial resistance is improbably to be caused either by a specific modification of a target site or by a by-pass of a metabolic process. It rather arises from a process causing the decrease of the concentration of biocide inside the bacterial cell under the threshold that is toxic to the bacterium. Multiple mechanisms based on this principle have been well-described, including a change in cell envelope, change in permeability, efflux and enzymatic degradation. It is probably that these mechanisms work synergistically although very few studies investigating multiple bacterial mechanisms of resistance following exposure to sub-lethal concentration of biocides have been performed **(SCENIHR, 2009**).

2.6.1 Intrinsic staphylococcal resistance mechanism

The cell wall of the genus *Staphylococcus* is composed mainly of peptidoglycan and teichoic acid. None of these appears to act as an effective barrier to the entry of antiseptics and disinfectants. Since high molecular- weight substances can readily cross the cell wall of staphylococci, this may explain the sensitivity of these organisms to many antibacterial agents including quaternary ammonium compounds (QACs) and chlorhexidine **(Russell, 1991; Russell, 1995; Russell and Chopra, 1996)**. Nevertheless, the plasticity of the bacterial cell envelope is a well-known phenomenon **(Poxton, 1993)**. The growth rate of organisms and any growth limiting nutrient will affect the physiological state of the bacterial cells. Under such conditions, the thickness and degree of cross linking of peptidoglycan are probably being modified and hence the cellular sensitivity to disinfectants and antiseptics will be changed.

In the nature, *S. aureus* may exist as mucoid strains, with the cells enclosed by a slime layer. Strains without this layer are killed more rapidly than mucoid strains by chloroxylenol, cetrimide, and chlorhexidine, but there is little difference in killing by phenols or chlorinated phenols; if the slime layer is removed by washing in saline broth or sub-culturing in Brain Heart Infusion (BHI) broth, the cells become sensitive. Therefore, the slime layer plays a protective role, either as a physical barrier to disinfectant penetration or as a loose layer interacting with or absorbing the biocide molecules. **Kolawole (1984)** investigated the effects of commercial preparations of some disinfectants and antiseptics on mucoid-grown *S. aureus* and found a substantial reduction in their killing efficiencies in the presence of mucoid-grown staphylococci, but not with non-mucoid organisms. This indicated that protection by the extra-cellular slime covering is an effective resistance mechanism of mucoid-grown staphylococci.

2.6.2 Acquired staphylococcal resistance mechanism

As can be seen with antibiotics, acquired resistance to disinfectants and antiseptics can increase by either mutation or the acquisition of genetic material in the form of plasmids or transposons. An increase in an antibiotic MIC can have significant consequences, often indicating that the target organism is unaffected by its antimicrobial action. Increased biocide MICs due to acquired mechanisms have also been reported and in some case misinterpreted as indicating resistance **(McDonnell and Russell, 1999)**.The role of plasmids in encoding resistance (or increased tolerance) to antiseptics and disinfectants was examined by **Chopra (1987)**; this topic was considered further by **Russell (1985)**. It was noticed that some biocides, for instance silver, other metals, and organomercurials, plasmids were not normally

responsible for the elevated levels of antiseptic or disinfectant resistance associated with certain species or strains. In contrast, there have been several reports linking the presence of plasmids in bacteria with increased tolerance to different types of disinfectants like chlorhexidine, QACs, and triclosan, in addition to diamidines, acridines and ethidium bromide **(Russell, 1997)**. It has been thought for numerous years that some antiseptics and disinfectants are always less inhibitory to *S. aureus* strains that contain plasmid carrying genes encoding resistance to the aminoglycoside antibiotic gentamycin. These biocidal agents include chlorhexidine, diamidines, and quaternary ammonium compounds (QACs), together with ethidium bromide and acridines **(Sasatsu et al., 1992)**. The genetic aspects of plasmid-mediated antiseptic and disinfectant resistant mechanisms have been investigated only in staphylococci species **(Sasatsu et al., 1985)**. These mechanisms are encoded in *S. aureus* strains by at least three separate multidrug resistance determinants. **Reverdy et al. (1992)** noticed an increase in the MIC values of antiseptics against MRSA strains and two gene families (*qacAB* and *qacCD*) of determinants were detected.

2.7 Induction of bacterial resistance to biocides with sub-lethal concentration

The induction of bacterial nonsusceptibility (resistance) mechanisms after exposure to a sub-lethal concentration of a biocide has been recorded in several studies for a number of biocides **(SCENIHR, 2009)**. Concentration is central to the definition of bacterial resistance in practice **(Maillard and Denyer 2009)**. The determination of the efficacy of a biocide with low concentration will indicate, by comparison to a reference strain, whether a bacterial strain is insusceptible (i.e. intrinsically resistant) or has acquired resistance to a biocide or not. The determination of minimum bactericidal concentrations (MBCs) is also another method that allows the comparison of lethality between a reference strain and clinically and/or environmentally resistant isolates. In some conditions, a phenotypic change leading to the incidence of resistance to several unrelated compounds in vitro has been reported, following exposure to a low concentration of a biocide **(Moken et al., 1997)**. It is possible that a biocide (triclosan) induces a stress response followed by, or in addition to, the expression of mechanisms that reduce the deleterious effect of the biocide **(Gilbert et al., 2002)**. A decrease in growth rates in *Escherichia coli* and *Pseudomonas aeruginosa* has been described following exposure to sub-lethal concentrations of triclosan, which indicates the generation of a stress to the organisms **(Gomez Escalada et al., 2005)**.

Bailey et al. (2009) found that triclosan induced bacterial resistance through the over-expression of efflux pumps via activation of mar and ram, over-expression and mutagenesis of fab1, expression of regulatory genes involved in the control of antibiotic resistance cascades (activator of drug efflux, decrease of membrane permeability) and fatty acid

metabolism in a number of bacterial genera **(Jang et al., 2008)**. **McBain et al. (2004)**, however, failed to demonstrate a biologically significant induction of drug resistance in a number of bacterial species exposed to sub-lethal concentrations of triclosan, suggesting that triclosan-induced drug resistance is not generally readily inducible nor is it transferred across bacterial species.

2.8 Antimicrobial resistance and *S. aureus* mastitis

2.8.1 Evidence of emerging antimicrobial resistance

Antibiotic therapy is considered as one of the most important tool of the five point plan for mastitis control. The treatments become more effective when they are directed by veterinarians; for example selection of a correct drug can be enhanced by using antimicrobial susceptibility test. The misuse or intensive use of antibiotics can lead to the development of resistance among different bacterial strains and contamination of foodstuff, with animal and human health implications **(Lingaas, 1998)**. Introduction of antibiotics such as tetracycline, aminoglycosides and macrolides into the scheme of bovine mastitis treatment has been attended by an incidence of resistance in bovine *S. aureus* strains **(Myllys et al., 1998)**. **Monecke et al. (2007)** and **Moon et al. (2007)** found that some strains of *S. aureus* were resistant to all β-lactams antibiotics and in this case *S. aureus* called methicillin resistance due to acquisition of modified penicillin-binding proteins (PBP). This property has been uncommon among bovine *S. aureus* isolates to date.

For a mastitis treatment to be successful, it must include the specific antibiotic agent. Drugs most commonly used are beta-lactams, aminoglycosides, macrolides, tetracyclines, chloramphenicol and lincosamides. The β-lactam antimicrobial agents, that include penicillins and cephalosporins, affect the bacterial cell wall production producing bacteria lysis. B-Lactam antibiotics are considered time-dependent drugs for their bactericidal effect. Aminoglycosides exerts their action through binding with specific receptor proteins on the 30S bacterial ribosomal subunit, inhibiting normal bacterial protein synthesis. Aminoglycosides are considered as concentration-dependent drugs for their bactericidal action. Macrolides and lincosamides are bacteriostatic, and impair protein synthesis in bacteria by binding to the 50S ribosomal subunits **(Barragry, 1994)**. Resistance of mastitis causing bacteria to antimicrobial agents is a well-documented challenge in dairy cows **(Pitkala et al., 2004)**. The World Health Organization (WHO) has reported that the resistance of antimicrobial drugs to different types of mastitis pathogens was associated with any use of antimicrobial agents **(WHO, 1997)**. This has called for more researches into the use of different antibacterial drugs in dairy animals and the determination of important factors

that affect the level of resistance in mastitis causing agents **(Østerås et al., 1999; Trolldenier, 1999; Aarestrup, 2005)**. Regional differences in resistance patterns of mastitis pathogens to antimicrobial agents exist in Germany and nearly all areas of the world **(De Oliveira et al., 2000)**. Not only early researches on resistance, but also some recent ones **(Erskine et al., 2002; Makovec and Ruegg, 2003)** were depended on the agar disk diffusion method, which has been shown not to correlate well with the MIC determined by dilution methods **(Kibsey et al., 1994; Kelly et al., 1999)**.

Treatment of bovine mastitis with penicillin during the dry period has been supposed to exert selection pressure toward penicillin resistant *S. aureus* strains **(Østerås et al., 1999)**. Resistance of mastitis pathogens to antimicrobial agents has 2 relevant aspects: The first is a reduction in cure rates after treatment of clinical mastitis cases **(Owens et al., 1997; Sol et al., 2000)**. The second aspect is the potential impact of transmission of resistant bacteria to humans via the food chain **(Ungemach, 1999)**. This is impossible to occur with milk from clinical cases of mastitis, because this milk is prevented from human consumption. In contrast, clinical cases may turn into subclinical cases or latent infections. Resistant bacteria from these infections are present in the bulk tank milk and may therefore be transmitted to humans via raw milk products.

S. aureus displays resistance to a wide variety of antimicrobial agents including chemical disinfectants **(Bjørland et al., 2001)**. In the Nordic countries of Europe, mastitis-causing *S. aureus* is less resistant to antimicrobial agents than in many other countries. In Norway and Sweden, the percentage of penicillin resistant isolates has stayed below 10% **(SVARM, 2002; NORM-VET, 2006)**. In the rest of the Europe, the percentage of penicillin resistant pathogens has ranged from 23% **(DANMAP, 2003)** up to 69% **(Nunes et al., 2007)**, additionally, in the United States it ranged from 38 to 61% **(Erskine et al., 2002)** as well as 40% in Argentina **(Gentilini et al., 2000)**. Impaired treatment response of bovine *S. aureus* strains has been associated with penicillin resistance **(Taponen et al., 2003)**. However, the connection is not direct, which may indicate that some other bacterial factors could be involved in the phenomenon **(Barkema et al., 2006)**. The most wide antimicrobial resistance studies involving mastitis isolates have examined *S. aureus.* From about forty three years ago **Jones et al. (1967)** observed that *S. aureus* isolates had relatively high MIC values for penicillin and ampicillin, and implied that beta-lactamase enzymes produced by some strains of *S. aureus* which lead to inactivation of the drugs. Beta-lactamase production is induced in some bacteria when exposed to Beta-lactam drugs.

Watts and Salmon (1997) noticed higher MIC values for *S. aureus* isolates that produced Beta-lactamase enzyme as compared with those isolated that did not. No evidence exists to

suggest that this adaptation of *S. aureus*, or resistance to other classes of antibacterial drugs, is any different from those noted thirty-five years ago. The MIC values and disk diffusion results demonstrate that ampicillin and penicillin are the antimicrobial drugs to which *S. aureus* are most commonly resistant. However, comparing values within tables from one time period to another should be avoided. Any comparison of this kind should be done with incredulity because of the differences in geography, numbers of isolates used within a study, and inconsistencies in laboratory methods. As an example, two studies performed in the same year by **Costa et al. (2000) and Gentilini et al. (2000)** in Argentina reported the percentage of oxacillin resistant strains of *S. aureus* as 42.0 and 0%, respectively.

2.8.2 Mechanisms of action of antimicrobial agents

There are five major mechanisms of action produced by antimicrobial agents: inhibition of the cell wall, protein and nucleic acid synthesis as well as inhibition of a metabolic pathway **(Neu, 1992)**. Disruption of the cell membrane function may be a fifth, although less well characterized mechanism of action. It is postulated that polymyxins exert their inhibitory effects by increasing bacterial membrane permeability, causing leakage of bacterial contents **(Storm et al., 1977)**. The β-lactams antimicrobial drugs such as the penicillins and cephalosporins work by inhibiting bacterial cell wall synthesis **(Neu, 1992; McManus, 1997)**. B-Lactam drugs inhibit the synthesis of the bacterial cell wall by interfering with the enzymes required for the synthesis of the peptidoglycan layer **(McManus, 1997)**. Macrolides, aminoglycosides, tetracyclines and chloramphenicol exert their antimicrobial effects by inhibiting protein synthesis **(Neu, 1992; McManus, 1997)**. Bacterial ribosomes differ in structure from their counterparts in eukaryotic cells. Antimicrobial agents take advantage of these differences to selectively inhibit bacterial growth. Macrolides, aminoglycosides, and tetracyclines bind to the 30S subunit of the ribosome, whereas chloramphenicol binds to the 50S subunit. **Drlica and Zhao (1997)** studied the antibacterial action of fluoroquinolones and found that they exert action by inhibiting DNA synthesis and causing lethal double-strand DNA breaks during DNA replication, whereas sulphonamides and trimethoprim (TMP) block the pathway for folic acid synthesis, which ultimately prevents DNA synthesis (**Yao and Moellering, 2003; Petri, 2006)**. The common antibacterial drug combination of TMP, a folic acid analogue, plus sulphamethoxozole (a sulphonamide) inhibits 2 steps in the enzymatic pathway for bacterial folate synthesis.

2.8.3 Resistance of bacteria to antimicrobial agents

Bacteria continue to surprise us with new mechanisms of resistance to antimicrobial agents. Bacteria are capable of acquiring or developing a wide-range of defense mechanisms

against antibiotics by acquisition of new genes from other bacteria or by mutations in their own existing genes. The emergence of resistance was observed almost simultaneously with the introduction of antibiotics and it is thought to be an evolutionary adaptation to the presence of antibiotics. The genetic determinants of defense mechanisms may derive from other bacteria e.g. antibiotic producing organisms. Bacteria which were susceptible to the substances produced by other bacteria or fungi had a handicap in the fight for resources. Thus they had to acquire some kind of resistance mechanism. Antibiotic producer microbes possess defense mechanisms against their own products and genes of these mechanisms usually reside in their chromosomes. Since bacterial genetic systems are very plastic, these genes can probably be integrated into mobile genetic elements and spread by horizontal transfer to other bacteria. Resistance genes transferred into the new hosts may undergo mutations, resulting in a wide diversity of structurally heterogeneous, but functionally homologous resistance determinants. As a result of single or multi-step mutations in genetic determinants of bacterial enzymes taking part in physiological cell metabolism change the substrate spectrum of enzymes, and they can degrade certain antibiotics **(Davies, 1994)**. Bacteria gain resistance to antibiotics by modifying their target structures by single- or multi-step mutations so that antibiotics cannot bind to them **(Storz and Hengge-Aronis, 2000)**.

2.9 Cross-resistance between biocides and antibiotics

The association between biocide nonsusceptibility (resistance) and antibiotic resistance is still unclear. Most investigators were able to demonstrate cross-resistance between antibiotics and biocides. But, when cross-resistance was demonstrated, it was often shown for second-line drugs or drugs not usually used for therapy **(Rogers, 2005)**. The possible linkage of biocide and antibiotic resistance in bacteria has been reported by several researchers and has fuelled recent debates as to whether the use of biocides selects for antibiotic resistance **(Maillard, 2002)**. Recently, several studies have been carried out to evaluate whether clinical or environmental isolates that show resistance (reduced susceptibility) to sub-inhibitory concentrations of biocides also exhibit resistance to antibiotics. Despite of some laboratory studies suggesting that the development of biocide and antibiotic resistance can be linked, other studies indicate no such link **(Russell et al., 1998; McDonnell and Russell, 1999)**.

2.9.1 Studies of reduced susceptibility to antibiotics in biocide-resistant bacteria

Antibiotic-susceptible *S. aureus* and other staphylococci are usually antiseptic-sensitive, whereas strains for which MICs indicated intermediate or high antiseptic resistance were also more resistant to a wide variety of antibiotics **(Reverdy et al., 1992)**. In one study, 310

Gram-positive strains isolated from quarter milk of dairy cows were investigated by **Martin and Maris (1995)**. They revealed positive links between chlorhexidine usage and resistance to the five tested antibiotics (ampicillin, kanamycin, streptomycin, tetracycline, gentamycin) in *Streptococcus* species, and between hexachlorophene and oxacillin in *Bacillus*. These studies enhance the need to develop research and surveillance programmes in the area of animal husbandry. **Irizarry et al. (1996)** and **Mitchell et al. (1998)** observed an increase in the MICs for MRSA strains for some biocides including chlorhexidine, benzalkonium chloride (BAC), cetrimide, hypochlorite, triclosan, parahydroxybenzoates and betadine. In another study, the MIC of triclosan was determined against 186 strains of MRSA and MSSA **(Bamber and Neal 1999)** and it indicated that the MICs of 14 isolates (7.5%) were increased, and these were equally distributed between MRSA and MSSA strains. Recently, **Karatzas et al. (2007)** described the effect of the bisphenol triclosan-resistant *Salmonella enterica* on emerging bacterial cross-resistance. The authors clarified that triclosan-selected strains are less susceptible to antibiotics than the wild type original strain. A more recent study described the survival of *Salmonella enterica serovar Typhymurium* after prolonged exposure to different types of disinfectants with sub-lethal concentrations on emerging cross-resistance to antibiotic profile **(Randall et al., 2007)**. When cross-resistance was examined, growth of *Salmonella* with sub-inhibitory concentrations of biocides favours the emergence of strains resistant to different classes of antibiotics.

2.9.2 Studies of no change in susceptibility to antibiotics in biocide-resistant bacteria

In contrast, several investigations have failed to make a direct association between biocide exposure and antibiotic resistance, although the antibiotic susceptibility of the bacterial strain was altered **(Nomura et al., 2004; Thomas et al., 2005)**. The chlorhexidine sensitivity of 33 clinical isolates of *Enterococcus faecium* sensitive to vancomycin and gentamycin was evaluated by **Baillie et al. (1992)**. The results showed no increase in resistance to chlorhexidine as indicated by the evaluation of MICs. Interestingly, another study of 67 ciprofloxacin-resistant isolates of *Pseudomonas aeruginosa* was done by **Baillie et al. (1993).** It was observed that 4 isolates were hypersensitive to chlorhexidine whilst none were found amongst 179 ciprofloxacin-sensitive isolates. Moreover, a series of antibiotic-resistant clinical and environmental isolates, including *Pseudomonas aeruginosa, Klebsiella* species*, E. coli, S. aureus* and *S. epidermidis*, were not less susceptible to the bactericidal activity of disinfectants, which included a phenol and a quaternary ammonium disinfectant, chloroxylenol, cetrimide and povidone iodine **(Rutala et al., 1997; Payne et al., 1999)**. The link between adaptive nonsusceptibility (resistance) to biocides and cross-resistance to antibiotics in *E. coli* O157 and *Salmonella enterica* was investigated by **Braoudaki and Hilton (2004)**. Four bacterial strains (*E. coli* O157, *Salmonella enterica serovar Enteritidis*,

Salmonella serovar Typhimurium, and *Salmonella serovar Virchow*) were adapted to grow in erythromycin, benzalkonium chloride (BKC), or other biocides by serial passage in sub-lethal concentrations of the antimicrobial. The authors found that no antibiotic cross-resistance was seen with benzalkonium chloride (BKC)-adapted *Salmonella serovar Enteritidis* or Typhimurium. Nevertheless, erythromycin-adapted *Salmonella serovar Typhimurium* was nonsusceptible to both chlorhexidine and triclosan. In contrast, the authors describe a high degree of cross-resistance between antibiotics for both *E. coli* and *Salmonella serovar Virchow*. **Lear et al. (2006)** demonstrated that environmental isolates with an increased MIC to triclosan remained susceptible to other biocides and antibiotics. **Jurgens et al. (2008)** determined if the exposure of *Pseudomonas aeruginosa* biofilms to chloraminated drinking water could lead to individual bacteria with resistance to antibiotics. It was observed that exposure to chloramine does not increase antibiotic resistance in this bacterial species. **Birošová and Mikulášová (2009)** reported that the emerging antibiotic resistance in *S. enterica* serovar Typhimurium after continuous exposure to sub-inhibitory concentrations of triclosan did not increased. Additionally, the antibiotic susceptibility of triclosan tolerant *S. aureus* strains was investigated by **Cottell et al. (2009)** and they stated that these strains remain susceptible to antibiotics used in clinical settings.

CHAPTER 3: MATERIALS AND METHODS

The main objective of the current study was to determine in vitro the efficacy and the resistance induction of *S. aureus* strains against two types of teat disinfectants (Ujosan® dip and Eimü Chlorhexidin® dip), as well as to check the antibiotic resistance patterns of bovine mastitis isolates of *S. aureus* and CNS. A further objective was to find a possible link between reduced susceptibility to teat dips and antibiotic resistance.

3.1 Materials

3.1.1 Milk samples and animals

Quarter milk samples were collected from six dairy herds with high prevalence of *S. aureus* in the federal state of Brandenburg, Germany, using standard procedures described by the **National Mastitis Council (2001)**. Of each herd, 32 cows in different stages of lactation and different age groups were chosen for sampling. Animals were divided according to udder teat dipping schemes into three groups. Teats of the first group were dipped with postmilking teat disinfectant Ujosan® dip (Nonoxinol–(9) Iodine–Complex); the second group was dipped in the Eimü Chlorhexidin® dip, while the third group was kept without dipping (a negative control group). Before sample collection, teat ends were cleaned with warm water and dried before 10 to 15 ml of milk was drawn and discarded. The teat ends were then scrubbed with a cotton or paper towel containing 70% ethanol; one towel was used for each teat before the sample was collected, and then the milk samples were collected every two weeks and transported on ice, frozen, and maintained at -20 °C until analysis.

3.1.2 Bacterial strains

A total of 70 isolates of *S. aureus* and CNS had been isolated from quarter milk of cows with subclinical mastitis from a survey carried out for a half year. Isolation of all isolates was performed according to the National Mastitis Council recommendations on the examination of quarter-milk samples. Preliminary identification of *S. aureus* strains was by colony morphology, hemolysis, and Gram staining. Creamy, grayish-white, or golden-yellow pigmented colonies that were catalase and coagulase- positive with gram-positive cocci that exhibited complete, incomplete, or both complete and incomplete hemolysis were identified as *S. aureus*. Specific identification of *S. aureus* strains and CNS were done phenotypically by the tube coagulase test and the Staph ID 32 API systems and genotypically by the Polymerase Chain Reachtion (PCR) and mass spectrally by using the matrix assisted laser desorption/ionization-time of flight- mass spectrometry (MALDI-TOF-MS). The reference

S. aureus strains used for each trial were American Type Culture Collection 25923 (ATCC 25923) and German Collection of Microorganisms (Deutsche Sammlung von Mikroorganismen 799 (DSM 799)).

3.1.3 Teat dips

30 isolates of *Staphylococcus* species (17 coagulase positive *S. aureus* and 13 CNS) stemmed from the first group of cows which were regularly dipped with the preparation Ujosan® dip (2700 ppm as Nonoxinol–(9)Iodine–Complex and glecrol > 8%), purchased from Company, Kesla, Germany; another 30 isolates of *Staphylococcus* species (29 coagulase positive *S. aureus* & one CNS) stemmed from the second group of cows that were regularly dipped with the preparation Eimü Chlorhexidin® dip (3500 ppm as Chlorhexidindigluconat, as well as glycerol, polysorbate 20, sodiumsalt (E141), Chlorophyllin-a-copper complex, purified water), purchased from the company of Eimermacher, Germany, and 10 isolates of *Staphylococcus species* were isolated from the third group of cows without teat dipping (control group).

3.1.4 Culture media

- **Tryptose Soya Broth (CASO Broth)**

CASO Broth is often used for the tube dilution method of antibiotic and disinfectant susceptibility testing. The medium will support a luxuriant growth of many fastidious organisms without the addition of serum.

Composition:

Ingredients	Grams/Litre
Casein peptone	17.0
Soy peptone	3.0
Sodium chloride	5.0
Dipotassium hydrogen phosphate	2.5
Glucose	2.5

Preparation:

Suspend 30 g of dehydrated media (Oxoid. LTD, Basingstoke, Hampshire, England) in 1 litre of purified filtered water. Sterilize at 121°C for 15 minutes. Cool to 45-50°C. Mix gently and

dispense into sterile Petri dishes or sterile culture tubes. Store prepared media below 8°C, protected from direct light. Store dehydrated powder in a dry place, in tightly-sealed containers at 2-25°C.

- **Tryptose Soya Agar (CASO Agar)**

CASO Agar is a medium very rich in nutrients for general use in microbiological laboratories. It supports the abundant growth of different organisms such as *S. aureus*. It is very useful for determination of hemolytic reactions.

Composition:

Ingredients	Grams/Litre
Casein peptone (pancreatic digest)	15.0
Soy peptone (papaic digest)	5.0
Sodium chloride	5.0
Agar	15.0
Final pH	7.3 ± 0.2

Preparation:

Suspend 40 g of the medium (Oxoid. LTD, Basingstoke, Hampshire, England) in one liter of deionized or distilled water. Heat with frequent agitation and boil for one minute until complete dissolution. Sterilize in an autoclave between 118 and 121°C for 15 minutes. In the case of large volume preparation, increase the time of sterilization but not the temperature or pressure. Cool and pour into Petri dishes.

- **Mueller-Hinton Agar**

Mueller-Hinton Agar is used in the tests for organism susceptibility to antimicrobial agents by the disk diffusion method.

Composition:

Ingredients	Grams/Litre
Beef Infusion	2.0
Corn Starch	1.5
Acid Casein Peptone (H)	17.5
Agar	17.0
pH	7.4 ± 0.2

Preparation:

Suspend 38 g of the medium (Oxoid. LTD, Basingstoke, Hampshire, England) in one liter of distilled or deionized water. Mix well and heat with frequent agitation. Boil for one minute and sterilize at 121°C for 15 minutes. Cool to 40-45°C. Pour the freshly prepared and cooled medium in flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates. The agar medium is cooled to room temperature and stored in the refrigerator (2 to 8°C) until use.

- **Columbia Blood Agar**

Columbia blood agar base media are typically supplemented with 5-10% sheep, rabbit, or horse blood for use in isolating, cultivating and determining hemolytic reactions of fastidious pathogenic microorganisms. Without enrichment, Columbia Blood Agar Base is used as a general purpose media.

Preparation:

Suspend 43 g of the medium in one litre of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium then autoclave at 121°C for 15 minutes. Prepare 5 - 10% blood agar by aseptically adding the appropriate volume of sterile defibrinated blood to melted sterile agar medium, cooled to 45 - 50°C.

3.1.5 Solutions

- **PCR master-mix**

PCR amplification was carried out by using master-mix which consisted of:

ReddyMix™ PCR buffer	22.5 µl	Mec-Forward (mec-F)	0.25 µl
Purified water	0.5 µl	Mec-Rear (mec-R)	0.25 µl
Nuc-Forward (nuc-F)	0.25 µl	16S rRNA-Forward	0.25 µl
Nuc-Rear (nuc-R)	0.25 µl	16S rRNA-Rear	0.25 µl

- **McFarland turbidity standard**

McFarland standard is used to adjust the turbidity of the inoculum and 0.5 McFarland may be prepared by adding 0.5 ml of a 1.175% (wt/vol) barium chloride dihydrate (BaCl2•2H2O) solution to 99.5 ml of 1% (vol/vol) sulfuric acid. The turbidity standard is then aliquoted into

test tubes identical to those used to prepare the inoculum suspension. Seal the McFarland standard tubes with wax, parafilm, or some other means to prevent evaporation. McFarland standards may be stored for up to 6 months in the dark at room temperature (22° to 25°C). Discard after 6 months or sooner if any volume is lost. Before each use, shake well, mixing the fine white precipitate of barium sulfate in the tube.

- **Physiological saline (Sodium chloride, NaCl)**

Physiological saline solution is used to adjust the turbidity of the inoculum and 0.5 McFarland and is prepared by dissolving 8.5 g of NaCl in 1 liter of distilled water and then sterilized by autoclaving. Store at ambient temperature for up to 6 months with caps tightened to prevent evaporation.

3.1.6 Instruments

- **Nephelometer**

An instrument (TREK Diagnostic Systems, East Grinstead, UK) used for measuring the concentration of bacteria in suspension by the amount of light that is scattered by the suspended particles.

- **Nexttec™ DNA isolation system**

Isolation of pure genomic DNA from staphylococci was obtained by the nexttec™ DNA isolation system (nexttec™ cleanColumns) in only 4 minutes* using one step for purification (nexttec Biotechnologie GmbH, Hemmelrather Weg 201, D-51377 Leverkusen, Germany).

- **Professional thermocycler PCR**

PCR amplification of all isolates of *Staphylococcus* species was done by using professional thermocycler (Biometra GmbH, Rudolf-Wissell-Str. 30, D-37079 Goettingen, Germany).

- **Microflex LT (MALDI-TOF-MS)**

Rapid identification of *S. aureus* and CNS was done by using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) (Brucker Daltonik GmbH, Leibzig, Germany).

- **Sensititre AutoReader**

The microtitre plates used for antibiotic susceptibility testing were read by a sensititre automatic reader (Trek Diagnostic Systems, East Grinstead, UK). The AutoReader plays an important role in the automatic transfer of test results to a data management system for processing, interpretation, and report generation to increase lab productivity.

3.1.7 Statistical methods

The data imported into SAS, and all calculations were performed using SAS, version 9.1 (SAS, Cary, NC, USA) according to **Carey et al. (1993)**.

3.2 Methods

3.2.1 Identification of *S. aureus* and CNS

3.2.1.1 Phenotypic identification

3.2.1.1.1 Tube coagulase test

The tube coagulase test is a generally accepted method for differentiating *S. aureus* from other Micrococcaceae **(Harmon et al., 1990)**. *S. aureus* is known to produce coagulase, which can clot plasma into gel. This test is useful in differentiating *S. aureus* from other CNS. The tube coagulase test was performed in sterile glass tubes (13 mm diameter) by adding 3 to 5 colonies of bacterial culture to 0.5 ml of reconstituted rabbit plasma (Becton, Dickinson and Company, Sparks, Maryland, USA)). After mixing by gentle rotation, the tubes were incubated at 37°C. Clotting was evaluated at 30 min intervals for the first 4 h of the test and then after 24 h incubation. The reaction was considered positive, if any degree of clotting was visible within the tube when tilted. At the time of use, both positive and negative control cultures were tested to confirm performance of the coagulase plasma, techniques and the methodology.

3.2.1.1.2 Staph ID 32 API system

Most of the species are determined based on various phenotypic characteristics, such as colony morphology and haemolysis patterns, and various biochemical reactions. Identification based on these conventional tests is time-consuming and costly, and therefore test series like API Staph (BioMe´rieux, France), for rapid identification of staphylococcal

species, are commonly used. The Staph ID 32 system strip (API System, BioMe´rieux, Paris, France) consists of 32 cupules, 26 of which contain dehydrated biochemical media for colorimetric tests. The tests included acid production from urea (URE), L-arginine (ADH), L-ornithine (ODC), esculin (ESC), D-glucose (GLU), D-fructose (FRU), D-mannose (MNE), D-maltose (MAL), D-lactose (LAC), D-trehalose (TRE), D-mannitol (MAN), D-raffinose (RAF), D-ribose (RIB), D-cellobiose (CEL), potassium nitrate (NIT), sodium pyruvate (VP), 2-naphthy- ßD-galactopyranoside (ßGAL), L-arginine ß-naphthyamide (ArgA), 2-naphthyl phosphate (PAL), pyroglutamic acid-ß-naphthylamide (PyrA), novobiocin (NOVO), sucrose (SAC), N-acetylglucosamine (NAG), D-turanose (TUR), L-arbianose (ARA) and 4-nitrophenyl-ßD-glucuronide (ßGUR). The manufacturer's recommended procedures (API System, BioMe´rieux) were followed. Briefly, the bacterial suspensions were prepared from overnight cultures on blood agar plates (5% horse blood). They were standardized with a turbidity equivalent to 0.5 McFarland standards in 6 ml of sterile distilled water. The ampule of inoculated API suspension medium was homogenized and 55 µl of the suspension were dispensed in each cupule of the strip. The tests URE, ADH and ODC were covered with 2 drops of mineral oil. After an incubation period of 24 h at 37°C, reagents were added for the nonspontaneous tests. Strain profiles were read and identified with Automatic Testing Bacteriology (ATB) Expression and were interpreted with API laboratory (LAB) software. This software gives the probability of the identification result in a range of 10 to 100%.

3.2.1.2 Genotypic identification

3.2.1.2.1 Isolation of genomic DNA

To apply the PCR test for detection of *S. aureus* isolated from milk of bovine subclinical mastitis, DNA was extracted from all bacterial isolates. 0.5 ml of bacterial culture was transferred to a 1.5 ml reaction tube and centrifuged (6,000x g, 1 min), then removed and discarded the supernatant. 90 µl Buffer B1, 10 µl Lysozyme and 20 µl RNase A were added to the bacterial cell pellet, cells resuspend by thorough vortexing and incubated with shaking (60°C, 1200 rpm, 10 min) in a thermomixer (Univortemp, Universal Labortechnik GmbH & Co. KG). 2.5 µl Buffer B2, 87.5 µl purified water and 10 µl Buffer B3 were added to each sample and then vortexed and incubated with shaking (60°C, 1200 rpm, 30 min) in a thermomixer. Nexttec ™ cleanColumn (nexttec GmbH Biotechnologie, Leverkusen, Germany) was equilibrated by adding 350 µl Prep Buffer to a nexttec ™ cleanColumn, incubated for at least 5 min at room temperature and centrifuged at 350x g for 1 min to remove excess buffer. The waste collection tube was discarded; the nexttec ™ cleanColumn was placed into a new DNA collection tube, and store equilibrated nexttec ™ cleanColumns closed at +2°C to +8°C and used within one week. 120 µl of the lysate was transferred to the equilibrated nexttec ™

cleanColumn and incubated for 3 min at room temperature and then centrifuged at 700x g for 1 min and the nexttec ™ cleanColumn discarded, the eluate contains the purified DNA.

3.2.1.2.2 PCR amplification

PCR amplifications were performed with a pair of primers specific for the nuc gene which encodes of the *S. aureus* -specific region of the thermonuclease gene *S. aureus*; mecA, a determinant of methicillin resistance and a genus-specific 16S rRNA sequence used as an internal amplification control for staphylococcal DNA. PCR primers specific for *S. aureus* nuc gene, mecA gene and 16S rRNA gene were designed according to the sequences published in Biomers, Ulm, Germany (http://www.biomers.net/de/index/impressum.html). The primer sequences were as follows: nuc forward primer, nuc1, 5´ TCAGCAAATGCATCACAAACAG 3´ and reverse primer, nuc2, 5´ CGTAAATGCACTTGCTTCAGG 3´; mecA forward primer, mecA1, 5´ GGGATCATAGCGTCATTATTC 3´ and reverse primer, mecA2, 5´ AACGATTGTGACACGATAGCC 3´; 16S rRNA forward primer, 16S rRNA1, 5´ GTGCCAGCAGCCGCGGTAA 3´ and reverse primer 16S rRNA2, 5´ AGACCC GGGAACGTATTCAC 3´; the three pairs of primers amplify 255-bp nuc gene fragment, 527-bp mecA gene fragment and 886-bp 16S rRNA gene fragment, respectively. PCR amplification was carried out in 0.5 ml tubes in a final reaction volume of 24 µl. The PCR master-mix consisted of 22, 5 µl reddy-mix, 0.5 µl purified water, nuc-F (0.25 µl), nuc-R (0.25 µl), mec-F (0.25 µl), and mec-R (0.25 µl). A DNA sample of 1 µl was used as the target in the PCR.

The amplification was performed with an automated thermocycler (Biometra GmbH). The PCR cycles consisted of pre-heating at 95°C for 10 min, denaturation at 94°C for 1 min, annealing at 55 °C for 0.5 min, and extention at 72°C for 1, 5 min. The amplification was performed for 37 cycles with a final extention step at 72°C for 5 min. The DNA fragments were separated by electrophoresis on a 1.5% agarose gel stained with TBE buffer (pH 8.3; 0.09 M Tris, 0.09 M boric acid, 2.0 mM EDTA) and with 0.003% (wt/vol) ethidium bromide incorporated for DNA staining. The sizes of PCR products (8 µl aliquot) were determined by comparison to the marker. Gels were run in 1x TBE buffer at 108V for 60 min. One positive control containing reference strain *S. aureus* ATCC 29213 and one negative control containing water were included in each experiment. The PCR products were visualized and photographed on a BioRad (Biometra GmbH & Co. KG).

3.2.1.3 Mass spectral identification

All 70 staphylococcal species were confirmly identified by MALDI-TOF-MS. Cells from a single colony of fresh overnight culture (Columbia agar supplemented with 5% horse blood (bioMérieux)), incubated 24 or 48 h at 37°C.) were used for each isolate to prepare samples according to the microorganism profiling ethanol/acid formic extraction procedure, as recommended by the manufacturer. After centrifugation at maximum speed for 2 min, one µL of each supernatant containing the bacterial extract was allowed to dry after overlaying it with 1 µl of a chemical matrix (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) on a polished steel MALDI target plate. Then, the samples were processed in the microflex LT (Bruker Daltonik GmbH, Leipzig, Germany) mass spectrometer equipped with a 20-Hz nitrogen laser. The spectra were recorded in the positive linear mode as described elsewhere (**Carbonnelle et al., 2007)**. Each spectrum was the sum of the ions obtained from 200 laser shots performed in 5 different regions of the same well. The spectra have been analyzed in a range of 1000 to 11000 m/z. The analysis was performed with the flex analysis software and calibrated with the protein calibration standard T (Protein I, Bruker Daltonics). The data obtained with the 2 replicates were added to minimise random effect. The presence and absence of peaks were considered as fingerprints for a particular isolate. The profiles were analysed and compared using the software BGP-database available on the website http: // sourceforge.net/projects/bgp.

3.2.2 *In vitro* susceptibility of *S. aureus* to commercial teat dips using broth macrodilution method

The MIC for all strains was determined using the broth macrodilution method which is indicative of the guideline for examination of chemical disinfectants in the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft (DVG)). Serial dilutions of disinfectant are made in a liquid medium as tryptose soya broth (TSB) which is inoculated with a standardized number of *S. aureus* and incubated for a prescribed time. The turbidity of the actively growing broth culture is adjusted by Nephelometer (TREK Diagnostic Systems, East Grinstead, UK) with sterile saline (NaCl) to obtain turbidity, optically comparable to that of the 0.5 McFarland standards (ca 1×10^8 KbE/ml). The lowest concentration (highest dilution) of disinfectant preventing appearance of turbidity is considered to be the MIC. This method was repeated two times in the same manner for Ujosan® dip, Eimü Chlorhexidin® dip and the negative control group. Although the tube dilution test is fairly precise, the test is laborious because serial dilutions of the disinfectant must be made and only one isolate can be tested in each series of dilutions.

3.2.3 Induction of *S. aureus* resistance to chemical disinfectants with sub-lethal concentration using broth macrodilution method

A good measure of resistance is the minimum concentration needed to kill the micro-organisms. An increase in the amount of biocide needed indicates that the microorganisms are becoming resistant to it **(SCENIHR, 2009)**. 10 different isolates of *S. aureus* previously tested with the preparations Ujosan® dip and Eimü Chlorhexidin® dip were included in this study. In the present study, attempts of sensitivity reduction (resistance) for *S. aureus* isolates were conducted through the repeated passage of these isolates in growth media with sub-lethal active substance concentration of Ujosan® dip or Eimü Chlorhexidin® dip (concentration of each disinfectant below the MIC, where the isolates still show growth (12.5 % & 25 % for Ujosan® dip and Eimü Chlorhexidin® dip, respectively)).

The basis of this investigation was to compare MIC for each active agent before and after 10 passages. All strains were passed for 10 times in a liquid medium (TSB) with sub-lethal concentration of a disinfectant within an 72h interval under complete hygienic conditions to avoid contamination; subsequently the MIC value for these isolates after the 10th passage was again detected, and then compared with the original MIC value before passages. The purity of the cultures was checked by streaking on to selective agar media for *S. aureus* (Mueller-Hinton agar). The stability of disinfectant resistance was determined by continuous subculture of the resistant strains in disinfectant –free nutrient broth (TSB). Subcultures were performed every 24 h for 10 passages and the MIC determined after the 10th passage. A check of culture purity was performed at each stage.

3.2.4 Antimicrobial drug resistance *of S. aureus* strains and CNS using agar disk diffusion method

The agar disc diffusion test was carried out to determine the drug susceptibility for all strains. This test was conducted and interpreted according to the recommendations and criteria of the National Committee of Clinical Laboratory Standards **(NCCLS, 1999)** for bacteria isolated from animals (Table 1). The following disks (Company, Mast Diagnostika, Reinfeld, Deutschland) were used: penicillin G, 10 I.U.; gentamycin, 10 µg; oxacillin, 5 µg; erythromycin, 15 µg; tetracycline 30 µg; chloramphenicol, 30 µg. Each culture to be tested should be streaked onto a non-inhibitory agar medium (tryptose soy agar) to obtain isolated colonies.

Preparation:

After incubation at 37°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline and vortex thoroughly. The turbidity of the actively growing broth culture is adjusted by using Nephelometer (TREK Diagnostic Systems, East Grinstead, UK) with sterile saline (NaCl) to obtain turbidity optically comparable to that of the 0.5 McFarland standards (ca 1×10^8 KbE/ml). Within 15 minutes after adjusting the turbidity of the inoculum suspension, dip a sterile cotton swab into the suspension. Pressing firmly against the inside wall of the tube just above the fluid level, rotate the swab to remove excess liquid. Streak the swab over the entire surface of a Mueller-Hinton agar plate using a sterile swab three times, rotating the plate approximately 60 degrees after each application to ensure an even distribution of the inoculum. Apply the antimicrobial disks to the plates as soon as possible, but no longer than 15 minutes after inoculation. Place the disks individually with sterile forceps, and then gently press down onto the agar.

In general, place no more than 3 disks on each plate. This prevents overlapping of the zones of inhibition and possible error in measurement. Diffusion of the drug in the disk begins immediately; therefore, once a disk contacts the agar surface, the disk should not be moved. After the disks are placed on the plate, invert the plate and incubate at 37°C for 16 to 18 hours. After incubation, measure the diameter of the zones of complete inhibition and record it in millimeters.

Table 1: Zone Diameter Interpretative Standards for different antibiotics against *S. aureus* according to the National Committee of Clinical Laboratory Standards **(NCCLS, 1999)**

Antibiotic	Disc content	Zone diameter (mm)		
		Susceptible	Intermediate	Resistant
Penicillin G	10 units	≥ 29	--	≤ 28
Tetracycline	30 µg	≥ 19	15-18	≤ 14
Gentamycin	10 µg	≥15	13-14	≤12
Oxacillin	5 µg	≥13	11-12	≤12
Erythromycin	15 µg	≥ 23	14-22	≤13
Chloramphenicol	30 µg	≥ 18	13-17	≤12

3.2.5 Test of the cross-resistance between teat dips and antibiotic resistance using broth microdilution method

The present study investigated whether Ujosan® dip and Eimü Chlorhexidin® dip adapted *S. aureus* strains were also resistant to antibiotics. 10 parent (original) strains of *S. aureus* isolated from quarter milk of cows with subclinical mastitis were adapted to grow in Ujosan® dip and Eimü Chlorhexidin® dip by serial passage through sub-inhibitory concentrations of the biocide. 9 strains became nonsusceptible to Ujosan® dip and only one strain became nonsusceptible to Eimü Chlorhexidin® dip after 10 sub-lethal exposures and 10 stable passages without active substance. The effect of biocides on antibiotic susceptibility in bacteria has been measured indirectly, whereby a bacterial population is treated first with a biocide and the surviving bacteria then investigated for their susceptibility to antibiotics. Cross-resistance towards a panel of antibiotics (Table 2) was determined by using the broth microdilution method in the Federal Institute for Risk Assessment in accordance with instructions M7-A8 of the Clinical Laboratory Standards Institute **(CLSI, 2009)** with Sensititre® plates (TREK Diagnostic Systems, East Grinstead, UK). All strains were tested against the following antimicrobials (concentration ranges tested expressed in mg/l): clindamycin (0.12-4); erythromycin (0.25-8); tetracycline (0.5-16); ciprofloxacin (0.25-8); rifampicin (0.016-,5); cefoxtin (0.5-16); streptomycin (4-32); thiamulin (1-4); linezolid (1-8); fusidic acid (0. 5-4); synercid (0.5-4): mupirocin (0.5-4); benzyl penicillin (0.12-2); vancomycin (1-16); sulphamethoxozole (64-512); chloramphenicol (4-64); gentamycin (1-16); kanamycin (4-64) and trimethoprim (2-32). Quality control testing was carried out using *S. aureus* strain ATCC 25923 and DSM 799. For all antimicrobials, MICs were interpreted using criteria published by the **CLSI (2009)**.

Briefly, Mueller-Hinton agar plates were streaked with bacterial cryobank to obtain isolated colonies. After incubation at 37°C overnight, select 4 or 5 well-isolated colonies with an inoculating needle or loop, and transfer the growth to a tube of sterile saline of NaCl and vortex thoroughly. The turbidity of the actively growing broth culture is adjusted by using Nephelometer (TREK Diagnostic Systems, East Grinstead, UK) with sterile saline to obtain turbidity optically comparable to that of the 0.5 McFarland standards (ca 1×10^5 KbE/ml) and then inoculate 11 ml cation adjusted Mueller-Hinton broth tube with 15-50 µl of the adjusted broth culture. Screw the dosing head on the tube and inoculate 50 µl in each well of the microtitre plate (European Susceptibility Testing (EUST)). The microtitre plates were sealed with a foil and then incubated 18-24 h at 37°C. Reading the plates using the sensititre automatic reader (TREK Diagnostic Systems, East Grinstead, UK).

Table 2: MIC values in mg/l for *S. aureus* according to European Susceptibility Testing (EUST) of different types of antibiotics used from National Reference Laboratory for Antibiotic Resistance (NRL-AR), Federal Institute for Risk Assessment (BFR)

Letter	1	2	3	4	5	6	7	8	9	10	11	12
A	CLI	CLI	CLI	CLI	CLI	CLI	ERY	ERY	ERY	ERY	ERY	ERY
	0.12	0.25	0.5	1	2	4	0.25	0.5	1	2	4	8
B	TET	TET	TET	TET	TET	TET	CIP	CIP	CIP	CIP	CIP	CIP
	0.5	1	2	4	8	16	0.25	0.5	1	2	4	8
C	RIF	RIF	RIF	RIF	RIF	RIF	FOX	FOX	FOX	FOX	FOX	FOX
	0.016	0.03	0.06	0.12	0.25	0.5	0.5	1	2	4	8	16
D	STR	STR	STR	STR	TIA	TIA	TIA	TIA	LZD	LZD	LZD	LZD
	4	8	16	32	0.5	1	2	4	1	2	4	8
E	FUS	FUS	FUS	FUS	SYN	SYN	SYN	SYN	MUP	MUP	MUP	MUP
	0.5	1	2	4	0.5	1	2	4	0.5	1	2	4
F	PEN	PEN	PEN	PEN	PEN	VAN	VAN	VAN	VAN	VAN	SMX	SMX
	0.12	0.25	0.5	1	2	1	2	4	8	16	64	128
G	CHL	CHL	CHL	CHL	CHL	GEN	GEN	GEN	GEN	GEN	SMX	SMX
	4	8	16	32	64	1	2	4	8	16	256	512
H	KAN	KAN	KAN	KAN	KAN	TMP	TMP	TMP	TMP	TMP	NEG Con	POS Con
	4	8	16	32	64	4	8	16	32	64		

CHL = Chloramphenicol, **CIP** = Ciprofloxacin, **CLI** = Clindamycin, **ERY** = Erythromycin, **FOX** = Cifoxtin, **FUS** = Fusidic acid, **GEN** = Gentamycin, **KAN** = Kanamycin, **LZD** = Linezolid, **MUP** = Mupirocin, **PEN** = Benzyl penicillin, **RIF** = Rifampicin, **SMX**, Sulphamethoxozole, **STR** = Streptomycin, **SYN** = Synercid, **TET** = Tetracycline, **TIA** = Thiamulin, **TMP** = Trimethoprim, **VAN** = Vancomycin, NEG **Con** – negative control; **POS Con** – positive control

CHAPTER 4: RESULTS

Seventy strains of *S. aureus* and CNS had been isolated from 6 dairy herds with subclinical mastitis after dipping the udder of cows with two types of chemical disinfectants during a survey carried out for a half year in the federal state of Brandenburg, Germany. Isolation of all strains was performed according to the National Mastitis Council recommendations on examination of quarter-milk samples. The main aim of the current trial was to determine in vitro the efficacy and attempt of resistance induction of the identified strains of *S. aureus* against two types of teat disinfectants (Ujosan® dip and Eimü Chlorhexidin® dip). Another objective was to check the antibiotic resistance patterns of bovine mastitis isolates of *S. aureus* and CNS. A further trial was to test the probability of cross-resistance between reduced susceptibility to teat disinfectants and different types of antibiotics commonly used in treatment of *S. aureus* bovine mastitis. Preliminary identification of *S. aureus* strains was done in the department of reproduction, Faculty of Veterinary Medicine, Free University of Berlin by colony morphology, hemolysis, and Gram staining. Creamy, grayish-white, or golden-yellow pigmented colonies that were catalase-positive and coagulase- positive gram-positive cocci and that exhibited complete, incomplete, or both complete and incomplete hemolysis were identified as *S. aureus*. The isolates were stored in cryobank at -80°C for further investigation.

In addition, all isolates were identified, phenotypically by the tube coagulase test and Staph ID 32 API system. Positive results for the tube coagulase test were recorded in 56 (80%) of 70 bacterial samples and would have been identified as *S. aureus*, while the rest (14 strains) were identified as CNS. According to the API results, 14 strains of CNS were correctly identified and the predominant species were identified as 7 strains of *S. xylosus*, 5 strains of *S. equorum*, one strain of *S. haemolyticus* and one strain of *S. epidermidis.* Genotypical identification was done by using polymerase chain reaction (PCR). The PCR amplifications were performed with a pair of primers specific for the nuc gene which encodes of the *S. aureus*-specific region of the thermonuclease gene; mecA, a determinant of methicillin resistance and a genus-specific 16S rRNA sequence were used as an internal amplification control for staphylococcal DNA. The sensitivity and specificity of the universal 16S rRNA primer set, the nuc gene primer set and the mecA gene primer set were 100%, 80% and 0%, respectively. A more rapid and accurate method for identification of both *S. aureus* and CNS was done by using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS), which was used as a confirmatory method for PCR (Table 3).

Table 3: Phenotypic, genotypic and mass spectral identification of *S. aureus* and CNS strains isolated from cows with subclinical mastitis

S. species	No. of isolates identified	Phenotypic identification		Genotypic identification			Mass spectral identification
		Coagulase test	Staph. ID 32 API	nuc gene	mec A	16S rRNA	
S. aureus	56	+		+	-	+	+
S. xylosus	7	-	+	-	-	+	+
S. equorum	5	-	+	-	-	+	+
S. epidermidis	1	-	+	-	-	+	+
S. haemolyticus	1	-	+	-	-	+	+

4.1 *In vitro* susceptibility of *S. aureus* to commercial teat dips

Quarter milk samples were collected from three groups of dairy cows using standard procedures described by the National Mastitis Council. Before samples collection, teats of the first group of cows were dipped in Ujosan® dip (Nonoxinol (9) Iod. Complex) and the second group were dipped in Eimü Chlorhexidin® dip, while the third group was kept without dipping (a negative control group). After isolation and identification of all isolates, 56 strains of *S. aureus* were used in this study. Seventeen isolates of *S. aureus* stemmed from the first group of cows which were regularly dipped with the preparation Ujosan® dip; another twenty nine isolates stemmed from the second group of cows that were regularly dipped with the preparation Eimü Chlorhexidin® dip, and another ten isolates isolated from the third group of cows without teat dipping (control group).

The minimum inhibitory concentration (MIC) for all strains was determined using the broth macrodilution method which is indicative of the guide line for examination of chemical disinfectants in the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft, DVG). This method was repeated two times in the same manner for Ujosan® dip, Eimü Chlorhexidin® dip and control group. Serial dilutions were obtained from both teat dips (100%, 50%, 25%, 12.5%, 6.25%, 3.12%, 1.56% and 0.78%). The primary results showed that the MIC values of Ujosan® dip and Eimü Chlorhexidin® dip which inhibit the multiplication of all *S. aureus* strains in control and dipped groups were 50% and 100%, respectively. Therefore serial dilutions were done for Ujosan® dip from 50% to 10% and for Eimü Chlorhexidin® dip from 100% to 50%. After determination the MIC values for both disinfectants, the growth of some strains in both control and dipped groups was inhibited by

40% of Ujosan® dip and the other strains were inhibited by 50%. While the MIC values of Eimü Chlorhexidin® dip were 100% in some strains and 90% in other strains. To detect exactly the MIC value of both teat dips, serial dilutions were made from 50% to 40% for Ujosan® dip and from 100% to 90% for Eimü Chlorhexidin® dip. As can be seen in the control group (Figure 5), the MIC of Ujosan® dip which inhibit the growth of 3 strains of *S. aureus* (B, D and E) was 41%; while strains G, H and I were inhibited by 42% of Ujosan® dip. The MIC was relatively increased in strains A (43%), C and J (44%). However marked increase in MIC was noticed in strain F (46%). From these results, it was found that the MIC values of Ujosan® dip in the control group were fluctuated from 41% to 46%.

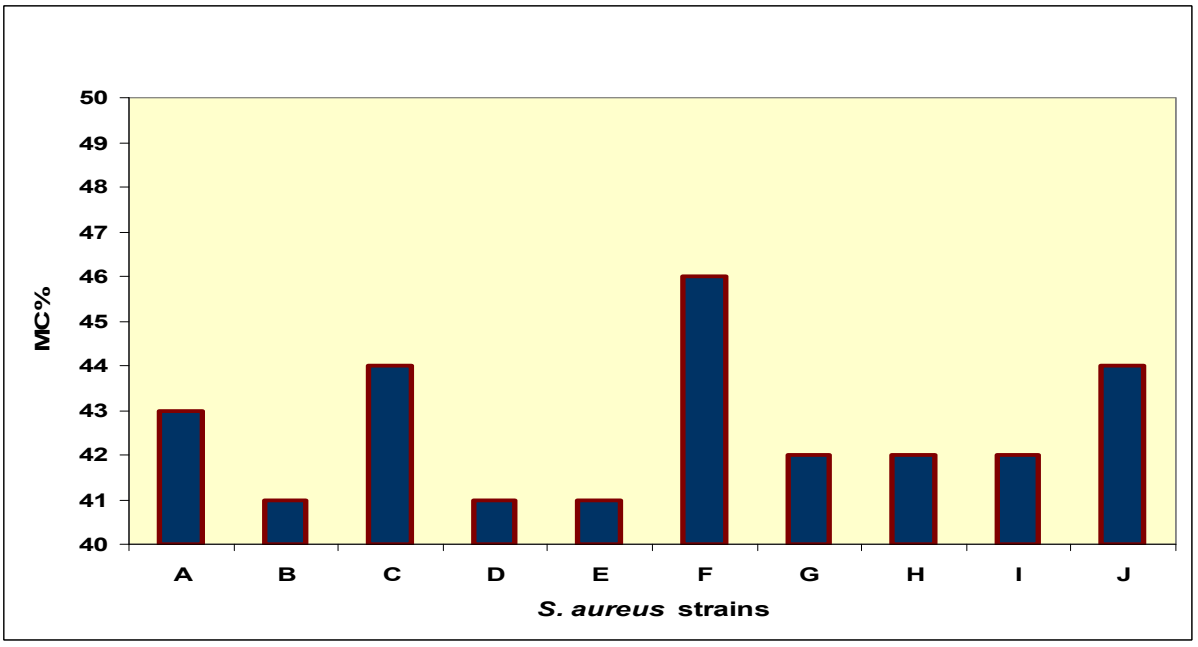

Figure 5: The MIC values of Ujosan® dip against 10 strains of *S. aureus* (control group)

In relation to the dipped group (Figure 6), the MIC values were determined in 17 strains of *S. aureus* stemmed from udder of cows dipped in the field with Ujosan® dip. It was noticed that three of them (123, 139 and 143) were inhibited by 49% of Ujosan® dip. In the other strains, the MIC values were decreased gradually to become 41% in strain number 133. From the previous results, the MIC values of Ujosan® dip were changed according to the type of *S. aureus* strain. After statistical analysis by using statistical analysis system (SAS), the mean MIC value of Ujosan® dip for dipped and control group were 45.70 ± 2.54% and 42.6 ± 1.64% (Figure 7), respectively. From these results, there was no significant difference ($P<0.05$) between dipped and control group.

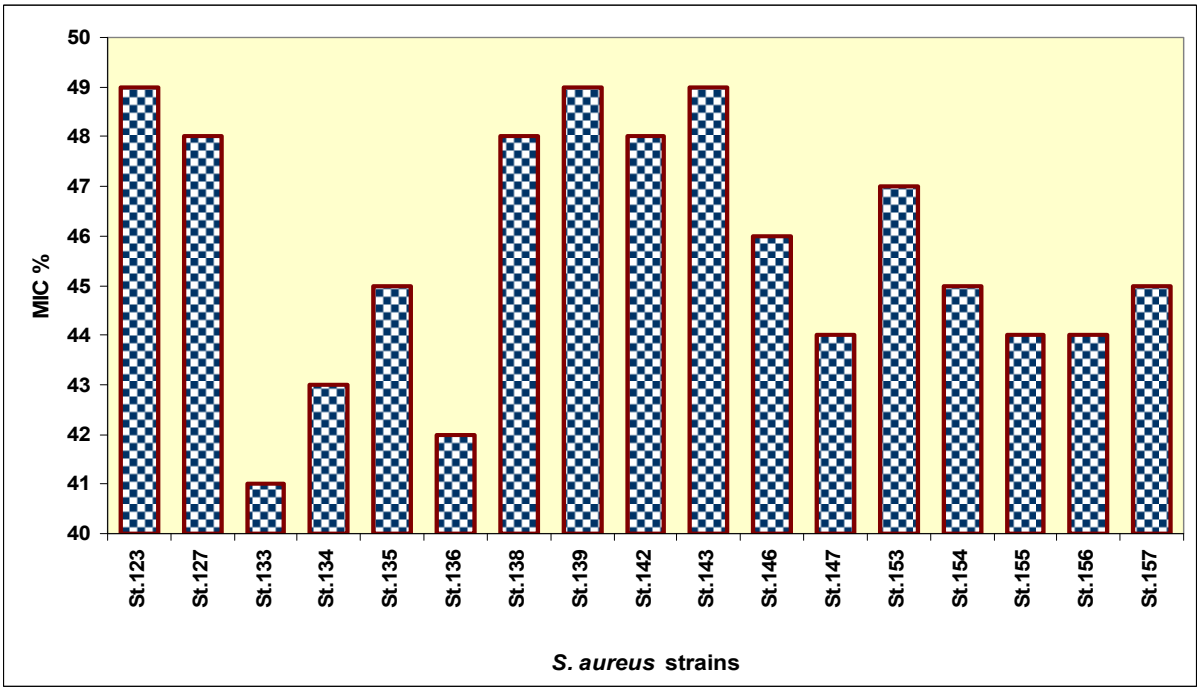

Figure 6: The MIC values of Ujosan® dip against 17 strains of *S. aureus* (dipped group)

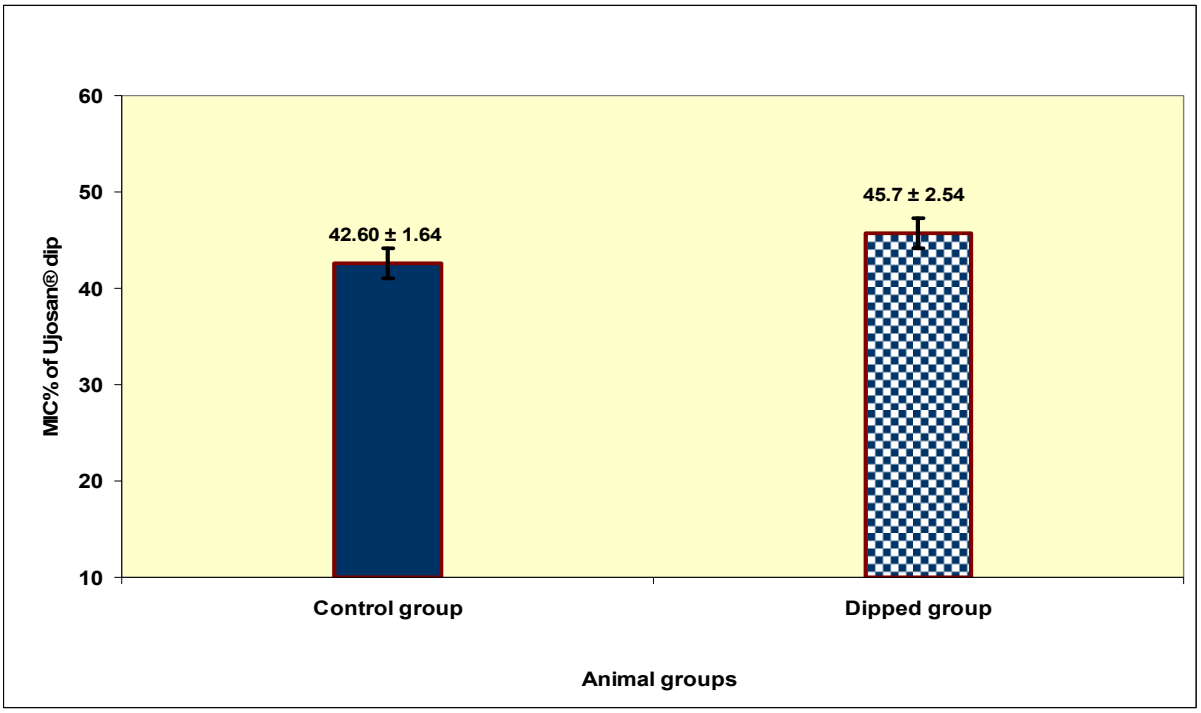

Figure 7: The mean MIC values of Ujosan® dip against *S. aureus* in control and dipped groups

Moreover, the MIC values of Eimü Chlorhexidin® dip in the control group which inhibited the growth of 10 strains of *S. aureus* were fluctuated from 96% to 98%. As can be seen in figure 8, four strains (B, D, F and H) were inhibited by 96% of Eimü Chlorhexidin® dip. Another four strains (A, G, I and J) were inhibited by 97% and only two strains (C and E) were killed by 98% Eimü Chlorhexidin® dip.

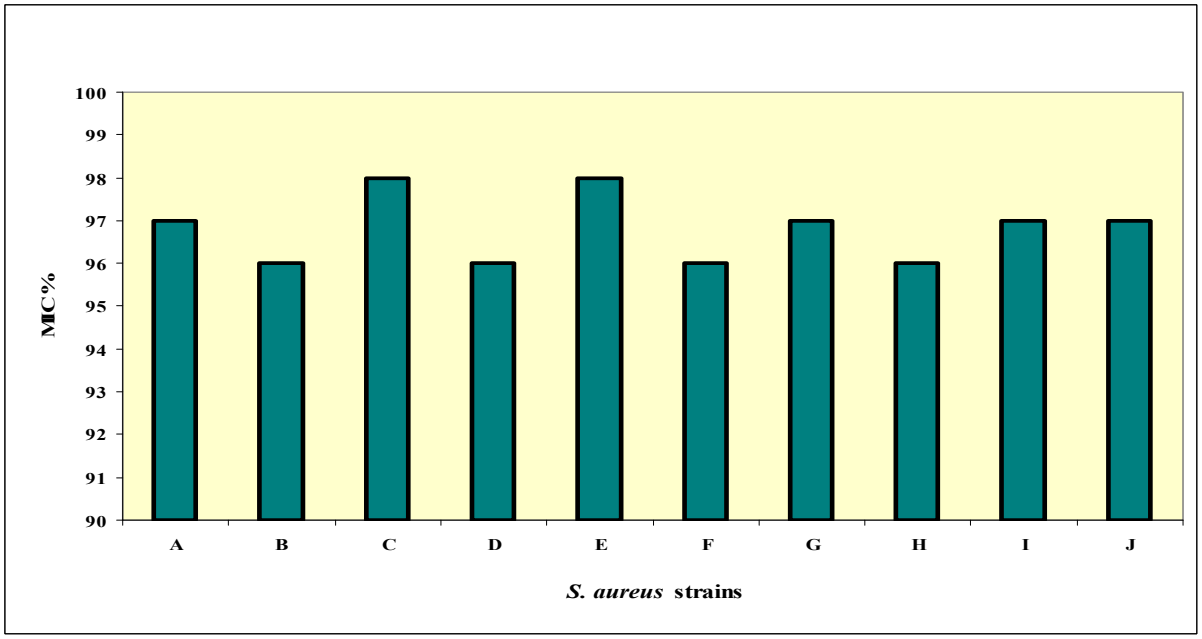

Figure 8: MIC values of Eimü Chlorhexidin® dip against 10 strains of *S. aureus* (control group)

Additionally, the susceptibility of 29 strains of *S. aureus* stemmed from the udder of cows previously dipped in the field was tested in vitro against Eimü Chlorhexidin® dip. After MIC values determination, it was noticed that these values changed from 95% to 99%. In figure 9, more than 40% of *S. aureus* strains were inhibited by 97% of Eimü Chlorhexidin® dip, while the growth of the other strains were inhibited by 95%, 96%, 98% and 99%. After statistical analysis, the mean MIC values of Eimü Chlorhexidin® dip in the dipped and control group were 97.51 ± 0.98% and 96.8 ± 0.78% (Figure 10), respectively. As can be seen, the antimicrobial action of Ujosan® dip against *S. aureus* strains (contagious mastitis pathogens) is rapid, even at low concentrations when compared to the Eimü Chlorhexidin® dip which needs high concentrations to exert its action. However, there was no significant difference ($p<0.05$) between the group dipped in Ujosan® dip, Eimü Chlorhexidin® dip and the control group.

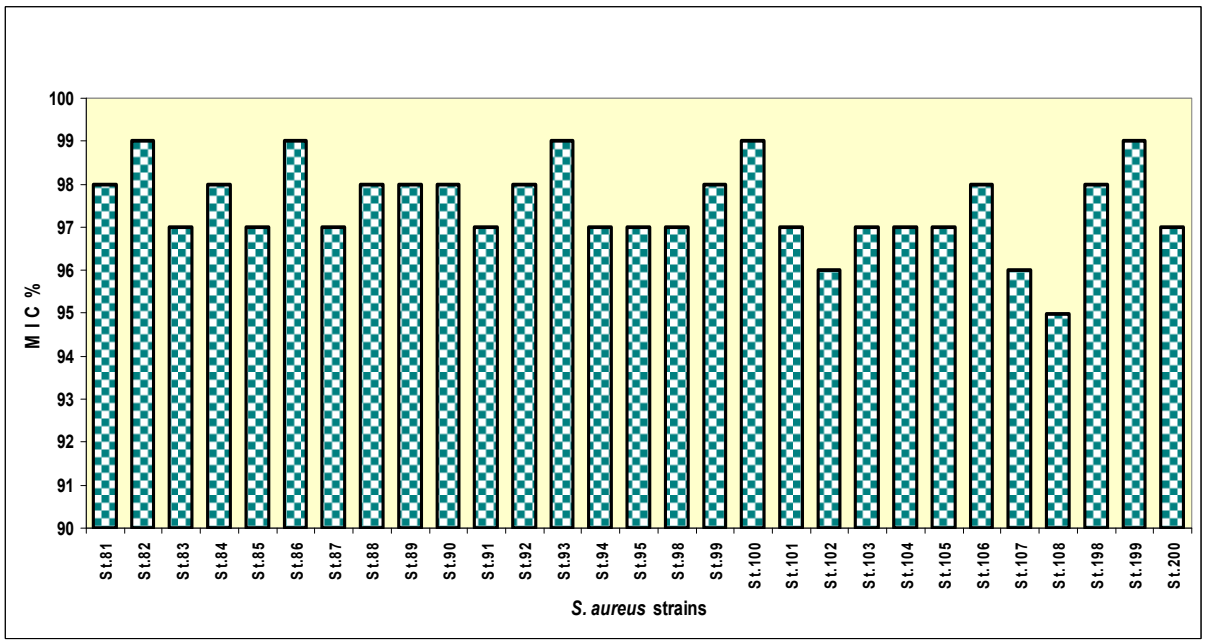

Figure 9: MIC values of Eimü Chlorhexidin® dip against 29 strains of both *S. aureus* and CNS (dipped group)

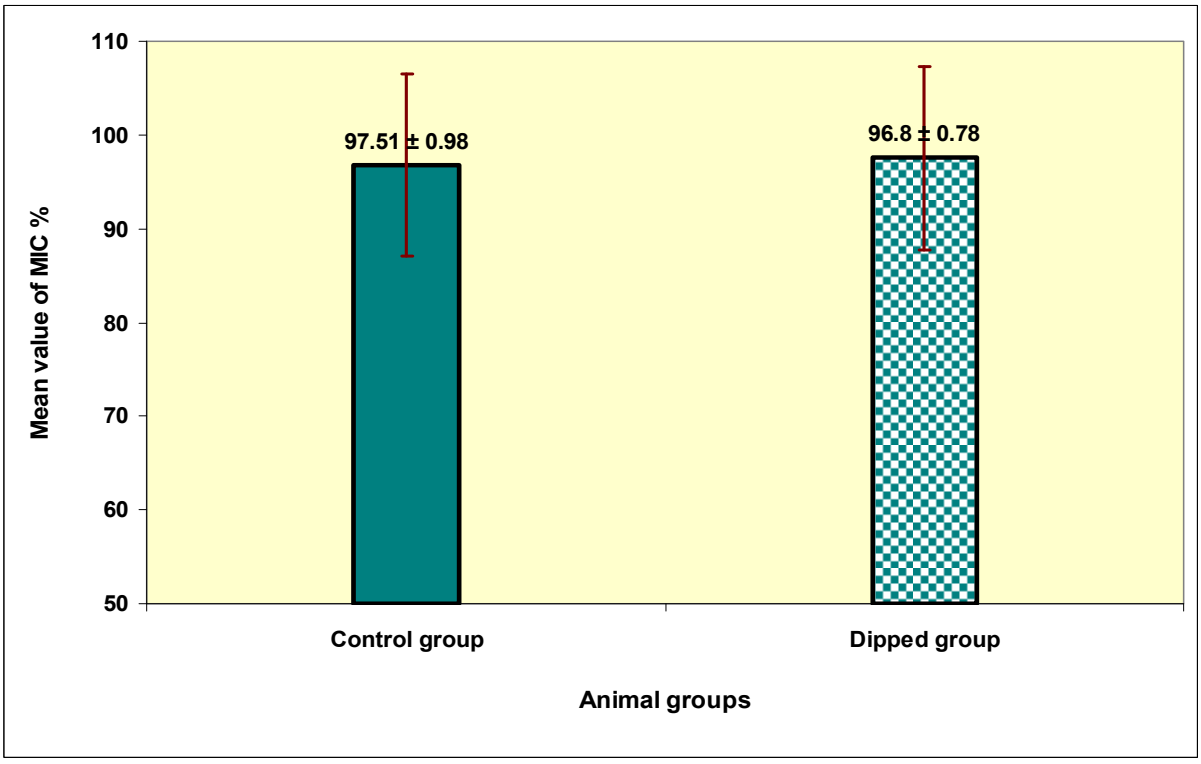

Figure 10: The mean MIC values of Eimü Chlorhexidin® dip against *S. aureus* in control and dipped groups

4.2 Induction of *S. aureus* resistance to chemical disinfectants with sub-lethal concentrations

MIC determinations have been used in many studies as an indicator of bacterial sensitivity change to a biocide. Bacteria showing an increased low-level of resistance/tolerance to a biocide might be selected by a low concentration of a biocide. After testing the efficacy of Ujosan® dip and Eimü Chlorhexidin® dip against 56 strains of *S. aureus*, 10 different isolates of *S. aureus* were selected for each disinfectant. Their level of resistance can increase through selection, for example by repeated exposure to a low concentration of a biocide, due to an increase of the concentrations of a biocide. The 10 isolates were passed ten times in sub-lethal concentrations of each disinfectant within a 72h interval for each passage. Subsequently the MIC value for all isolates after the 10th passage was again determined and then compared with the original MIC value before passages.

After statistical analysis using SAS, 9 strains of *S. aureus* showed a strong susceptibility reduction to Ujosan® dip and only one strain (G) showed a weak susceptibility reduction (Table 6 (Appendix) & Figure 11). In contrast, susceptibility was not changed in all strains exposed to sub-lethal concentrations of Eimü Chlorhexidin® dip except for strain F which showed a significant susceptibility reduction (Table 6 (Appendix) & Figure 13). All isolates with increased MICs were passed 10 stable passages without active substance every day for 10 days in growth media (tryptose soya broth) in the absence of selective pressure, to check whether the acquired resistance was stable or not. The MIC after the 10th stable passage was again detected and compared with the MIC value before passages. The stability of the acquired resistance was noticed in all Ujosan® dip and Eimü Chlorhexidin® dip adapted *S. aureus* strains.

From the previous results, the percentages of *S. aureus* strains which showed stable resistance against Ujosan® dip and Eimü Chlorhexidin® dip 90% and 10%, respectively. Therefore the attempt of resistance induction to disinfect agents in the current study clarified that using the sub-lethal concentrations of Ujosan® dip led to increases of the mean MIC value from 42.60% to 48.70% and the properties acquired were stable in most cases (Figure 12). In contrast, using of sub-lethal concentrations of Eimü Chlorhexidin® dip led to an insignificant increase of MIC from 96.8% to 97% and the properties acquired were stable only in one strain (Figure 14). It proved e impossible to increase resistance to chlorhexidine after serial passage in vitro of most strains of *S. aureus*. In general, results of the current study support the hypothesis that prolonged exposure to commercial teat dips alters the germicidal susceptibility of *S. aureus.* The exception to this conclusion was that *S. aureus* did not exhibit enhanced tolerance to Eimü Chlorhexidin® dip like it did to Ujosan® dip.

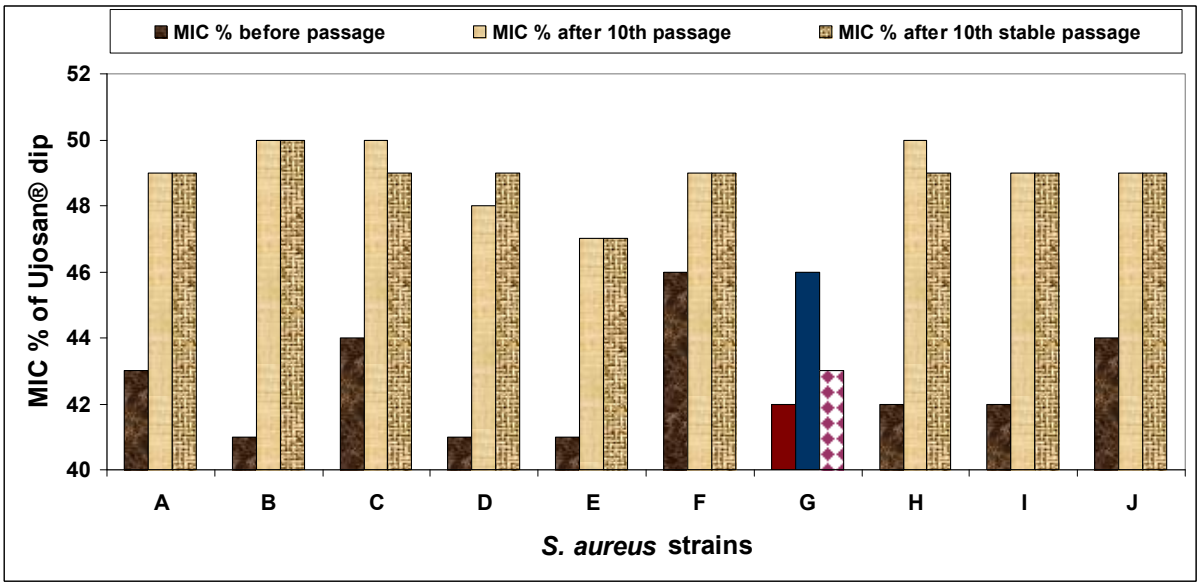

Figure 11: MIC values of Ujosan® dip with sub-lethal concentrations before, after 10th passage and after 10th stable passage of 10 *S. aureus* strains

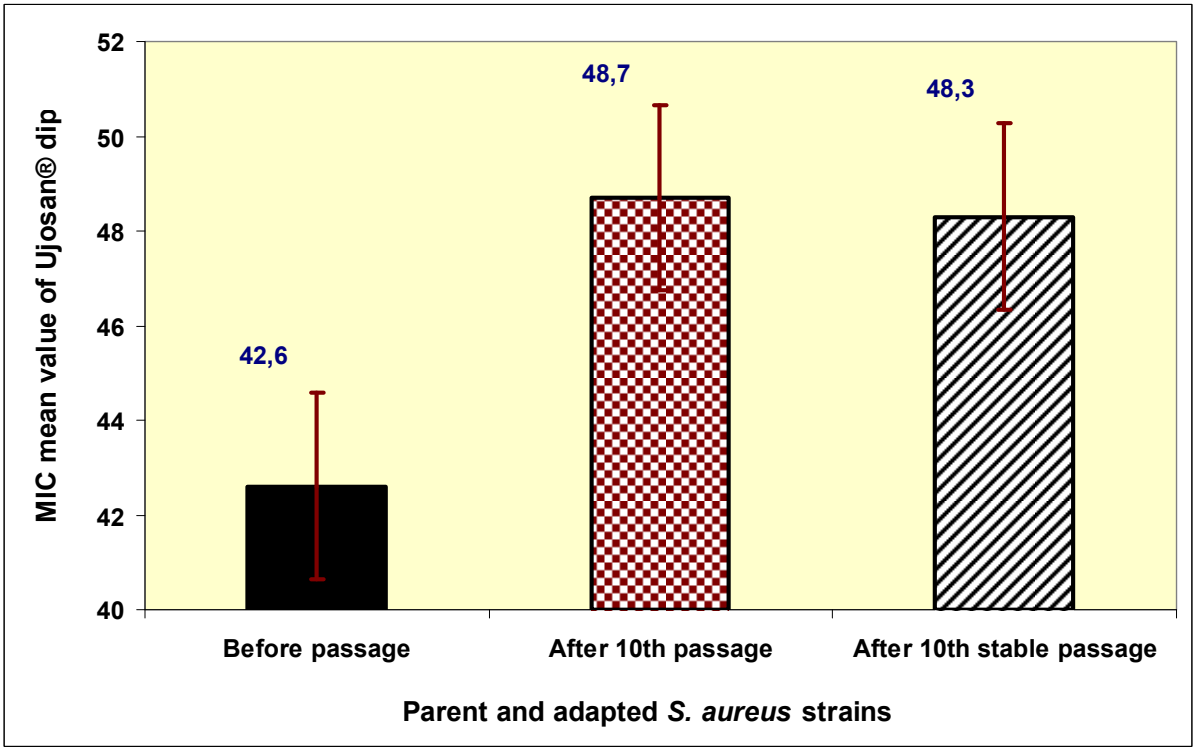

Figure 12: Mean MIC values of Ujosan® dip with sub-lethal concentrations before, after 10th passage and after 10th stable passage of 10 *S. aureus* strains

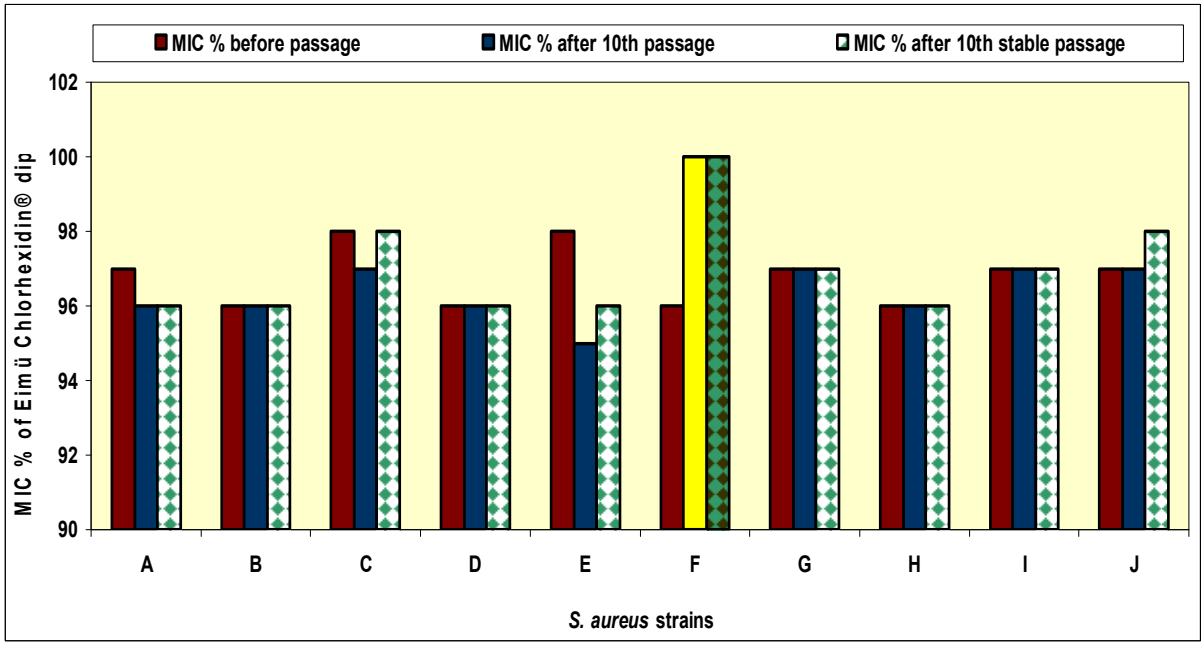

Figure 13: The MIC values of Eimü Chlorhexidin® dip with sub-lethal concentration before, after 10th passage and after 10th stable passage of 10 *S. aureus* strains

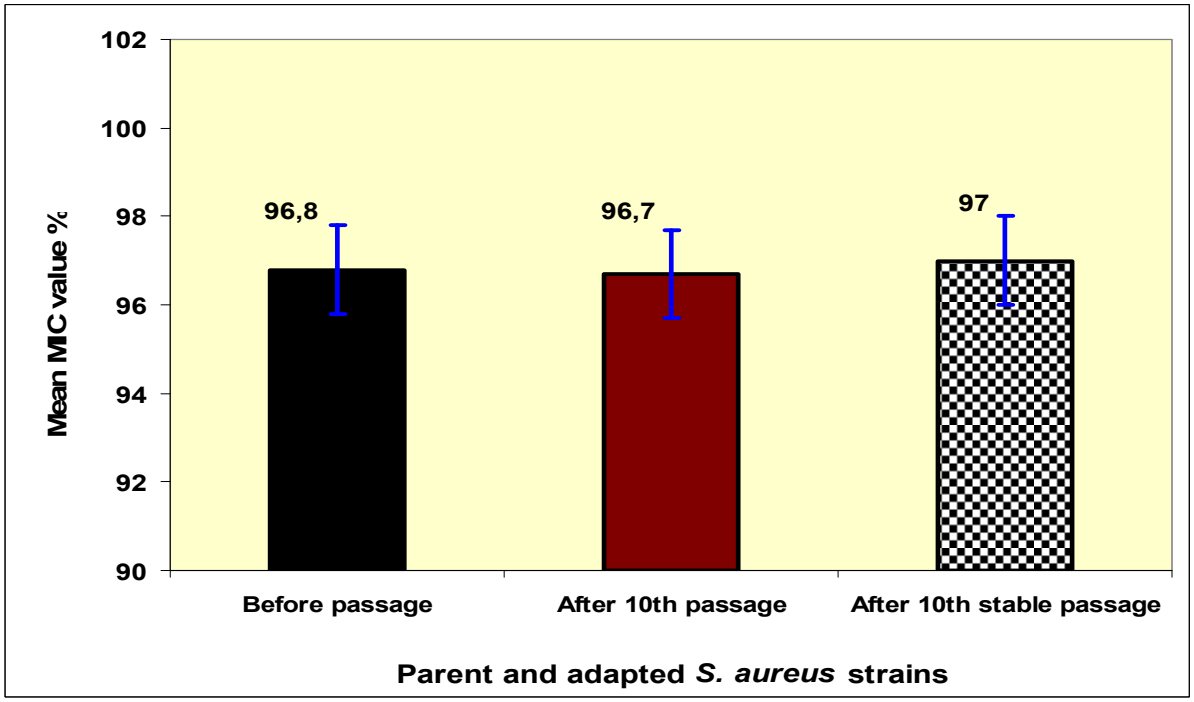

Figure 14: The mean MIC value of Eimü Chlorhexidin® dip before, after 10th passage and 10th stable passage of 10 *S. aureus* strains

4.3 Antimicrobial drug resistance *of S. aureus* and CNS

A total of fifty six *S. aureus* and fourteen CNS strains were used in this study. *S. aureus* and CNS were tested against 6 different antimicrobial agents. The MICs of antibiotics and the susceptibility of *S. aureus* and CNS strains isolated from cows with subclinical mastitis are shown in tables 7-8 (Appendix). According to the results, the susceptibility of *S. aureus* was 100% for oxacillin and erythromycin tested but was 96.44% for chloramphenicol, 89.28% for tetracycline, 85.72% for gentamycin and only 14.29% for penicillin G. The susceptibility of CNS was 100.0% for two antimicrobials (oxacillin and erythromycin), but was lower for tetracycline, gentamycin and chloramphenicol (92.86%) and penicillin G (71.43%).

As can be seen, *S. aureus* isolates showed the highest *in vitro* resistance rate to penicillin G (85.72%), while CNS isolates were lower in resistance (28.57%). In addition, 7.14% of *S. aureus* and CNS were resistant to tetracycline and only 10.71% of *S. aureus* and 7.14% of CNS were resistant to gentamycin. While the percentage of resistant *S. aureus* and CNS to chloramphinicol was 1.78% and 7.14%, respectively (Table 8 (Appendix) & Figure 15-16). On the other hand, all staphylococci were susceptible to oxacillin and erythromycin.

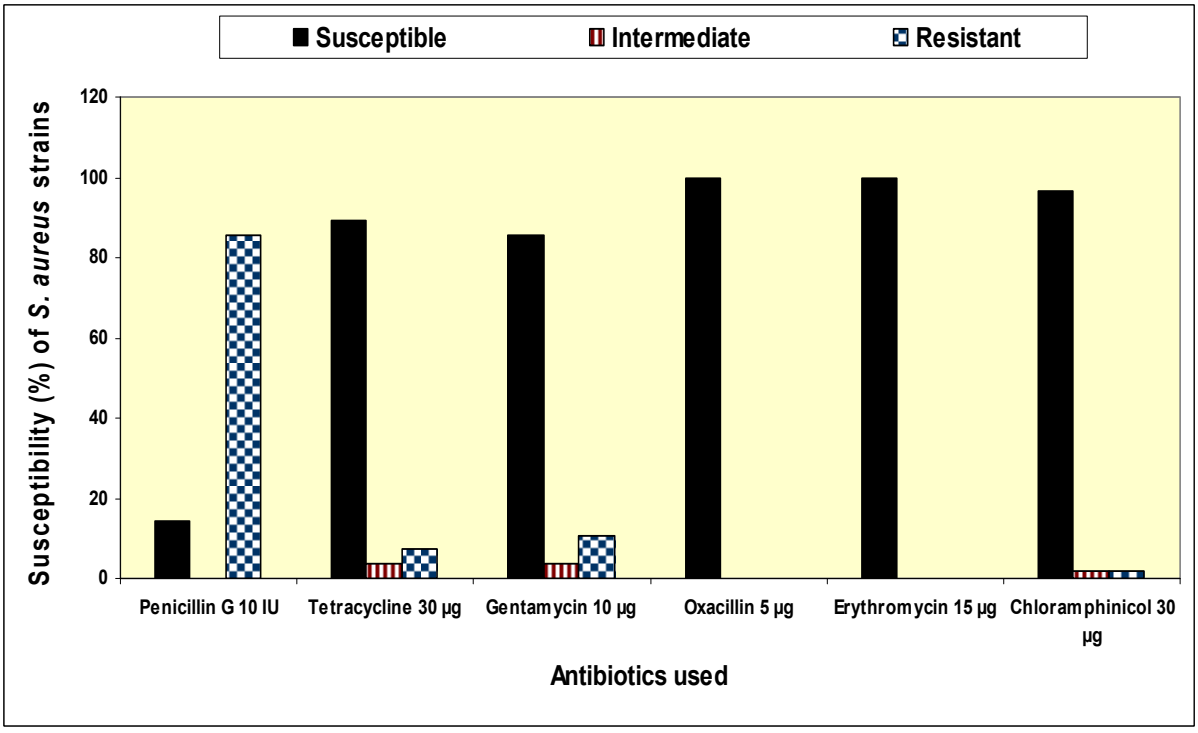

Figure 15: Percentages of susceptible, intermediate and resistant *S. aureus* to 6 different antimicrobial drugs commonly used in treatment of bovine mastitis

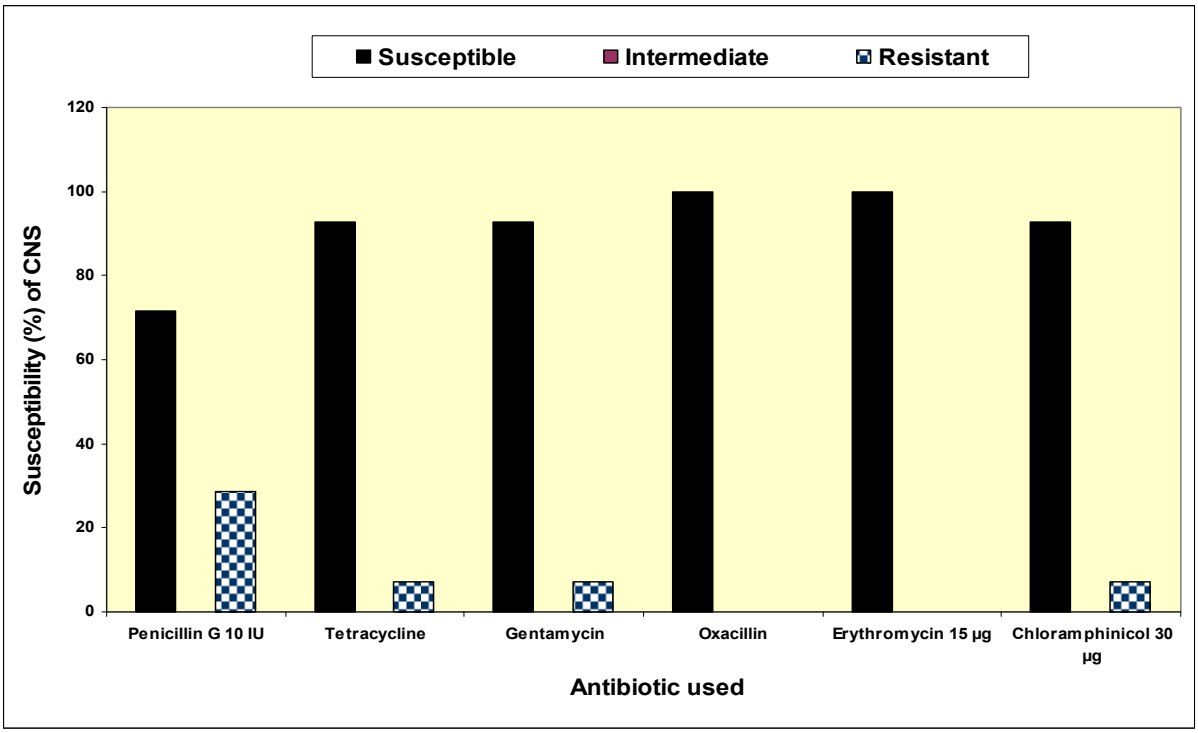

Figure 16: Percentages of susceptible, intermediate and resistant CNS to 6 different antimicrobial drugs commonly used in treatment of bovine mastitis

4.4 Possibility of cross-resistance between biocides and antibiotic resistance

A large number of studies have been carried out to evaluate whether clinical or environmental isolates that show reduced susceptibility to biocides also exhibit resistance to antibiotics. Alternatively, these same studies have looked for reduced susceptibility to biocides in antibiotic resistant isolates. Although some laboratory findings suggest that the development of biocide and antibiotic resistance can be associated, other studies indicate no such link **(IFH, 2000)**. Despite of limited knowledge about biocide mechanisms of action and their role in cross-resistance to antibiotics, research in this area is increasing. The study described below, suggests that it is relatively not easy for bacteria to become less susceptible to antibiotics after growth in amounts of a biocide that is not lethal to bacteria (sub-lethal).

Notably, resistance to low-to-high concentrations of Ujosan® dip and Eimü Chlorhexidin® dip occurred after exposure to sub-lethal doses. Moreover, biocide nonsusceptibility was often stable. The study investigated whether Ujosan® dip and Eimü Chlorhexidin® dip-adapted *S. aureus* strains were also resistant to antibiotics. 10 parent strains of *S. aureus* isolated from quarter milk of cows with subclinical mastitis were adapted to grow in Ujosan® dip and Eimü

Chlorhexidin® dip by serial passage through sub-lethal concentration of each biocide. 9 strains became nonsusceptible to Ujosan® dip and only one strain became nonsusceptible to Eimü Chlorhexidin® dip after 10 sub-lethal exposures and after 10 stable passages without active substance. Resistance or sensitivity to an antibiotic for a respective isolates was determined by measuring the MICs values (measured in mg/l) of the parent strains and comparing them with the mean MIC value of Ujosan® dip and Eimü Chlorhexidin® dip-adapted *S. aureus* strains.

The MIC is defined as the lowest concentration of an antimicrobial agent that results in inhibition of visible growth. Thus, the lower the MIC values, the higher the antimicrobial activity. This assay can be performed in a 96-microwell plate (Figure 17), where each row is inoculated with the tested micro-organism and each column contains different concentrations of the antimicrobial agents. When cross-resistance was examined, Ujosan® dip and Eimü Chlorhexidin® dip-adapted *S. aureus* strains were susceptible to most of the antibiotics tested. Therefore, exposure to chemical disinfectant did not increase antibiotic resistance in nearly all cases.

The antibiotic susceptibility rates of *S. aureus* isolated from bovine mastitis are detailed in Table 9 (Appendix) according to the National Reference Laboratory for Antibiotic Resistance (NRL-AR), Federal Institute for Risk Assessment (BFR). As can be seen, all isolates showed in vitro susceptibility to all types of antibiotics that are commercially available for the treatment of bovine *S. aureus* mastitis (Table 4). Resistance to antibiotics was demonstrated only in a minority of cases; Ujosan® dip-resistant *S. aureus* F and H demonstrated decreased susceptibility only to Benzyl penicillin from a panel of different antimicrobial agents in which MICs ranged between 0.25 to 2 mg/l. No activity or resistance to most of the antimicrobial agents was observed. However, only one parent strain was resistant to ciprofloxacin, cifoxtin, fusidic acid, mupirocin, rifampicin, sulphamethoxozole, synercid, thiamulin and trimethoprim. Additionally, the only tested Eimü Chlorhexidin® dip-adapted *S. aureus* strain F (Table 9 (Appendix) demonstrated increased susceptibility to all antibiotics used in the treatment of bovine *S. aureus* mastitis.

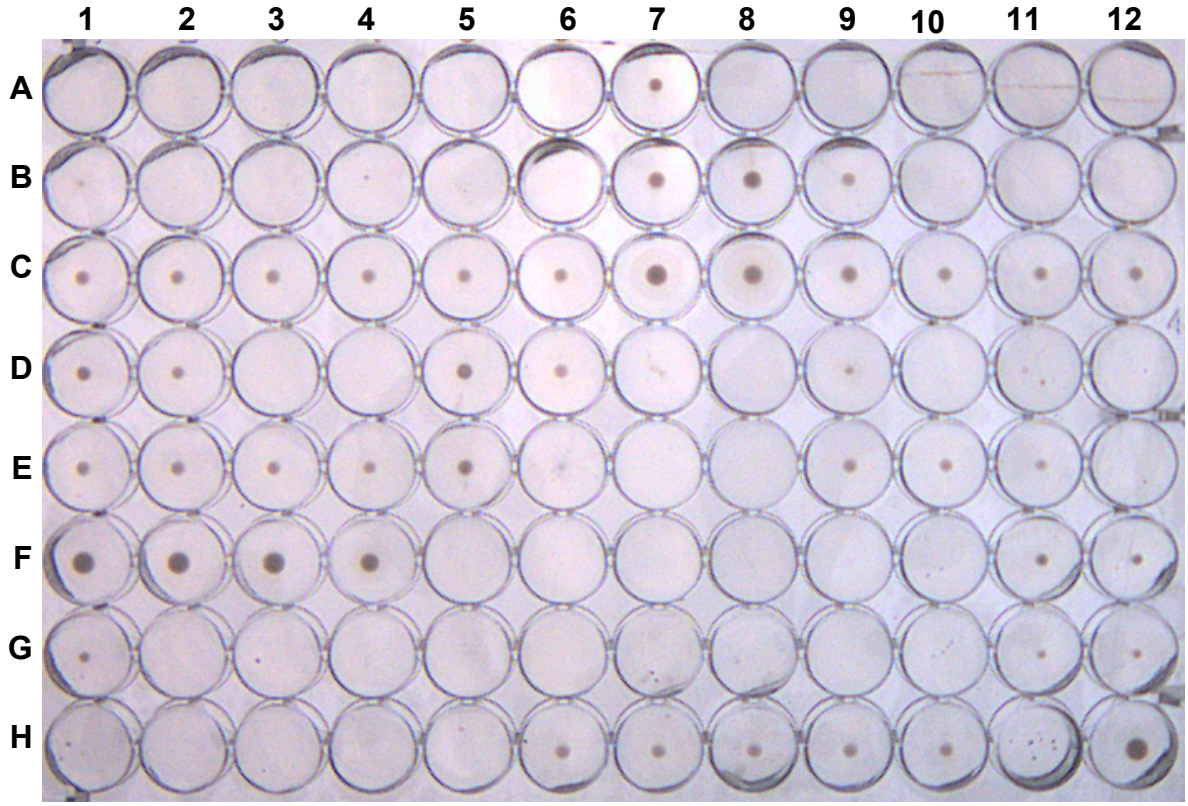

Figure 17: Growth (turbidity) occurs in these wells with antibiotic concentrations below the MIC. Column number 11 was the negative control as seen in table 2 (i.e. inoculated medium in absence of antibiotic).

Table 4: Mean MICs (mg/l) of original and Ujosan® dip-adapted strains of *S. aureus* to 19 antimicrobial agents that are commercially available for the treatment of bovine *S. aureus* mastitis

Antibiotic	Mean MIC (mg/l)			epidemiological cut off value ECV≤	clinical breakpoint CB EUCAST >	Resistance %		
	Before passage 9 original strains	After passage 9 adapted strains	After stable passage 9 adapted strains			before passage	after 10th passage	after 10th stable passage
Chloramphinicol	8	8	7.55	16	-	0	0	0
Ciprofloxacin	0.611	0.41	0.41	1	1	11.1	0	0
Clindamycin	0.12	0.12	0.12	0.25	0.5	0	0	0
Erythromycin	0.5	0.5	0.44	1	2	0	0	0
Cifoxtin	5.11	3.77	4	4	4	11.1	0	0
Fusidic acid	1.33	0.5	0.5	0.5	1	11.1	0	0
Gentamycin	1	1	1	2	-	0	0	0
Kanamycin	4	4	4	8	-	0	0	0
Linezolid	2	2	1.33	4	4	0	0	0
Mupirocin	28.5	0.5	0.5	0.5	-	11.1	0	0
Benzylpenicillin	0.73	0.26	0.17	0.12	-	44.4	22.2	22.2
Rifampicin	0.06	0.1	0.016	0.016	-	11.1	11.1	0
Sulphamethoxozole	142.2	71.1	71.11	128	-	22.2	0	0
Streptomycin	8.88	8.44	8	16	-	0	0	0
Synercid	1.11	0.5	0.5	1	2	11.1	0	0
Tetracycline	1	0.5	0.55	1	2	0	0	0
Thiamulin	1.22	0.94	0.83	2	-	11.1	0	0
Trimethoprim	5.55	2.59	2	4	4	11.1	0	0
Vancomycin	1.11	1.33	1.55	2	2	0	0	0

CHAPTER 5: DISCUSSION

Mastitis is the most important disease in the dairy industry worldwide. It accounts for significant losses due to reduced yield, treatment costs and loss of income if milk is discarded as a result of reduced quality, bacterial contamination and antibiotic residues. The industry is facing many challenges in attempting to manage this disease. Increasing demands for milk require that farming operations are more intensive which in turn places increasing pressure on dairy farms to produce a high quality product that meets legislative requirements **(Petrovski et al., 2006)**. From this point of view, control of *Staphylococcus* (*S*) *aureus* mastitis can be achieved through the correct diagnosis, teat dipping, segregation of infected animals, dry cow therapy, treatment during lactation and culling programs **(Wilson et al., 1995)**. Cumbersome preventive and control measures have to be taken on farms with *S. aureus* mastitis problems, and the treatment of *S. aureus* mastitis is associated with poor success **(Sutra and Poutrel, 1990)**, leading to a relatively high culling rate.

Reliable and rapid methods for the identification of *S. aureus* isolated from quarter milk of mastitic animals are very important tools for the control of this disease and for economically sound udder health management. Historically, bacterial identification was achieved by using phenotypic based techniques. However, those techniques still are time consuming and sometimes of limited value **(Carretto et al., 2005)**. Moreover, polymerase chain reaction (PCR) amplifications used for the identification of different types of bacteria remain also time consuming, expensive and technically demanding. With the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) technique, sample preparation and analysis are simple and can be performed within minutes. No special lysis step is necessary beyond the exposure to the matrix solution, and the instrument does not require a specialist operator. Only a loopful of cells is needed for MALDI-TOF-MS analysis, and the profile is generated with minimal consumables and cost **(Carbonnelle et al., 2007)**. For one sample, MALDI-TOF-MS analysis is achieved in a few minutes (versus 1 day for the Staph ID 32 API system and at least several hours for the molecular biology techniques). Multiple samples can be tested per day, and furthermore the cost of the analysis is inexpensive compared to other techniques (in the range of a few cents).

Furthermore, postmilking teat disinfection is one of the fundamentals of the mastitis control five-point plan and is crucial in the control of staphylococcal mastitis **(Jones and Ohnstad, 2002)**. Today, the strategy of mastitis control includes a combination of post-milking dipping and dry cow therapy associated with good veterinary practice of application of antimicrobials to prevent or treat new infections in the farm. However, this control strategy is not always fully successful. Some studies state that the failure of control programs of bovine mastitis can

be partly attributed to the teat disinfectants and/or antibiotics which do not afford sufficient protection against the multiple pathogens that cause mastitis, particularly *S. aureus*, *Strept. agalactiae* and CNS. Depended on the previous facts that post milking teat disinfectants and antibiotic therapy are very important tools in the control of bovine *S. aureus* mastitis, the current study concentrated on studying the susceptibility and induction of *S. aureus* resistance to different types of post milking teat disinfectants as well as on the possibility of cross-resistance between these teat dips and different types of antibiotics commonly used in the treatment of bovine *S. aureus* mastitis

5.1 *In vitro* susceptibility of *S. aureus* strains to commercial teat dips

The current trial used two types of teat disinfectants, Ujosan® dip and Eimü Chlorhexidin® dip in six commercial dairy herds with histories of subclinical mastitis during a survey carried out for a half year. After determining the susceptibility of *S. aureus* strains to commercial teat dips, it was observed that no significant difference occurred between treated quarters with Ujosan® dip and Eimü® Chlorhexidin-dip and the control group. A similar finding was obtained by **Edinger et al. (2000)** who investigated 149 Holstein–Frisian heifers to determine the effect of teat dipping with a barrier teat sealant on intra mammary infection (IMI) and clinical mastitis. They found no significant differences between treated and control quarters with regards to IMI and the incidence of clinical mastitis.

The lack of significant differences in the current study can be discussed by the fact that many of these pathogen have already been established in the udder before using teat dipping (**Compton et al., 2007; Østerås et al., 2008; Parker et al., 2008)** as well as it is not expected that post milking teat dips would have any effect on already established infections which is reported by **Whist et al. (2007)**, who noticed a higher somatic cell count in older cows with a high prevalence of *S. aureus* dipped with iodine postmilking teat dip. The respective outcomes of the present study and especially the effect of Ujosan® dip and Eimü Chlorhexidin® dip on already infected quarters need to be investigated further.

5.3 Induction of *S. aureus* resistance to chemical disinfectants with sub-lethal concentrations

Induction of resistance for *S. aureus* was readily achieved by repeated passage in sub-lethal concentrations of Ujosan® dip and Eimü Chlorhexidin® dip. Exposure to a relatively low concentration of Ujosan® dip led to a high-level of resistance within ten passages for most strains (90%). Firstly, all strains were primarily extremely sensitive to the low concentration of Ujosan® dip. Subsequently, most did acquired a high level of resistance following ten sub-

lethal exposures. Therefore, the attempt of resistance induction to disinfecting agents by the use of sub-lethal active substance concentrations led to a significant increase (P>0.001) of the MIC value of Ujosan® dip in most strains, while such resistance was not present for Eimü Chlorhexidin® dip, except one strain with a significant increase of the MIC value after 10 stable passages.

Similar findings were reported in several studies for a number of biocides **(SCENIHR, 2009)**. These studies clarified that exposure of bacteria to sub-inhibitory concentrations of biocides led to a high-level of resistance to these microorganisms. In addition, **Moken et al. (1997)** and **Gilbert et al. (2002)** reported that a phenotypic change leading to the emergence of resistance to several biocides in vitro follows exposure to a low concentration of a biocide. Moreover, **Gomez Escalada et al. (2005)** found a decrease in growth rates in *Escherichia coli* and *Pseudomonas aeruginosa* following exposure to sub-lethal concentrations of a biocide triclosan, which indicates the generation of a stress to the organism. However, the current results were different from the results obtained by **Hogan and Smith (1989)**, who tested eight strains of *S. aureus* to determine in vitro if prolonged exposure (15 times) to sub-lethal concentrations of four commercial teat dips could enhance bacterial tolerance. They found that the growth responses of *S. aureus* to chlorhexidine, sodium hypochlorite, and iodophor were not affected by prolonged exposure to these teat dips. Reports concerning increased resistance to antiseptics and disinfectants are also numerous. Irrational use of antimicrobial drugs as well as of biocides in human and veterinary practice (needless use, incorrect choice, low dosage, short contact, irregular application) is mostly responsible for the emergence of resistant bacteria of many species, including staphylococci **(Schwarz and Chaslus-Dancla, 2001; Collignon, 2002; Yilmaz and Kaleta, 2009)**.

In vitro exposure of bacteria to sub-lethal concentrations of a chemical disinfectant by repeated sub-passages can result in the development of resistance within a bacterial population **(Kirchhoff, 1962; Wille, 1976)**. It was thought that chemical disinfectants have multiple target sites against microbial cells. Thus, the emergence of general bacterial resistance is improbably to be caused either by a specific modification of a target site or by a by-pass of a metabolic process. **SCENIHR (2009)** reported that bacterial resistance emerges from a mechanism causing the decrease of the intracellular concentration of a biocide under the threshold that is harmful to the bacterium. Furthermore, **McDonnell and Russell (1999)** clarified that resistance is either a hereditary natural property of an organism or is acquired by mutation or acquisition of plasmids or transposons.

However, mechanisms by which bacteria resist killing by different types of antibiotics and biocides are still poorly defined, prolonged *in vitro* exposure to sub-lethal concentrations of

antimicrobial agents undoubtedly contributes to their development **(Braoudaki and Hilton, 2004)**. With Ujosan® dip, it was possible through several passages to reach high levels of resistance in nine of ten tested *S. aureus* strains. In contrast, the use of sub-lethal concentration of Eimü Chlorhexidin® dip could insignificantly increase the MIC value of only one strain of the total of 10 strains. The acquired tolerance of *S. aureus* to these teat dips was measured after bacteria were subcultured 24 hour in a media void of germicide. The development of strains that retain resistance to a germicide in absence of the germicide was postulated to be due to selection or the emergence of stable mutants **(Koshiro and Oie, 1979)**, as well as acquired stable tolerance of *S. aureus* to iodine and chlorhexidine also has been shown **(Prince et al., 1978)**.

At present, it is unknown which mechanisms are contributing to the adaptive resistance observed in the strains under study; however, this resistance is likely due to the presence of active efflux. It has gained increased recognition as a resistance mechanism over the past decade. Efflux pumps decrease the intracellular concentration of toxic compounds **(Levy, 2002; Poole, 2002; Piddock, 2006)**. Efflux pumps are an important mechanism by which bacteria can evade the effect(s) of antimicrobial agents. This resistance mechanism has received considerable attention in recent years **(Huet et al., 2008)**. The role of efflux pumps in the development of bacterial resistance to biocides might be considered modest since the increase in bacterial susceptibility to selected biocides as the results of the expression of efflux pumps is usually measured as an increase in MICs rather than as resistance to a high concentration of an active ingredient. Efflux pumps have been shown to decrease the efficacy of a large number of biocide, including quaternary ammonium compounds (QACs), phenolics parabens and intercalating agents **(Davin-Regli et al., 2006; Randall et al., 2007)** observably in *S. aureus* with identified pumps such as QacA-D **(Wang et al., 2008)**, QacG **(Heir et al., 1999)** and QacH **(Heir et al., 1998)**. Historically, it has been known that some antiseptics and disinfectants, on the basis of MIC, are somewhat less inhibitory to *S. aureus* strains that contain a plasmid carrying gene encoding resistance to the aminoglycoside antibiotic gentamycin **(Mcdonnell and Russell, 1999)**.

Moreover, **Kolawore (1984)** reported that the extra-cellular slime covering mucoid-grown *S. aureus* protected the bacterial cells from disinfectants by both serving as a physical barrier and inactivating bactericidal agents. Presence of a slime layer may interfere with the expression and detection of extra-cellular and cell wall proteins. Unlike antibiotic resistance, the issues relating to biocide resistance in the healthcare environment are considered to have a very low profile and priority **(Cookson, 2005)**. Despite the widespread use of disinfectants and antiseptics in healthcare settings, acquired resistance to biocides in bacteria isolated from clinical specimens or the environment is not routinely characterised.

Emerging bacterial resistance to biocides has been well described in vitro, but evidence in practice is still lacking **(Maillard and Denyer 2009)**. Based on the previous data, resistance of bacteria to chemical disinfectants may be more probably to develop if they are used at concentrations lower than required for optimal biocidal effect. This reinforces the importance of always using disinfectants at the recommended concentrations and according to the label directions.

5.3 Antimicrobial drug resistance *of S. aureus* strains and CNS

S. aureus has been the main subject of several studies on antimicrobial resistance because of its prevalence and importance for mastitis in dairy cows. The occurrence of bovine mastitis has serious consequences for animal and public health. Antimicrobial susceptibility of *S. aureus* isolated from bovine mastitis varies widely by region **(Makovec and Ruegg, 2003; Gill et al., 2006)**. In the present study, results indicated that *S. aureus* isolates exhibited the highest degree of resistance to penicillin G (85.72%), whereas there was only a limited occurrence of resistance to other antimicrobial agents. This result was nearly in agreement with those obtained by **Werckenthin et al. (2001), Malinowski et al. (2002)** and **Shi et al. (2010)**, who isolated 206 *S. aureus* strains in the Inner Mongolia, China, which were found to be resistant to penicillin with a resistance rate of 87.30%.

Major differences in the occurrence of penicillin resistance have also been observed between countries. Thus, previous reports have, as also noticed in this study; found high frequencies of penicillin-resistance in the USA, England and Ireland and Finland. In contrast, **Aarestrup and Jensen (1998)** recorded a low penicillin resistance 10% in the Scandinavian countries (Denmark, Norway and Sweden) **(SVARM, 2002)**. In the rest of the Europe, the proportion of penicillin-resistant isolates has ranged from 23% **(DANMAP, 2003)** up to 69% **(Nunes et al., 2007)**, in the United States from 38 to 61% **(Erskine et al., 2002)** and was reported to be 40% in Argentina **(Gentilini et al., 2000)**. Large scale studies on antimicrobial resistance of bovine *S. aureus*, involving up to 5240 isolates per year, conducted as part of the national monitoring programme in Germany during 1992–1997 revealed resistance to penicillin in 38–57% of the isolates **(Trolldenier, 1996; Werckenthin et al., 2001)**.

The high rate of penicillin resistance amongst *S. aureus* is likely due to the wide use of intramammary preparations containing combinations and broad-spectrum antimicrobials **(Pitkala et al., 2004)**. Numerous factors can influence the overall susceptibility patterns of mastitis pathogens. Scar tissue in the udders of cattle chronically infected by *S. aureus* is an important factor which prevents the penetration of antimicrobial agents **(De Oliveira et al., 2000)**. Moreover, penicillin resistance is due to the expression of inducible β-lactamase

encoded by the *blaZ* gene, which causes hydrolysis of the β-lactam ring of penicillin. The first reports on the ability of *S. aureus* to break down penicillin were published in 1940, a year before the antimicrobial was introduced for therapeutic use. Impaired treatment response has been associated with penicillin resistance of the infectious *S. aureus* strain **(Taponen et al., 2003)**. **Jones et al. (1967)** noted over thirty years ago that *S. aureus* isolates had relatively high MIC values for penicillin and ampicillin, and referred this to Beta-lactamase inhibition of the antimicrobial drugs. Beta-lactamase production is induced in some bacteria when exposed to Beta-lactam drugs. The importance of prolonged Beta-lactamase-related resistance in *S. aureus* was underscored by the **Watts and Salmon (1997)** report of higher MIC values for isolates that produced this enzyme as compared to isolates that did not. No evidence exists to suggest that this adaptation of *S. aureus*, or resistance to other classes of antibacterial drugs, is different from those noted thirty-five years ago. The MIC values and disk diffusion results demonstrate ampicillin and penicillin to be consistently the antimicrobial drugs to which *S. aureus* are most commonly resistant. However, comparing values within tables from one time period to another should be avoided. Any comparison of this kind should be done with skepticism because of the differences in geography, numbers of isolates used within a study, and inconsistencies in laboratory methods. As an example, two studies performed in the same year in arjentina by **Costa et al. (2000)** and **Gentilini et al. (2000)** reported the proportion of oxacillin resistant strains of *S. aureus* as 42.0 and 0%, respectively.

Sing and Buxi (1982) stated that resistance to penicillin among *S.* species isolated from mammary glands is wide spread. **Anderson (1983)** found that there were three types of resistance to antibiotics in staphylococci. Of these, penicillinase production mediated by plasmids is considered one of the most common forms of penicillin resistance among staphylococci, although the percentages of such strains vary between countries. **Iqbal et al. (1984)** found that 92.86% of *S. aureus* isolates froms cow milk was resistant to penicillin. In 84.6% of these isolates, resistance to penicillin was associated with penicillinase production.

Also this study indicated that ofloxacin and chloramphenicol had the highest sensitivity (100%) to the *S. aureus* isolates. This apparently high level of sensitivity to ofloxacin and chloramphenicol appears to suggest that these two antimicrobial drugs could be the best drugs of choice for treating infections caused by *S. aureus* in the study area, especially at the present time, when *S. aureus* strains resistant to other commonly used antibiotics has been reported. This result was in agreement with those obtained by **Chalita et al. (2004)** and **Obi et al. (1996)**. Although resistance level provide important information towards the development of effective prevention and treatment strategies for this disease, eradication of

S. aureus mastitis nevertheless has not been possible. Use of a vaccination program to protect against *S. aureus* mastitis would be most beneficial in the future **(Shi et al., 2010)**.

5.4 Possibility of cross-resistance between biocides and antibiotics

Recently, several studies has been carried out to evaluate whether clinical or environmental isolates that show reduced susceptibility to biocides also exhibit resistance to antibiotics. Despite of some laboratory investigations suggesting that the emergence of biocide and antibiotic resistance can be closely associated, other studies indicate no such link **(Russell et al., 1998; McDonnell and Russell, 1999)**. The potential for biocide-selected cross-resistance to clinically important antimicrobial drugs is the subject of some discussion in several literatures **(Levy, 2000; Russell et al., 1999; Russell, 2000; Schweizer, 2001)**. Studies that biocide resistant (i.e. efflux) genes do not predominate in versus methicillin resistant *S. aureus* (MRSA) versus methicillin susceptible *S. aureus* (MSSA) **(Bamber and Neal 1999; Suller and Russell 1999)** and that biocides such as triclosan are effective at killing clinical MRSA isolates **(Webster et al., 1994; Zafar et al., 1995)** suggest that, clinically at least, biocide–antibiotic cross-resistance is not a problem in *S. aureus*. This is supported by observations that *S. aureus* triclosan-resistance in the laboratory does not lead to antibiotic resistance **(Suller and Russell 2000)**.

In the current study, cross-resistance of Ujosan® dip and Eimü Chlorhexidin® dip -resistant *S. aureus* to a panel of antibiotics was investigated in 29 strains of *S. aureus*. When MIC for original and adapted strains were determined by the microdilution method, Ujosan® dip and Eimü Chlorhexidin® dip-adapted *S. aureus* strains were susceptible to all tested antibiotics. The difference in MIC did not affect the classifications of all strains, which were all sensitive according to EUCAST breakpoint guidelines. The same finding was reported by **Baillie et al. (1992); Baillie et al. (1993); Rutala et al. (1997); Payne et al. (1999); Nomura et al. (2004); Thomas et al. (2005); Lear et al. (2006); Jurgens et al. (2008); Birošová and Mikulášová (2009)** and **Cottell et al. (2009)**. However our result was not in agreement with the results obtained by **Reverdy et al. (1992), Bamber and Neal (1999), Martin and Maris (1995); Irizarry et al. (1996), Mitchell et al. (1998), Karatzas et al. (2007)** and **Randall et al. (2007)**. They discovered cross-resistance between bacterial species with reduced susceptibility to biocides and antibiotics.

There is no indication from these results to suggest that Ujosan® dip and Eimü Chlorhexidin® dip-resistant strains are resistant to antibiotics according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The association between biocide nonsusceptibility and antibiotic resistance is still unclear. Most of investigators were able to

demonstrate cross-resistance between antibiotics and biocides. But, when cross-resistance was demonstrated, it was often reported for second-line drugs or drugs not usually used for therapy. Moreover, nearly all researches describe laboratory experiments whose relationship to the real world situation is not defined. These studies only examined antibiotic and biocide sensitivities in vitro. The lack of cross-resistance of the Ujosan® dip and Eimü Chlorhexidin® dip resistant mutants to the antibiotics tested in this study suggested that these biocides do not share their resistance mechanism(s) with different types of antibiotics.

Although bacterial susceptibilities to antimicrobial drugs are truly well characterized, currently the relevance of a change in the MIC of an antiseptic is unknown. Even so, **Rogers (2005)** recorded the fact that growing clinical isolates in sub-lethal concentrations of biocides can lead to a change in the profile of antibiotic susceptibility, especially if changes in biocide susceptibilities can be related to therapeutic levels of antibiotics. Multiple studies suggested that an efflux mechanism was involved in the biocide nonsusceptibility. Current knowledge of efflux mechanisms suggests that these pumps can utilize a variety of substrates, including both antibiotics and biocides, and therefore, may become a problem. Currently, there is incomplete understanding as to whether the use of biocides might select resistance to current antibiotics or prevent development of new antibiotics. Clearly, more research is needed to characterize the relationship between biocide nonsusceptibility and antibiotics resistance.

CHAPTER 6: SUMMARY

Susceptibility of *Staphylococcus aureus* strains isolated from cows with subclinical mastitis to different types of disinfectants and antibiotics

The primary objective of the current study was to determine in vitro the efficacy of two teat dips, Ujosan® dip and Eimü Chlorhexidin® dip against 56 *Staphylococcus* (*S.*) *aureus* strains isolated from subclinical cases of bovine mastitis. A further main objective was an attempt of resistance induction of selected strains of *S. aureus* against the same two types of teat disinfectants. Another objective was to test the antibiotic resistance patterns of bovine mastitis isolates of *S. aureus* and coagulase negative staphylococci (CNS). The last objective was to check the possibility of cross-resistance between reduced susceptibility to disinfectants and different types of antibiotics that are commercially available for the treatment of bovine *S. aureus* mastitis. Quarter milk samples were collected from six dairy herds with high prevalences of *S. aureus* in the federal state of Brandenburg, Germany. Of each herd, 32 cows in different stages of lactation and different age groups were chosen for sampling. Cows were divided according to the udder teat dipping scheme into three groups. Teats of the first group were dipped in the postmilking teat disinfectant Ujosan® dip; the second group was dipped in Eimü Chlorhexidin® dip, while the third group was kept without dipping (a negative control group). A total of seventy isolates of *S. aureus* and CNS were identified phenotypically by the tube coagulase test and the Staph ID 32 API system; genotypically by using the polymerase chain reaction (PCR) and the mass spectrally by matrix assisted laser desorption/ionisation- time of flight-mass spectrometry (MALDI-TOF-MS), which was used as a confirmatory method for PCR.

After identification of all strains, the minimum inhibitory concentration (MIC) of Ujosan® dip and Eimü Chlorhexidin® dip against *S. aureus* strains was determined, using the broth macrodilution method which is indicative of the guideline for examination of chemical disinfectants in the German Veterinary Association (Deutsche Veterinärmedizinische Gesellschaft, DVG). All strains were inoculated in a liquid medium (tryptose soya broth, TSB), serially diluted with the two teat dips. The mean MIC values of Ujosan® dip and Eimü Chlorhexidin® dip for dipped and control groups were 45.70 ± 2.54%; 42.6 ± 1.64% and 97.51 ± 0.98%; 96.8 ± 0.78%, respectively. This study showed that there was no significant difference ($p<0.05$) between dipped and negative control groups for both Ujosan® dip and Eimü Chlorhexidin® dip.

The main objective was to induce in vitro sensitivity reduction (resistance) of the same two commercial teat dips with sub-lethal concentrations against ten different strains of *S. aureus*. For each disinfectant, 10 strains were repeatedly passed 10 times in growth media with sub lethal concentrations of Ujosan® dip and Eimü Chlorhexidin® dip. The MIC values of the teat

dip after passages were determined and compared with the original MIC values before passages. According to the results, 9 strains (90%) became nonsusceptible to Ujosan® dip and only one strain (10%) became nonsusceptible to Eimü Chlorhexidin® dip. All isolates with a significant increase (p>0.001) of MICs were passed every day for 10 days in tryptose soya broth (TSB) without disinfectant (active substance), to check whether the acquired resistance was stable or not. Stability of acquired resistance was noticed in all Ujosan® dip and Eimü Chlorhexidin® dip adapted *S. aureus* strains. Furthermore, the sensitivity of 6 selected antimicrobial agents against 70 coagulase positive *S. aureus* and CNS strains was checked using the agar disk diffusion test. 85.71% of *S. aureus* strains and 28.57% of CNS were resistant to Penicillin G, 7.14% of *S. aureus* and CNS were resistant to tetracycline and only 10.71% of *S. aureus* and 7.14% of CNS were resistant to gentamycin. The percentages of resistant *S. aureus* and CNS to chloramphinicol were 1.78% and 7.14%, respectively. No resistance was detected for the other tested antimicrobial agents (oxacillin and erythromycin).

Finally the current study investigated whether Ujosan® dip-and Eimü Chlorhexidin® dip-adapted *S. aureus* strains were also resistant to antibiotics commercially available for the treatment of bovine *S. aureus* mastitis. According to the results obtained from the Federal Institute for Risk Assessment (Berlin, Germany), all Ujosan® dip and Eimü Chlorhexidin® dip-adapted *S. aureus* strains showed in vitro the highest susceptibility to all types of antibiotics. Therefore, prolonged exposure of sub-inhibitory concentrations of Ujosan® dip or Eimü Chlorhexidin® dip did not increase emerging antibiotic resistance in *S. aureus.* The current results and published data indicate that more detailed investigations on the cross-resistance between reduced susceptibility of chemical disinfectants and antibiotics are needed.

KAPITEL 6: ZUSAMMENFASSUNG

Empfindlichkeit von *Staphylococcus aureus* Stämmen isoliert von Kühen mit subklinischer Mastitis gegen verschiedene Arten von Desinfektionsmitteln und Antibiotika

Das Ziel der Studie war zunächst, die Wirksamkeit von zwei Zitzendippmitteln (Zitzendesinfektionsmittel), Ujosan® dip und Eimü Chlorhexidin® dip, gegenüber 56 *Staphylococcus* (S.) aureus-Stämmen, die von subklinischen Fällen boviner Mastitis isoliert wurden, zu bestimmen. Ein weiteres Ziel war, eine Resistenzinduktion bei ausgewählten Stämmen von *S. aureus* gegen Ujosan® dip und Eimü Chlorhexidin® dip zu versuchen. Ein drittes Ziel bestand darin, die Antibiotikaresistenz von *S. aureus* und koagulasenegativen Staphylokokken (KNS)-Isolaten von Kühen mit Mastitis zu testen. Viertes Ziel war es, die Möglichkeit der Kreuzresistenz zwischen verminderter Empfindlichkeit gegenüber Ujosan® dip und Eimü Chlorhexidin® dip und verschiedenen Arten von Antibiotika, die für die Behandlung von boviner *S. aureus*-Mastitis kommerziell verfügbar sind, zu testen. Viertelmilchproben wurden aus sechs Milchkuhbeständen mit hoher Prävalenz von *S. aureus* im Bundesland Brandenburg (Deutschland) gesammelt. Von jeder Herde wurden 32 Kühe in verschiedenen Stadien der Laktation und unterschiedlichen Altersgruppen für die Probenahme ausgewählt. Die 32 Kühe lassen sich in drei Gruppen aufteilen. Die Zitzen der Kühe aus der ersten Gruppe wurden nach dem Melken in das Zitzendesinfektionsmittel Ujosan® dip getaucht, in der zweiten Gruppe wurde dafür Eimü Chlorhexidin® dip verwendet, in der dritten Gruppe wurde kein Zitzendipmittel angewendet. Insgesamt 70 Isolate von *S. aureus* und KNS wurden phänotypisch durch Röhrchen-Koagulase-Test und Staph ID 32 API-System identifiziert. Auch wurden diese Isolate mit Hilfe von Polymerase-Kettenreaktion und der Matrix-unterstützten Laserdesorptions/ionisations-Flugzeit-Massenspektrometrie, (Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry, MALDI-TOF-MS), die als ein Bestätigungsverfahren für die PCR-Methode verwendet wurde, genotypisch identifiziert.

Nach der Identifizierung aller Stämme, wurde die minimale Hemmkonzentration (MHK) von Ujosan® dip und Eimü Chlorhexidin® dip gegen *S. aureus*-Stämme mit Benutzung der Bouillon-Makrodilutionsmethode entsprechend den Richtlinien zur Prüfung von chemischen Desinfektionsmitteln der Deutschen Veterinärmedizinischen Gesellschaft (DVG) von 2000 ermittelt. Die Mittelwerte der MHK von Ujosan® dip und Eimü Chlorhexidin® dip für die Dip-Gruppen- und Kontrollgruppen waren respektiv 45,70 ± 2,54%; 42,6 ± 1,64% und 97,51 ± 0,98%; 96,8 ± 0,78%. Diese Studie zeigte, dass es keine signifikanten Unterschiede ($p < 0,05$) zwischen den Isolaten der Gruppen mit Ujosan® dip und Eimü Chlorhexidin® dip und den Isolaten aus der Kontrollgruppe. Das Hauptziel war, in vitro die Reduktion der Empfindlichkeit (Resistanz) mit subletalen Konzentrationen von Ujosan® dip und Eimü Chlorhexidin® dip bei Stämmen von *S. aureus* zu induzieren. 10 Stämme wurden 10-mal in

Nährmedien mit subetaler Konzentration von Ujosan® dip und Eimü Chlorhexidin® dip passagiert. Die MHK-Werte der Zitzendipmittel wurden nach dem Passagieren bestimmt und mit den MHK-Werten vor den Passagen verglichen. 9 Stämme (90%) wiesen eine geringere Empfindlichkeit gegenüber Ujosan® dip auf und nur 1 Stamm (10%) hatte eine reduzierte Empfindlichkeit gegenüber Eimü Chlorhexidin® dip. Alle Isolate mit signifikantem Anstieg (p> 0,001) der MHK-Werten wurden 10 Tage hintereinander in Tryptose-Soja-Bouillon (TSB) ohne Ujosan® dip bzw. Eimü Chlorhexidin® dip I passagiert, um zu prüfen, ob die erworbene Resistenz stabil oder nicht stabil war. Die Stabilität der erworbenen Resistenz wurde in allen dem Ujosan® dip und dem Eimü Chlorhexidin® dip angepassten *S. aureus*-Stämmen festgestellt.

Weiterhin wurde die Empfindlichkeit von 6 ausgewählten antimikrobiellen Substanzen gegen 70 *S. aureus*-Stämme und KNS mit dem Agar-Disk-Diffusions-Test (Plättchendiffusionstest auf Agar) geprüft. 85,71% der *S. aureus*-Stämme und 28,57% der KNS-Stämme waren resistent gegen Penicillin G, 7,14% aller *S. aureus*-und KNS-Stämme resistent gegen Tetracyclin und nur 10,71% von *S. aureus*- und 7,14% der KNS- Stämmen waren resistent gegen Gentamycin sowie gegen Chloramphenicol respektive 1,78% und 7,14%. Es wurde keine Resistenz gegen die anderen getesteten Antibiotika (Oxacillin und Erythromycin) nachgewiesen.

Schließlich wurde untersucht, ob an Ujosan® dip und Eimü Chlorhexidin® dip angepasste *S. aureus*-Stämme auch gegen Antibiotika, die für die Behandlung von boviner *S. aureus*-Mastitis erhältech sind, resistent waren. Nach den Ergebnissen aus dem Bundesinstitut für Risikobewertung (Berlin, Deutschland) zeigten in vitro alle an Ujosan® dip und Eimü Chlorhexidin® dip angepassten *S. aureus*-Stämme die höchste Empfindlichkeit für alle getsteten Antibiotika. Daher hat eine anhaltende Exposition gegenüber subletalen Hemmkonzentrationen von Ujosan® dip oder Eimü Chlorhexidin® dip zu keiner Antibiotikaresistenz bei *S. aureus* geführt. Die aktuellen Ergebnisse und veröffentlichte Daten zeigen, dass mehr detaillierte Untersuchungen über Kreuzresistenzen zwischen verminderter Empfindlichkeit gegenüber chemischen Desinfektionsmitteln und Antibiotika erforderlich sind.

CHAPTER 8: REFERENCES

Aarestrup, F.M. (2005): Veterinary drug usage and antimicrobial resistance in bacteria of animal origin. Basic Clin. Pharm. Toxicol. **96:** 271–281.

Aarestrup, F.M.; Dangler, C.A. and Sordello, L.M. (1995): Coagulase gene polymorphism in *Staphylococcus aureus* isolated from dairy cattle with mastitis. Can. J. Vet. Res. **59:** 124.

Aarestrup, F.M. and Jensen, N.E. (1998): Development of penicillin resistance among Staphylococcus aureus isolated from bovine mastitis in Denmark and other countries. Microb. Drug Resist. **4:** 247-256.

Abera, M.; Demie, B.; Aragaw, K.; Regassa, F. and Regassa, A. (2010): Isolation and identification of *Staphylococcus aureus* from bovine mastitic milk and their drug resistance patterns in Adama town, Ethiopia. J. Vet. Med. and Animal Heal. **2** (3)**:** 29-34

Albandar, J.; Gjermo, P. and Preus, H. (1994): Chlorhexidine use after two decades of over-the-counter availability. J. Periodontol. **465:** 109-112.

Anderson, J.C. (1983): Veterinary aspects of staphylococci. In: Staphylococci and Staphylococcal Infections. Vol. 1. Adlam, C. and C. S. F. Easmon (eds), Academic Press, New York, USA, **pp:** 193-241.

Anderson, K.L. and Lyman, R.L. (2006): Long-term persistence of specific genetic types of mastitis-causing Staphylococcus aureus on three dairies. J. Dairy Sci., **89:** 4551-4556.

Anderson, R.L.; Holland, B.W.; Carr, J.K.; Bond, W.W. and Favero, M.S. (1990): Effect of disinfectants on pseudomonads colonized on the interior surface of PVC pipes. Am. J. Public Health **80:** 17–21.

Bailey, A.M.; Constantinidou, C.; Ivens, A.; Garvey, M.I.; Webber, M.A.; Coldham, N.; Hobman, J.L.; Wain, J.; Woodward, M.J. and Piddock, L.J.V. (2009): Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. J. Antimicrob. Chemo. **64:** 973-85.

Baillie, L.W.J.; Wade, J.J. and Casewell, M.W. (1992): Chlorhexidine sensitivity of *Enterococcus faecium* resistant to vancomycin, high levels of gentamycin or both. Journal of Hospital Infection **20:** 127-128.

Baillie, L.W.J.; Power, E.G.M. and Phillips, I. (1993): Chlorhexidine hypersensitivity of ciprofloxacin resistant variants of *P. aeruginosa* PAO. J. Antimicrob. Chemother. **31:** 219-225.

Bamber, A.I. and Neal, T.J. (1999): An assessment of triclosan susceptibility in methicillin resistant and methicillin sensitive *Staphylococcus aureus*. J. Hosp. Infect. **41:** 107-109

References

Barkema, H.W.; Schukken, Y.H. and Zadoks, R.N. (2006): Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. J. Dairy Sci. **89:** 1877-1895.

Barragry, T.B. (1994): Veterinary Drug Therapy. 1st ed. Lea & Febiger, Philadelphia, PA.

Barrett, D.J.; Healy, A.M.; Leonard, F.C. and Doherty, M.L. (2005): Prevalence of pathogens causing subclinical mastitis in 15 dairy herds in the Republic of Ireland. Irish Veterinary Journal **58:** 333-337.

Bennedsgaard, T.W.; Thamsborg, S.M; Aarestrup, S.M; Enevoldsen, C.; Vaarst1, M. and Christoffersen, A.B. (2006): Resistance to penicillin of *Staphylococcus aureus* isolates from cows with high somatic cell counts in organic and conventional dairy herds in Denmark. Acta Veterinaria Scandinavica **48:** 24.

Bernardo, K.; Pakulat, N.; Macht, M.; Krut., O.; Seifert, H.; Fleer, S.; Hünger, F. and Krönke, M. (2002): Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics **2:** 747-753.

Birošová, L. and Mikulášová, M. (2009): Development of triclosan and antibiotic resistance in *Salmonella enterica* serovar Typhimurium. J. Med. Microbiol. **58:** 436-441.

Bjørland, J.; Sunde, M. and Waage, S. (2001): Plasmid-borne *smr* gene causes resistance to quaternary ammonium compounds in bovine *Staphylococcus aureus*. J. Clin. Microbiol. **39:** 3999–4004.

Block, S.S. (2001): Definitions of terms. In: Block SS, (ed). Disinfection, Sterilization, and preservation. 5th ed. Philadelphia: Lippincott Williams & Wilkins, **pp:** 19-28

Boddie, R.L. and Nickerson, S.C. (1993): Permanent drying off of chronic mastitic quarters. La. Cattleman, **Nov:** 10-11

Boddie, R.L.; Nickerson, S.C. and Adkinson, R.W. (1997): Efficacies of Teat Germicides Containing 0.5% Chlorhexidine and 1% Iodine during Experimental Challenge with *Staphylococcus aureus* and *Streptococcus agalactiae*. J. Dairy Sci. **80:** 2809–2814.

Boddie, R.L.; Nickerson, S.C. and Adkinson, R.W. (2000): Efficacies of chlorine dioxide and iodophor teat dips during experimental challenge with *Staphylococcus aureus* and *Streptococcus agalactiae*. J. Dairy Sci. **83:** 2977-2981.

Bramley, A.J. (1981): The role of hygiene in preventing intramammary infection. In Mastitis control and herd management, **pp:** 53-66 Tech. Bull (Eds A.J. Bramley).

References

Bramley, A.J.; Cullor, J.S.; Erskine, R.J.; Fox, L.K.; Harmon, R.J.; Hogan, J.S.; Nickerson, S.C.; Oliver, S.P.; Smith, K.L. and Sordillo, L.M. (1996): Pages 1–3 in Current Concepts of Bovine Mastitis. 4th ed. National Mastitis Council, Madison, WI.

Braoudaki, M. and Hilton, A.C. (2004): Adaptive Resistance to Biocides in *Salmonella enterica* and *Escherichia coli* O157 and Cross-Resistance to Antimicrobial Agents. J. Clini. Micro. **42** (1)**:** 73–78.

Buzzola, F.R.; Quelle, L.; Gomez, M.I.; Catalano, M.; Steele-Moore, L.; Berg, D.; Gentilini, E.; Denamiel, G. and Sordelli, D.O. (2001): Genotypic analysis of *Staphylococcus aureus* from milk of dairy cows with mastitis in Argentina. Epidemiol. Infect. **126:** 445–452.

Capuco, A.V.; Bright, S.A.; Pankey, J.W.;Wood, D.L.; Miller, R.H. and Bitman, J. (1992): Increased susceptibility to intramammary infection following removal of teat canal keratin. J. Dairy Scc. **75:** 2126-2130.

Carbonnelle, E.; Beretti, J.L.; Cottyn, S.; Quesne, G.; Berche, P.; Nassif, X. and Ferroni, A. (2007): Rapid identification of staphylococci isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J. Clin. Microbiol. **45:** 2156-2161.

Carretto, E., Barbarini, D.; Couto, I.; De Vitis, D.; Marone, P.; Verhoef, J.; De Lencastre, H. and Brisse, S. (2005): Identification of coagulase-negative staphylococci other than *Staphylococcus epidermidis* by automated ribotyping. Clin. Microbiol. Infect. **11:** 177–184.

Carey, V.; Zeger, S.L. and Diggle, P.J. (1993): Modelling multivariate binary data with alternating logistic regressions. Biometrika **80:** 517–526.

Chalita, M.K.; Hofling-Lima, A.L.; Paranhos, A.; Schor, P. and Belfort. R. (2004): Shifting trends in vitro antibiotic susceptibilities for common ocular isolates during a period of 15 years. Am. J. Ophthalmol. **137**(1)**:** 43-51.

Chang, S.L. (1971): Modern concept of disinfection. J. Sanit. Eng. Div. Proc. ASCE. **97:** 689.

Chaves, C.J.; Tirante, L.; Pol., M.; Bas, D.; Vandoni, R. and Olivieri, R. (2001): Prevalence of intramammary infections in 74 dairy herds located in Argentina. 40th Annu. Meet. Natl. Mastitis Council, Reno, Nevada, 205-206.

Chopra, I. (1987): Microbial resistance to veterinary disinfectants and antiseptics. Blackwell Scientific Publications Ltd., 43–65.

CLSI, (2009): Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard- Eighth Edition, M7-A8, volume (29), No. 2

References

Cole, E.C.; Addison, R.M. Rubino, J.R.; Leese, K.E.; Dulaney, P.D.; Newell, M.S.; Wilkins, J.; Gaber, D.J.; Wineinger, T.; Criger, D.A. (2003): Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of bacterial users and nonusers. J. Appl. Microbiol. **95:** 664-76.

Collignon, P.J. (2002): Antibiotic resistance. Med. J. Aust. **177:** 225−229.

Compton, C.W.; Heuer, C.; Parker, K. and McDougall, S. (2007): Risk factors for peripartum mastitis in pasture-grazed dairy heifers. J. Dairy Sci. **90:** 4171–4180.

Cookson, B. (2005): Clinical significance of emergence of bacterial antimicrobial resistance in the hospital environment. J. Appl. Microbiol. **99:** 989-996.

Costa, E.O.; Benites, N.R. and Guerra, J.L. (2000): Antimicrobial susceptibility of *Staphylococcus* spp. isolated from mammary parenchymas of slaughtered dairy cows. J. Vet. Med. B. Infect. Dis. Vet. Public Health **47:** 99-103.

Cottell, A.; Denyer, S.P.; Hanlon, G.W. and Maillard, J-Y. (2009): Triclosan-tolerant bacteria: Changes in susceptibility to antibiotics. Journal of Hospital Infection **72:** 71-76.

DANMAP (2003): DANMAP - Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, foods and humans in Denmark. http://www.danmap.org/pdfFiles/Danmap_2003.pdf.

Da Silva Santos, O.C.; Barros, E.M.; Brito, M.A.V.P; Bastos, M.C.F.; dos Santos, K.R.N. and deMarval, M.G. (2008): Identification of coagulase-negative staphylococci from bovine mastitis using RFLP-PCR of the groEL gene. Vet. Microbiol. **130:** 134–140.

Davidson, I. (1961): The epidemiology of staphylococcal mastitis. Vet. Rec. **73:** 1015-1018.

Davies, J. (1994): Inactivation of antibiotics and the dissemination of resistance genes. Science **264** (5157)**:** 375 - 382

Davin-Regli, A.; Chollet, R.; Bredin, J.; Chevalier, J.; Lepine, F. and Pagès, J.M. (2006): Enterobacter gergoviae and the prevalence of efflux in parabens resistance. J. Antimicrob. Chemother. **57:** 757-60.

Denton, G.W. (2001): Chlorhexidine. In: Block SS editor Disinfection, sterilization, and preservation 5th edition, Chapter 15. Lippincott, Williams and Wilkins, Philadelphia, 321-336.

Denyer, S.P. (1995): Mechanisms of action of antibacterial biocides. Int. Biodeterior. Biodegrad. **36:** 227–245.

De Oliveira, A.P.; Watts, J.L.; Salmon, S.A. and Aarestrup, F.M. (2000): Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Europe and the United States. J. Dairy Sci. **83:** 855–862.

References

De Vliegher, S.; Laevens, H.; Devriese, L.A.; Opsomer, G.; Leroy, J.L.M.; Barkrama, H.W. and de Kruif, A. (2003): Prepartum teat apex colonization with *Staphylococcus chromogenes* in dairy heifers associated with low somatic cell count in early lactation. Vet. Microbiol. **92:** 245-252.

DeWitte, M.; Eberhart, R.J. and Griel, L.C. (1980): Mastitis caused by teat dipping error. Vet. Med. Small Anim. Clin. **75** (10)**:**1613-1616.

Dos Santos Nacimento, J.S.; Fagundes, P.C.; Brito, M.A.P.V.; Santos, K.R.N. and Bastos, M.C.F. (2005): Productions of bacteriocins involved in bovine mastitis. Vet. Microbiol. **106** (1-2)**:** 61-71.

Drlica, K. and Zhao, X. (1997): DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol. Biol. Rev. **61:** 377–392.

Dychdala, G.R. (1968): Chlorine and chlorine compounds Page 286 in Disinfection, sterilization, and preservation. Lawrence, C.A. and Block, S.S. (eds). Lea and Febiger, Philadelphia, PA.

Edinger, D.; Tenhagen, B.A.; Kalbe, P.; Klünder, G.; Baumgärtner, B. and Heuwieser, W. (2000): Effect of teat dipping with a germicide barrier teat dip in late gestation on intramammary infection and clinical mastitis during the first 5 days post-partum in primiparous cows. J. Vet. Med. A Physiol. Pathol. Clin. Med. **47:** 463–468.

El-Moug, T.; Rogers, D.T.; Furr, J.R.; El-Falaha, B.M.A. and Russell, A.D. (1985): Antiseptic-induced changes in the cell surface of a chlorhexidine sensitive and a chlorhexidine-resistant strain of Providencia stuartii. J. Antimicrob. Chemother. **16:** 685–689.

Ericsson Unnerstad, H.; Lindberg, A.; Persson Waller, K.; Ekman, T.; Artursson, K.; Nilsson Öst, M. and Bengtsson, B. (2008): Microbial etiology of acute clinical mastitis and agent specific risk factors. Veterinary Microbiology, (Article in Press).

Erskine, R.J.; Walker, R.D.; Bolin, C.A.; Bartlett, P.C. and White, D.G. (2002): Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. J. Dairy Sci. **85:** 1111–1118.

Farnsworth, R.J., Wyman, L. and Hawkinson, R. (1980): Use of a teat sealer for prevention of intramammary infections in lactating cows. J. Am. Vet. Med. Assoc. **177:** 441.

Flachowsky, G., Schöne, F., Leiterer, M., Bemmann, D., Spolders, M. and Lebzien, P. (2007): Influence of an iodine depletion period and teat dipping on the iodine concentration in serum and milk of cows. J. Anim. Feed Sci. **16:** 18-25.

Foster, T.J.; O'Reilly, M. and Bramley, A.J. (1990): Genetic studies of *Staphylococcus aureus* virulence factors. Pages, 35-46 in Pathogenesis of Wound and Biomaterial Associated Infections. Wadstrom, T., Eliasson, I., Holder, I. and Ljungh, A., (eds). Springer-Verlag, London.

Fox, L.K.; Ferens, W.A.; Bohach, G.A.; Bayles, K.W. and Davis. W.C. (2000): *Staphylococcus aureus*: Super mastitis pathogen. Pages 98-103 *in* Proc. 39th Annu. Meet. Natl. Mastitis Counc., Inc. Atlanta, GA.

Fox, L.K.; Gershman, M.; Hancock, D.D. and Hutton, C.T. (1991): Fomites and reservoirs of *Staphylococcus aureus* causing intramammary infections as determined by phage typing – the effect of milking time hygiene practices. Cornell Vet. **81:** 183-193.

Gentilini, E.; Denamiel, G.; Llorente, P.; Godaly, S.; Rebuelto, M. and DeGregorio, O. (2000): Antimicrobial susceptibility of *Staphylococcus aureus* isolated from bovine mastitis in Argentina. J. Dairy Sci. **83:** 1224–1227.

Gershenfeld, L. (1977): Iodine in Disinfection, sterilization, and preservation. 2nd ed. S. S. Black, (ed), Lea & Febiger, PA.

Ghorbanpoor, M.; Seyfiabad, S.M.; Moatamedi, H.; Jamshidian, M. and Gooraninejad, S. (2007): Comparison of PCR and bacterial culture methods for diagnosis of dairy cattle's subclinical mastitis caused by *Staphylococcus aureus.* J.Vet. Res. **62** (4)**:** 87-91.

Gianneechini, R.E.; Concha, C. and Franklin, A. (2002): Antimicrobial susceptibility of udder pathogens isolated from dairy herds in the West Littoral region of Uruguay. Acta Vet. Scand. **43:** 31–41.

Giesecke, W.H.; Preez, J.H. and Petzer, I.M. (1994): Practice mastitis control in dairy herds. Butterworths, Durban.

Gilbert, P.; Allison, D.G. and McBain, A.J. (2002): Biofilms in vitro and in vivo: do singular mechanisms imply cross-resistance? J. Appl. Micro. Symp. Suppl. **92:** 98–110.

Gill, J.J.; Pacan, J.C.; Carson, M.E.; Leslie, K.E.; Griffiths, M.W. and Sabour, P.M. (2006): Efficacy and pharmacokinetics of bacteriophage therapy in treatment of subclinical *Staphylococcus aureus* mastitis in lactating dairy cattle. Antimicrob. Agents Chemother. **50:** 2912–2918.

Gjermo, P. (1974): Chlorhexidine in dental practice. J. Clin. Periodontol. **1**(39)**:** 143-152.

Gomez Escalada, M.; Harwood, J.L.; Maillard, J.Y. and Ochs, D. (2005): Triclosan inhibition of fatty acid synthesis and its effect on growth of *E. coli* and *Ps. aeruginosa*. J. Antimicrob. Chemother. **55:** 879-882.

Gottardi, W. (1983): Potentiometric evaluation of the equilibrium concentrations of free and complex bound iodine in aqueous solutions of polyvinylpyrrolidone-iodine (Povidone-iodine) [article in German]. Fresenius Z. Anal. Chem. **314:** 582-585.

Gottardi, W. (1991): Iodine and iodine compounds, p. 152–166. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation. 4th ed. Lea & Febiger, Philadelphia, Pa.

Gröhn, Y.; Wilson, D.J.; Gonzalez, R.N.; Hertl, J.A.; Schulte, H.; Bennett, G. and Schukken, Y.H. (2004): Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. **87:** 3358-3374

Gruet, P.; Maincent, P.; Berthelot, X. and Kaltsatos. V. (2001): Bovine mastitis and intramammary drug delivery: Review and perspectives. Adv. Drug Deliv. Rev. **50:** 245–259.

Guidry, A.J. (1985): Mastitis and the immune system of the mammary gland. Pages 240-251 in Lactation. B.L. Larson, (ed), Iowa State Univ. Press, Ames.

Guidry, A.J.; O'Brien, C.N.; Oliver, S.P.; Dowlen, H.H. and Douglass, L.W. (1994): Effect of whole *Staphylococcus aureus* and mode of immunization on bovine opsonizing antibodies to capsule. J. Dairy Sci. **77:** 2965.

Harmon, R.J; Eberhart, R.J.; Jasper, D.E.; Langlois, B.E. and Wilson, R.A. (1990): Microbiological procedures for the diagnosis of bovine udder infection. Arlington, VA: National Mastitis Council.

Haveri, M. (2008): *Staphylococcus aureus* in bovine intramammary infection: molecular, clinical and epidemiological characteristics. Academic dissertation, Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsiniki, Finland.

Heir, E.; Sundheim, G. and Holck, A.L. (1998): The Staphylococcus qacH gene product: a new member of the SMR family encoding multidrug resistance. FEMS Microbiol. Lett. **163:** 49-56.

Heir, E.; Sundheim, G. and Holck, A.L. (1999): The qacG gene on plasmid pST94 confers resistance to quaternary ammonium compounds in staphylococci isolated from the food industry. J. Appl. Microbiol. **86:** 378-88.

Hoblet, K.H.; Schnitkey, G.D.; Arbaugh, D.; Hogan, J.S.; Smith, K.L.; Schoenberg, P.S.; Todhunter, D.A.; Hueston, W.D.; Pritchard, D.E.; Bowman, G.L.; Heider, L.E.; Brockett, B.L. and Conrad. H.R. (1991): Cost associated with selected preventive practices and with episodes of clinical mastitis in nine herds with low somatic cell counts. J. Am. Vet. Med. Assoc. **199:** 190–196.

Hogan, J.S. and Larry Smith, K. (1989): Prolonged in Vitro Exposure of Staphylococcus aureus to Germicidal Teat Dips. J. Dairy Sci. **72:** 1052-1056.

Huet, A.A.; Raygada, J.L.; Mendiratta, K.; Seo, S.M. and Kaatz, G.W. (2008): Multidrug efflux pumps overexpression in *Staphylococcus aureus* after single and multiple in vitro exposures to biocides and dyes. Microbiol. **154:** 3144–3153.

Hugo, W.B. and Longworth, A.R. (1964): Some aspects of the mode of action of chlorhexidine. J. Pharm. Pharmacol. **16:** 655–662.

IFH, (2000): Microbial resistance and biocides. A review by the International Scientific Forum on Home Hygiene (IFH). http://www.ifh-homehygiene.org/2003/2public/antresFINAL.pdf.

Iqbal, J.; Hashmi, A.S. and Ashfaq, M. (1984): Incidence of penicillin resistant staphylococci. Pakistan Vet. J. **4:** 169-170.

Irizzary, L.; Merlin, T.; Rupp, J. and Griffith, J. (1996): Reduced susceptibility of methicillin resistant *Staphylococcus aureus* to cetylpyridinium chloride and chlorhexidine. Chemotherapy **42:** 248-252.

Jang H.J.; Chang, M.W.; Toghrol, F. and Bentley, W.E. (2008): Microarray analysis of toxicogenomic effects of triclosan on *Staphylococcus aureus*. Appl. Microbil. Biotechnol. **78:** 695-707.

Joklik, W.K. (1992): Sterilization and disinfection. Zinsser Microbiology. Appleton and Lange, Connecticut. Ch., **10:** 188-200.

Jones, A.; Higgs, T.M.; Neave, F.K. and Smith, A. (1967): The sensitivity of bovine staphylococci, streptococci and corynebacterium to cloxacillin and various other antibiotics. J. Dairy Res. **34:** 249-255.

Jones, T. and Ohnstad, L. (2002): Milking procedures recommended for the control of bovine mastitis. In Practice **24:** 502-511

Jurgens, D.J.; Sattar, S.A. and Mah, T.F. (2008): Chloraminated drinking water does not generate bacterial resistance to antibiotics in Pseudomonas aeruginosa biofilms. Lett. Appl. Microbiol. **46:** 562-567.

Karatzas, K.A.; Webber, M.A.; Jorgensen, F.; Woodward, M.J.; Piddock, L.J. and Humphrey, T.J. (2007): Prolonged treatment of Salmonella enterica serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. J. Antimicrob. Chemother. **60:** 947-955.

Kelly, L.M.; Jacobs, R.M. and Appelbaum, P.C. (1999): Comparison of agar dilution, microdilution, E-test, and disk diffusion methods for testing activity of cefditoren against *Streptococcus pneumoniae.* J. Clin. Microbiol. **37:** 3296–3299.

Kibsey, P.C.; Rennie, R.P. and Rushton, J.E. (1994): Disk diffusion versus broth microdilution susceptibility testing of *Haemophilus* species and *Moraxella catarrhalis* using seven oral antimicrobial agents: Application of updated susceptibility guidelines of the National Committee for Clinical Laboratory Standards. J. Clin. Microbiol. **32:** 2786–2790.

King, J.S.; Godinho, K.S. and Bramley, A.J. (1981): Page 159 *in* Testing and efficacy of teat skin disinfectants. H. C. Collins, (ed), Academic Press, New York, NY.

Kingwill, R.G. (1973): Effect of a mastitis control system on infections by the common pathogens. Page 50 in Proc. 12th Annu. Mtg., Natl. Mastitis Counc., Inc., Washington, DC.

Kingwill, R.G.; Neave, F.K.; Dodd, F.H.; Griffin, T.K.; Westgarth, D.R. (1970): The effect of a mastitis control system on levels of subclinical and clinical mastitis in two years. Vet. Rec. **87:** 94–100.

Kirchhoff, H. (1962): Untersuchungen über die Resistenzsteigerung von Bakterien gegen Desinfektionsmittel. Arch. Hyg. Bakteriol. **146:** 549-554.

Kloos, W.E. (1997): Taxonomy and systematics of staphylococci indigenous to humans. The molecular basis of pathogenesis. In: The staphylococci in human disease. Crossley, K. B. and G. L. Archer (eds.). Churchill Livingstone, N. Y., pp. 113-137.

Kolawole, D.O. (1984): Resistance mechanisms of mucoid-grown *Staphylococcus aureus* to the antibacterial action of some disinfectants and antiseptics. FEMS Microbiol. Lett. **25:** 205–209.

Koshiro, A. and Oie, S. (1979): Increased resistance in vitro of Pseudomonas aeruginosa to chlorhexidine. Bull. Yamaguchi Med. Sch. **26:** 73.

Kruse, W.C. (1970): Halogen action on bacteria, viruses and protozoa, In Proceedings of the National Special Conference on Disinfection. ASCE, Amherst, Mass. p. 113–136.

Lear, J.C.; Maillard, J-Y.; Dettmar, P.W.; Goddard, P.A. and Russell, A.D. (2006): Chloroxylenol- and triclosan-tolerant bacteria from industrial sources – susceptibility to antibiotics and other biocides. International Biodeterioration & Biodegradation. **57:** 51-56.

Leslie, K.E. and Dingwell, R.T. (2003): Background to dry cow therapy: What, where, why— Is it still relevant? Pages 5–17 in 42nd National Mastitis Council Annual Meeting, Fort Worth, Texas. Natl. Mastitis Counc., Madison, WI.

Leslie, K.E.; Petersson, C.S.; Vernooy, E. and Bashiri, A. (2005): Efficacy of an iodophor test disinfectant against *Staphylococcus aureus* and *Streptococcus agalactiae* in experimental challenge. J. Dairy Sci. **88:** 406-410.

Levy, S.B. (2000): Antibiotic and antiseptic resistance: impact on public health. Pediatric Infectious Disease Journal **19:** 120-122.

Levy, S.B. (2002): Antibacterial household products: cause for concern. Emerging Infect. Dis. **7:** 512-5.

Lingaas, E. (1998): The use of antimicrobials in animal production – a threat to humans? NKVet Symposium, Helsinki, Finland, **pp:** 26-27.

Longworth, A.R. (1971): Chlorhexidine, p. 95–106. In W. B. Hugo (ed.), Inhibition and destruction of the microbial cell. Academic Press, Ltd., London, England.

Maillard, J.Y. (2002): Bacterial target sites for biocide action. J. Appl. Microbiol. Symposium **92:** 16-27.

Maillard, J.Y and Denyer, S.P. (2009): Emerging bacterial resistance following biocide exposure: should we be concerned? Chemica Oggi. **27**(3)**:** 26-28.

Makovec, J.A. and Ruegg, P.L. (2003): Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8,905 samples (1994–2001). J. Am. Vet. Med. Assoc. **222:** 1582–1589.

Malinowski, E.; KŁossowska, A.; Kaczmarowski, M.; Henryka, L. and Krystyna, K. (2002): Antimicrobial susceptibility of staphylococci isolated from affected with mastitis cows. Bull. Vet. Inst. Pulawy **46:** 289-294.

Mallard, B.A. and Barnum, D.A. (1993): *Staphylococcus aureus* mastitis: Genetics and immunity. Pages 27-35 *in* Proc. 32nd Annu. Meet. Natl. Mastitis Counc., Inc. Kansas City, MO.

Martin, H. and Maris, P. (1995): Antiseptic and antibiotic resistance of 310 gram-positive strains isolated from udders after use of post-milking teat germicides. Vet. Res. **26**(1)**:** 43-56.

Matthews, K.R.; Rejman, J.J.; Turner, J.D. and Oliver, S.P. (1994): Proliferation of a bovine mammary epithelial cell line in the presence of bacterial virulence factors. J. Dairy Sci. **77**(10)**:** 2959-2964.

McBain, A.J.; Ledder, R.G.; Sreenivasan, P. and Gilbert, P. (2004): Selection for high-level resistance by chronic triclosan exposure is not universal. Journal of Antimicrobial Chemotherapy **53:** 772-777.

McDonnell, G. and Russell, A.D. (1999): Antiseptics and disinfectants: activity, action and resistance. Clinical Microbiology Reviews **12**(1)**:** 147-179.

McDougall, S.; Agnew, K.E.; Cursons, R.; Hou, X.X. and Compton, C.R.W. (2007): Parenteral treatment of clinical mastitis with tylosin base or penethamate hydriodide in dairy cattle. J. Dairy Sci. **90**(2)**:** 779–789

McManus, M.C. (1997): Mechanisms of bacterial resistance to antimicrobial agents. Am. J. Health Syst. Pharm. **54**(12)**:** 1420 –1433.

McMurry, L.M., Oethinger, M. and Levy, S.B. (1998): Triclosan targets lipid synthesis. Nature **394:** 531-532.

Mifflin, M. (2004): Bovine Mastitis- Definition of bovine mastitis in Medical Dictionary. The free dictionary by FARLEX, pp.15-20.

Mitchell, B.A.; Brown, M.H. and Skurray, R.A. (1998): QacA multidrug efflux pumps from *Staphylococcus aureus:* Comparative analysis of resistance to diamidines, biguanides and guanylhydrazones. Antimicrobial Agents and Chemotherapy **42**(2)**:** 475-477.

Moak, H. (1916): Control and eradication of infectious mastitis in dairy herds. Cornell Vet. **6:** 36.

Moken, M.C.; McMurry, L.M. and Levy, S.B. (1997): Selection of multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli* by using the disinfectant pine oil: Roles of the mar and acrAB loci. Antimicrob. Agents Chemother. **41**(12)**:** 2770-2772.

Monecke, S.; Kuhnert, P.; Hotzel, H.; Slickers, P. and Ehricht, R. (2007): Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. Vet. Microbiol. **125:** 128-140.

Moroni, P.; Pisoni, G.; Antonini, M.; Ruffo, G.; Carli, S.; Varisco, G. and Boettcher, P. (2005): Subclinical Mastitis and Antimicrobial Susceptibility of *Staphylococcus caprae* and *Staphylococcus epidermidis* isolated from Two Italian Goat Herds. J. Dairy Sci. **88:** 1694-1704.

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

Myllys, V.; Asplund, K.; Brofeldt, E.; Hirvela¨-Koski, V.; Honkanen-Buzalski, T.; Junttila, J.; Kulkas, L.; Myllykangas, O.; Niskanen, M.; Saloniemi, H.; Sandholm, M and Saranpää, T. (1998): Bovine mastitis in Finland in 1988 and 1995-Changes in prevalence and antimicrobial resistance. Acta Vet. Scand. **39**(1)**:** 119–126.

National Mastitis Council (2001): Summary of peer-reviewed publications on efficacy of premilking and postmilking teat disinfectants published since 1980, updated January 2001. NMC website nmconline.org.

Natzke, R.P.; Everett, R.W.; Guthrie, R.D.; Keown, J.F.; Meck, A.M.; Merrill, W.G.; Roberts, S.J. and Schmidt, G.H. (1972): Mastitis control program: Effect on milk production. J. Dairy Sci. **55**(9)**:** 1256.

References

NCCLS, (1999): Performance Standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard. NCCLS Document M31-A **19**(11)**:** 60.

Neave, F.K.; Dodd, F.H. and Kingwell, R.G. (1966): A method of controlling udder disease. Vet. Rec. **78**(15)**:** 521-523.

Neave, F.K.; Dodd, F.H.; Kingwill, R.G. and Westgarth. D.R. (1969): Control of mastitis in the dairy herd by hygiene and management. J. Dairy Sci. **52:** 696-707.

Neu, H.C. (1992): The crisis in antibiotic resistance. Science **257:** 1064–1073.

Newbould, F.H.S. and Barnum, D.A. (1960): The effect of dipping cows' teats in a germicide on the number of micrococci on the teat cup liners. J. Milk Food Technol. **21:** 348.

Nickerson, S.C. (1999): Role of vaccination and treatment programs. Pages 76-85 in Proc. 38th Annu. Meet. Natl. Mastitis Counc. Inc. Arlington, VA.

Nickerson, S.C. (2001): Choosing the best teat dip for mastitis control and milk quality. NMC-PDPW Milk Quality Conference Proceedings, pp 43.

NORM-VET (2006): Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Available at: http://www.scribd.com/doc/4866679/Usage-of-Antimicrobial-Agents-and-Occurance-Og-Antimicobial-Resistance-in-Norway

Nomura K, Ogawa, M.; Miyamoto, H.; Muratani, T. and Taniguchi, H. (2004): Antibiotic susceptibility of glutaraldehyde tolerant Mycobacterium chelonae from bronchoscope washing machine. Am. J. Infect. Control **32**(4)**:**185-188.

Nunes, S.F.; Bexiga, R.; Cavaco, L.M. and Vilela. C.L. (2007): Technical Note: Antimicrobial susceptibility of Portuguese isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* in subclinical bovine mastitis. J. Dairy Sci. **90**(7)**:** 3242-3246.

Obi, C.L.; Iyiegbuniwe, A.E.; Olukoya, D.K.; Babalola, C. Igunbor, E.O. and Okonta, A.A. (1996): Antibiogram and plasmids of *Staphylococcus aureus* and coagulase negative staphylococci isolated from different clinical sources. Cent. Afr. J. Med. **42**(9)**:** 258-61.

Olde Riekerink, R.G.M.; Barkema, H.W.; Veenstra, S.; Poole, D.E.; Dingwell, R.T. and Keefe, G.P. (2006): Prevalence of contagious mastitis pathogens in bulk tank milk in Prince Edward Island. Can. Vet. J. **47:** 567–572.

Oliver, S.P.; King, S.H.; Lewis, M.J.; Torre, P.M.; Mathews, K.R. and Dowlen, H.H. (1990): Our industry today: Efficacy of chlorhexidine as a postmilking teat disinfectant for the prevention of bovine mastitis during lactation. J. Dairy Sci. **73:** 2230.

O'Rourke, E.; Runyan, D.; O'Leary, J. and Stern, J. (2003): Contaminated iodophor in the operating room. Am. J. Infect. Control **31**(4)**:** 255-256.

Osterås, O.; Martin, S.W. and Edge, V.L. (1999): Possible risk factors associated with penicillin resistant strains of *Staphylococcus aureus* from bovine subclinical mastitis in early lactation. J. Dairy Sci. **82**(5)**:** 927–938.

Osterås, O.; Whist, A.C. and SØverØd, L. (2008): The influence of iodine teat dipping and an external teat sealant in heifers on bacterial isolation from quarter milk culture obtained post-calving. Livestock Science **119:** 129-136.

Owens, W.E. (1987): Isolation of *Staphylococcus aureus* L-forms from experimentally induced bovine mastitis. J. Clin. Microbio. **25**(10)**:** 1956-1961.

Owens, W.E.; Ray, C.H.; Watts, J.L. and Yancey, R.R. (1997): Comparison of success of antibiotic therapy during lactation and results of antimicrobial susceptibility tests for bovine mastitis. J. Dairy Sci. **80**(2)**:** 313–317.

Owens, W.E. and Watts, J.L. (1988): Antimicrobial susceptibility and β-lactamase testing of staphylococci isolated from dairy herds. J. Dairy Sci. **71**(7)**:** 1934.

Pankey, J.W.; Eperhart, R.J.; Cuming, A. L.; Daggett, R.D.; Farnsworth, R.J. and Mcduff, C.K. (1984): Our industry today. Uptake on postmilking teat antisepsis. J. Dairy Sci. **67:** 1336-1353.

Parker, K.I.; Compton, C.W.; Anniss, F.M.; Heuer, C. and McDougall, S. (2008): Quarter-level analysis of subclinical and clinical mastitis in primiparous heifers following the use of a teat sealant or an injectable antibiotic, or both, precalving. J. Dairy Sci. **91:** 169–181.

Paulson, D.S. (1993): Efficacy evaluation of a 4% Chlorhexidine gluconate as a full-body shower washes. Am. J. Infect. Control **21:** 205-209.

Payne, D.N.; Babb, J.R. and Bradley, C.R. (1999): An evaluation of the suitability of the European Suspension Test to reflect *in vitro* activity of antiseptics against clinically resistant organisms. Letters in Applied Microbiology **28:** 7-12.

Petri, W.A.J. (2006): Antimicrobial agents: sulphonamides, trimethoprim-sulphamethoxozole, quinolones, and agents for urinary tract infections. 11th ed. New York: McGraw-Hill, **pp:** 1111–1126.

Petrovski, K.R.; Trajcev, M. and Buneski, G. (2006): A review of the factors affecting the costs of bovine mastitis. J. S. Afr. Vet. Assoc. **77** (2)**:** 52-60.

Petzer, I.M. (2009): Efficacy of different dry-cow intramammary antimicrobial products on the prevalence of mastitis in high producing dairy herd. J. S. Afr. Vet. Assoc. **80**(1)**:** 23-30.

Philpot, W.N.; Boddie, R.L. and Pankey. J.W. (1978): Hygiene in the prevention of udder infections. IV. Evaluation of teat dips with excised cows' teats. J. Dairy Sci. **61:** 950.

Philpot, W.N. and Nickerson, S.C. (1992): Mastitis: Counter Attack. Babson Bros. Co., Naperville, IL.

Philpot, W.N., and Nickerson, S.C. (1999): Mastitis: Counter attack. Westfalia surge LLC: Illinois, USA.

Philpot, W.N. and Pankey, J.W. (1975): Hygiene in the prevention of udder infection. Ill. Effectiveness of 59 teat dips for reducing bacterial populations on teat skin. J. Dairy Sci. **58:** 209.

Piccinini, R.; Binda, E.; Zecconi, A. and Zanini, L. (2003): Prevalence study on bulk tank milk cultures in 1000 dairy herds in Lombardia (Italy). 42nd Ann. Meet. Natl. Mastitis Council, Fort Worth, pp. 396-397.

Piddock, L.J. (2006): Clinically relevant chromosomally encoded multidrug resistance efflux pump in bacteria. Clin. Microbiol. Rev. **19:** 382-402.

Pitkala, A.; Haveri, M.; Pyorala, S.; Myllys, V. and Honkanen- Buzalski, T. (2004): Bovine mastitis in Finland 2001 – Prevalence, distribution of bacteria and antimicrobial resistance. J. Dairy Sci. **87:** 2433–2441.

Poole, K. (2002): Mechanisms of bacterial biocide and antibiotic resistance. J. Appl. Microbiol. **92:** 55-64.

Poxton, I.R. (1993): Prokaryote envelope diversity. J. Appl. Bact. Symp. Suppl. **70:** 1–11.

Prince, H.N.; Nonemaker, W.S.; Norgard, R.C. and Prince, D.L. (1978): Drug resistance studies with topical antiseptics. J. Pharm. Sci. **68:** 1629.

Pyörälä, S. and Pyörälä, E. (1997): Accuracy of methods using somatic cell count and N-acetyl--D-glucosaminidase activity in milk to assess the bacteriological cure of bovine clinical mastitis. J. Dairy Sci. **80:** 2820-2825.

Pyörälä, S. and Taponen, S. (2009): Coagulase-negative staphylococci-Emerging mastitis pathogens. Veterinary Microbiology, **134:** 3-8.

Quinn, P.J.; Carter, M.E.; Markey, B. and Carter, G.R. (1994): California mastitis test (CMT). Clinical Veterinary Microbiology, Harcourt publishers Ltd.: London, **pp:** 333-334.

Quinn, P.J.; Carter, M.E.; Markey, B. and Carter, G.R. (1999): Clinincal Veterinary Microbiology, MOSBY, Harcourt publishers Ltd.: London, **pp:** 118-126.

Quinn, P.J. and Markey, B.K. (2001): Disinfection and disease prevention in veterinary medicine. In: Block SS, (editor). Disinfection, sterilization and preservation. 5th edition. Philadelphia: Lippincott, Williams & Wilkins. pp 1069-1103.

Radostitis, O.M.; Blood, D.C. and Gay, C.C. (1994): Veterinary Medicine: A text book of the diseases of cattle, sheep, pigs, goats and horses. 8th ed. Bailliere Tindall: London **8:** 563-613.

Radostitis, O.M.; Gay, C.C.; Blodd, D.C. and Hinchcliffk, W. (2000): Veterinary Medicine. Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses. 9th Edition, pp 603-700.

Randall, L.P.; Cooles, S.W.; NColdham, N.G.; Penuela, E.G.; Mott, A.C.; Woodward, M.J.; Piddock, L.J.V. and Webber, M.A. (2007): Commonly used farm disinfectants can select for mutant *Salmonella enterica* serovar Typhimurium with decreased susceptibility to biocides and antibiotics without compromising virulence. J. Antimicrob. Chemother. **60:** 1273-80.

Reverdy, M.E.; Bes, M.; Brun, Y. and Fleurette, J. (1992): E´ volution de la re´sistance aux antibiotiques et aux antiseptiques de souche hospitalie`res de *Staphylococcus aureus* isole´es de 1980 a` 1991. Pathol. Biol. **41:** 897–904.

Roberson, J.R. (1999): The Epidemiology of *Staphylococcus aureus* on Dairy Farms. National Mastitis Council 38th Annual Meeting, pp 38.

Roberson, J.R.; Fox, K.L.; Hancock, D.D.; Gay, J.M. and Besser, T.E. (1994): Ecology of *Staphylococcus aureus* isolated from various sites of dairy farms. J. Dairy Sci. **77:** 3354-3364.

Rogers, C.K. (2005): Consumer Antiseptic Drug Products: Antiseptic Handwash and Bodywash Center for Drug Evaluation and Research, Food and Drug Administration, 75N-0183H.

Russell, A.D. (1985): The role of plasmids in bacterial resistance to antiseptics, disinfectants and preservatives. J. Hosp. Infect. **6**(1)**:** 9-19.

Russell, A.D. (1991): Mechanisms of bacterial resistance to non-antibiotics: food additives and food and pharmaceutical preservatives. J. Appl. Bacteriol. **71:**191–201.

Russell, A.D. (1995): Mechanisms of bacterial resistance to biocides. Int. Biodeterior. Biodegrad. **36:** 247–265.

Russell, A.D. (1997): Plasmids and bacterial resistance to biocides. J. Appl. Microbiol. **82:** 155–165.

Russell, A.D. (2000): Do biocides select for antibiotic resistance? Journal of Pharmacy and Pharmacology **52:** 227–233.

Russell, A.D. and Chopra, I. (1996): Understanding antibacterial action and resistance. 2nd ed. Ellis Horwood, Chichester, England.

Russell, A.D.; Suller, M.T. and Maillard, J.-Y. (1999): Do antiseptics and disinfectants select for antibiotic resistance? Journal of Medical Microbiology **48:** 613–615.

Russell, A.D; Tattawasart, U.; Maillard, J.Y. and Furr, J.R. (1998): Possible link between bacterial resistance and use of antibiotics and biocides. Antimicrobial Agents and Chemotherapy **42:** 2151.

Rutala, W.A.; Steigel, M.M.; Sarubbi, F.A. and Weber, D.J. (1997): Susceptibility of antibiotic-susceptible and antibiotic-resistant hospital bacteria to disinfectants. Infection Control and Hospital Epidemiology **18:** 417-421.

Saei, H.D.; Ahmed, M.; Mardani, K. and Batavani, R.A. (2010): Genotyping of Staphylococcus aureus isolated from bovine mastitis based on PCR-RFLP analysis of the aroA gene. Comp. Clin. Pathol. **19:** 163-168.

Sanholm, M.; Hokanen-Buzalski, T.; Kaartinen, L. and Pyörälä, S. (1995): The bovine udder and mastitis. University of Helsiniki, Faculty of Veterinary Medicine.

Sasatsu, M.; Shibata, Y.; Noguchi, N. and Kono. M. (1992): High-level resistance to ethidium bromide and antiseptics in *Staphylococcus aureus*. FEMS Microbiology Letters **93** (2)**:** 109-113.

Sasatsu, M.; Shirai, Y.; Hase, M.; Noguchi, N.; Kono, M.; Behr, H.; Freney, J.; Schmidt, A.L; Oliver, S.P. and Fydenkevez, M.E. (1985): Germicidal persistence of teat dips by moditied excised teat procedure. J. Dairy Sci. **68:** 158.

Sauer, S.; Freiwald, A.; Maier, Th.; Kube, M.; Reinhardt, R.; Kostrzewa, M. and Geider, K. (2008): Classification and Identification of Bacteria by Mass Spectrometry and Computational Analysis. **3**(7)**:** 2843.

SCENIHR, (2009): Assessment of the Antibiotic Resistance Effects of Biocides. European Commission Health & Consumer Protection DG

Schwarz, S. and Chaslus-Dancla, E. (2001): Use of antimicrobials in veterinary medicine and mechanisms of resistance. Vet. Res. **32:** 201-225.

Schweizer, H.P. (2001): Triclosan: a widely used biocide and its link to antibiotics. FEMS Microbiology Letters **202:** 1–7.

Sears, P.M. and McCarthy, K.K. (2003): Management and treatment of Staphylococcal mastitis. Vet. Clin North Am. Food Anim. Pract. **19:** 171-185.

Seegers, H.; Fourichon, C. and Beaudeau, F. (2003): Production effects related to mastitis and mastitis economics in dairy cattle herds. Veterinary Research **34** (5)**:** 475-491.

Shi, D.; Hao, Y.; Zhang, A.; Wulan, B. and Fan, X. (2010): Antimicrobial Resistance of Staphylococcus Aureus Isolated From Bovine Mastitis in China. Transboundary and Emerging Diseases **57:** 221–224.

Sing, K.B. and Buxi, K.K. (1982): Studies on etiology, *in vitro* sensitivity and treatment of subclinical mastitis in milch animals. Indian Vet. J. **59:** 191-198.

Smith, T.H.; Fox, L.K. and Middleton, J.R. (1998): Outbreak of mastitis caused by one strain of Staphylococcus aureus in a closed dairy herd. J. Am. Vet. Med. Assoc. **212:** 553-556.

Sol, J. (2002): Cure of Staphylococcus aureus mastitis in Dutch dairy cows. Ph.D. Thesis. University of Utrecht.

Sol, J.; Sampimon, O.C.; Barkema, H.W. and Schukken, Y.H. (2000): Factors associated with cure after therapy of clinical mastitis caused by Staphylococcus aureus. J. Dairy Sci. **83:** 278-84.

Sordillo, L.M. and Streicher, K.L. (2002): Mammary gland immunity and mastitis susceptibility. J. Mammary Gland Biol. Neoplasia 7: 135–146.

Storm, D.R.; Rosenthal, K.S. and Swanson, P.E. (1977): Polymyxin and related peptide antibiotics. Annu. Rev. Biochem. **46:** 723–763.

Storz G. and Hengge-Aronis, R., (2000): Bacterial Stress Responses. Am. Soc. Microbiol., Washington, DC.

Suller, M.T. and Russell, A.D. (1999): Antibiotic and biocide resistance in methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococcus. Journal of Hospital Infection **43:** 281–291.

Suller, M.T. and Russell, A.D. (2000): Triclosan and antibiotic resistance in Staphylococcus aureus. Journal of Antimicrobial Chemotherapy **46:** 11–18.

Sutra, L. and Poutrel, B. (1990): Detection of capsular polysaccharide in milk of cows with intramammary infections caused by *Staphylococcus aureus*. Am. J. Vet. Res. **51:** 1857–1859.

Sutra, L. and Poutrel, B. (1994): Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. J. Med. Microbiol. **40:** 79-89.

SVARM (2002): Swedish Veterinary Antimicrobial Resistance Monitoring. Available at: http://www.sva.se/upload/pdf/Tjänster%20och%20produkter/Trycksaker/svarm2002.pdf.

Swinkels, J.M.; Hogeveen, H. and Zadoks, R.N. (2005): A partial budget model to estimate economic benefits of lactational treatment of subclinical *Staphylococcus aureus* mastitis. J. Dairy Sci. **88:** 4273-4287.

Szumala, A. and Pemak, J. (1986): The natural and laboratory resistance of gram-negative rods to quaternary ammonium chlorides with alkylthiomethyl radicals. Pharmazie **41:** 5221.

Talbot, B.G. and Lacasse, P. (2005): Progress in the development of mastitis vaccines. Livestock Production Science **98:** 101-113.

Taponen, S.; Jantunen, A.; Pyörälä, E. and Pyörälä, S. (2003): Efficacy of targeted 5-day combined parenteral and intramammary treatment of clinical mastitis caused by penicillin susceptible or penicillin-resistant *Staphylococcus aureus*. Acta Vet. Scand. **44:** 53-62.

Tattawasart, U.; Maillard, J.Y.; Furr , J.R. and others. (1999): Development of chlorhexidine and antibiotic resistance in Pseudomonas stutzeri. Proceedings of the 98th General Meeting of the American Society for Microbiology. p 533.

Tenhagen, B.A.; Köster, G.; Wallmann, J. and Heuwieser, W. (2006): Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. J. Dairy Sci. **89:** 2542-2551.

Thomas, L.; Russell, A.D. and Maillard, J-Y. (2005): Antimicrobial activity of chlorhexidine diacetate and benzalkonium chloride against Pseudomonas aeruginosa and its response to biocide residues. J. Appl. Microbiol. **98:** 533-43.

Trolldenier, H. (1996): Development of resistance in infectious agents of agricultural animals in Germany (1990–1994) – a review [in German], Dtsch. Tierärztl. Wochenschr. **103:** 256-260.

Trolldenier, H. (1999): Zur Resistenzproblematik in der Veterinaermedizin - Uebersicht aus bundesweit erfassten Daten. Tierarztl. Prax. **27** (G)**:** 317–323.

Ungemach, F.R. (1999): Einsatz von Antibiotika in der Veterinaermedizin: Konsequenzen und rationaler Umgang. Tierarztl. Prax. **27**(G)**:** 335–340.

Van Wely, K.H.; Swaving, J.; Freudl, R. and Driessen, A.J. (2001): Translocation of proteins across the cell envelope of Gram-positive bacteria. FEMS Microbial. Rev. **25:** 437-454.

Walhauser, K.H. (1984): Antimicrobial preservatives used by cosmetic industry. In: Kabara, J.J. (ed.): Cosmetic and drug preservation: principles and practice. Marcel Dekker, New York, 605-745.

Wang, J.T.; Sheng, W.H.; Wang, J.L.; Chen, D.; Chen, M.L.; Chen, Y.C. and Chang, S.C. (2008): Longitudinal analysis of chlorhexidine susceptibilities of nosocomial methicillin-resistant *Staphylococcus aureus* isolates at a teaching hospital in Taiwan. J. Antimicrob. Chemother. **62:** 514-7.

Watts, J.L. (1988): Etiology of bovine mastitis. Vet. Microbiology **16:** 41-66.

Watts J.L. and Salmon, S.A. (1997): Activity of selected antimicrobial agents against strains of *Staphylococcus aureus* isolated from bovine intramammary infections that produce Betalactamase. J. Dairy Sci. **80:** 788-791.

Webster, J.; Faoagali, J.L. and Cartwright, D. (1994): Elimination of methicillin-resistant *Staphylococcus aureus* from a neonatal intensive care unit after hand washing with triclosan. Journal of Paediatrics and Child Health **30:** 59–64.

Werckenthin, C.; Cardoso, M.; Martel, J.L. and Schwarz, S. (2001): Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. Vet. Res. **32:** 341-362.

Wesen, D.P. and Schultz, L.H. (1970): Effectiveness of a post-milking teat dips in preventing new udder infections. J. Dairy Sci. **53:** 1391.

Whist, A.C.; Østerås, O. and SØverØd, L. (2007): *Staphylococcus aureus* and *Streptococcus dysagalactiae* in Norwegian herds after introduction of selective dry cow therapy and teat dipping. J. Dairy Res. **74:** 1-8.

WHO (1997): Recommendations. Pages 11–16 in WHO-Proceedings: The medical impact of the use of antimicrobials in food animals. WHO/EMC/ZOO/97.4, WHO, Geneva, Switzerland.

Wille, B. (1976): Möglichkeiten einer Resistenzentwicklung von Mikroorganismen gegenüber Desinfektionsmitteln. Zentralbl. Bakteriol. [Orig B] **162:** 217−220.

Wilson, D.J.; Gonzales, R.N. and Das, H.H. (1997): Bovine mastitis pathogens in New York and Pennsylvania: Prevalence and effects on somatic cell count and milk production. J. Dairy Sci. **80:** 2592–2598.

Wilson, D.J.; Gonzales, R.N. and Sears, P.M. (1995): Segregation or use of separate milking units for cows infected with *Staphylococcus aureus*: effects on prevalence of infection and bulk tank somatic cell count. J. Dairy Sci. **78:** 2083-2085.

Windholz, M. (1976): Page 4903 *in* The Merck index. 9th ed. Merck & Co., Rahway, N.J.

Winicov, M. (1982): Diluted povidone - iodine kills bacteria faster. Hosp. Infect. Control. **9**(1)**:**1-3.

Yao, J. and Moellering, R.J. (2003): Antibacterial agents. 8th ed. Washington, DC: ASM Press, 1039 –1073.

Yilmaz, A. and Kaleta, E.F. (2009): Suitability of two commercial preparations for disinfections against methicillin-resistant *Staphylococcus aureus* in veterinary medicine. Tierärztliche Wochenschrift 116, Heft 5, 180–185.

Zafar, A.B.; Butler, R.C.; Reese, D.J.; Gaydos, L.A. and Mennonna, P.A. (1995): Use of 0.3% triclosan (Bacti-Stat) to eradicate an outbreak of methicillin-resistant *Staphylococcus aureus* in a neonatal nursery. American Journal of Infection Control **23:** 200–208.

Zhao, X., and Lacasse, P. (2008): Mammary tissue damage during bovine mastitis: Causes and control. J Anim. Sci. **86:** 57-65.

CHAPTER 9: APPENDIX

Table 5: The MIC of Eimü Chlorhexidin® dip and Ujosan® dip against *S. aureus* strains (dipped group) (a = first test; b = second test)

Eimü Chlorhexidin® dip			Ujosan® dip		
Strain no.	**a (%)**	**b (%)**	**Strain no.**	**a (%)**	**b (%)**
81	98	98	**123**	49	49
82	99	98	**127**	48	48
83	97	97	**133**	41	41
84	97	98	**134**	43	43
85	97	97	**135**	44	45
86	97	99	**136**	42	42
87	96	97	**138**	47	48
88	98	98	**139**	49	49
89	97	98	**142**	48	48
90	98	97	**143**	49	49
91	97	96	**146**	46	46
92	98	98	**147**	44	44
93	98	99	**153**	46	47
94	97	97	**154**	45	44
95	97	97	**155**	44	44
98	97	97	**156**	44	44
99	98	98	**157**	45	45
100	98	99			
101	97	96			
102	96	96			
103	97	97			
104	97	97			
105	97	96			
106	97	98			
107	96	96			
108	95	95			
198	98	98			
199	98	99			
200	96	97			

Appendix

Table 6: The MIC values of Ujosan® dip and Eimü Chlorhexidin® dip with sub-lethal concentration before, after 10th passage and 10th stable passage of 10 *S. aureus* strains (a = first test; b = second test)

Strain no.	Ujosan® dip						Eimü Chlorhexidin® dip					
	MIC% before passage		MIC% after 10th passage		MIC% after 10th stable passage		MIC% before passage		MIC% after 10th passage		MIC% after 10th stable passage	
	a	b	a	b	a	b	a	b	a	b	a	B
A	42	43	48	49	49	49	97	96	96	96	96	96
B	41	41	49	50	50	48	96	96	96	96	96	96
C	44	44	49	50	48	49	97	98	97	97	98	97
D	40	41	48	48	49	49	96	96	96	96	96	96
E	41	41	47	47	47	47	97	98	95	95	96	95
F	45	46	49	49	49	49	96	96	99	100	99	100
G	42	42	46	46	42	43	97	97	97	97	97	96
H	42	41	50	49	49	49	96	96	96	96	96	95
I	42	42	49	49	48	49	97	97	97	97	97	97
J	43	44	49	49	49	49	96	97	97	97	98	97

Table 7: Sensitivity of 70 *Staphylococcus* isolates isolated from subclinical bovine mastitis against 6 different types of antibiotics using agar disc diffusion test

Strain	T		PG		GM		OX		C		E	
	***S. aureus* strains**											
123	35	S	21	R	26	S	33	S	29	S	31	S
127	11	R	20	R	12	R	31	S	12	R	33	S
133	38	S	20	R	11	R	38	S	32	S	38	S
134	31	S	19	R	10	R	34	S	32	S	34	S
135	35	S	18	R	23	S	35	S	33	S	37	S
136	34	S	19	R	25	S	32	S	13	I	33	S
138	19	S	26	R	30	S	34	S	37	S	34	S
139	40	S	30	S	30	S	39	S	39	S	42	S
142	18	I	29	S	33	S	30	S	30	S	34	S
143	34	S	22	R	25	S	29	S	27	S	28	S
146	37	S	24	R	26	S	31	S	31	S	38	S
147	37	S	25	R	28	S	33	S	33	S	38	S
153	38	S	28	R	27	S	35	S	35	S	37	S
154	15	I	24	R	12	R	35	S	35	S	38	S
155	28	S	25	R	26	S	34	S	34	S	39	S
156	40	S	24	R	28	S	34	S	34	S	35	S
157	40	S	25	R	26	S	34	S	32	S	35	S
81	32	S	24	R	24	S	33	S	28	S	33	S
82	39	S	31	S	30	S	39	S	33	S	29	S
83	33	S	23	R	23	S	32	S	28	S	32	S
84	34	S	25	R	24	S	32	S	29	S	33	S
85	33	S	25	R	23	S	34	S	29	S	33	S
86	31	S	24	R	25	S	33	S	28	S	33	S
87	31	S	25	R	21	S	30	S	28	S	32	S
88	34	S	27	R	25	S	36	S	30	S	35	S
89	35	S	27	R	25	S	35	S	29	S	34	S
90	34	S	28	R	24	S	34	S	29	S	32	S
91	32	S	23	R	24	S	33	S	28	S	34	S
92	31	S	24	R	12	R	30	S	28	S	32	S
93	34	S	26	R	26	S	31	S	31	S	30	S
94	33	S	26	R	26	S	34	S	29	S	34	S
95	12	R	26	R	25	S	35	S	26	S	31	S
98	34	S	25	R	24	S	33	S	29	S	34	S
99	11	R	26	R	25	S	34	S	30	S	34	S
100	34	S	24	R	25	S	33	S	30	S	33	S
101	33	S	25	R	14	I	33	S	26	S	32	S

102	30	S	25	R	23	S	31	S	27	S	31	S
103	33	S	25	R	26	S	33	S	28	S	33	S
104	33	S	25	R	24	S	31	S	27	S	35	S
105	32	S	26	R	25	S	33	S	28	S	34	S
106	30	S	26	R	23	S	32	S	26	S	31	S
107	32	S	25	R	25	S	35	S	27	S	32	S
108	13	R	27	R	13	I	34	S	28	S	30	S
198	33	S	28	R	24	S	34	S	29	S	33	S
199	31	S	23	R	25	S	31	S	28	S	31	S
200	30	S	25	R	23	S	34	S	26	S	33	S
A	33	S	48	S	23	S	29	S	29	S	32	S
B	34	S	49	S	23	S	28	S	28	S	31	S
C	35	S	48	S	11	R	29	S	29	S	31	S
D	34	S	38	S	24	S	28	S	28	S	32	S
E	36	S	33	S	24	S	26	S	26	S	34	S
F	32	S	27	R	25	S	28	S	28	S	31	S
G	32	S	23	R	23	S	29	S	29	S	32	S
H	35	S	24	R	23	S	31	S	31	S	32	S
I	32	S	25	R	24	S	26	S	11	S	33	S
J	32	S	25	R	25	S	28	S	28	S	32	S
	CNS strains											
124	37	S	23	R	26	S	34	S	34	S	33	S
125	36	S	26	R	38	S	39	S	31	S	34	S
126	40	S	27	R	11	R	40	S	37	S	38	S
128	38	S	31	S	36	S	34	S	36	S	31	S
131	9	R	34	S	35	S	41	S	40	S	42	S
132	38	S	48	S	40	S	38	S	31	S	33	S
140	34	S	39	S	35	S	34	S	32	S	33	S
141	33	S	26	R	31	S	31	S	12	R	32	S
144	36	S	38	S	32	S	32	S	30	S	33	S
145	35	S	45	S	38	S	35	S	34	S	35	S
148	34	S	38	S	30	S	30	S	30	S	35	S
151	39	S	30	S	29	S	38	S	38	S	40	S
152	34	S	40	S	26	S	30	S	30	S	34	S
96	33	S	42	S	32	S	34	S	32	S	34	S

T = Tetracycline, **PG** = Penicillin **G**, **GM** = Gentamycin, **OX** = Oxacillin, **C** = Chloramphenicol, **E** = Erythromycin

R= Resistant

S= Susceptible

Appendix

Table 8: In vitro susceptibility percentage of 70 *Staphylococcus* species obtained from bovine subclinical mastitis to six selected antimicrobial agents (S = susceptible; I = intermediate; R = resistant)

Antibiotic	Disc content	Susceptibility of *S. aureus* (56 isolates) in %			Susceptibility of CNS (14 isolates) in %			Susceptibility of total isolates (70 isolates) in %		
		S	I	R	S	I	R	S	I	R
Penicillin G	10 units	14.29	0.00	85.71	71.43	0.00	28.57	25 .72	0.00	74.28
Tetracycline	30 µg	89.28	3.57	7.14	92.86	0.00	7.14	90.00	2.86	7.14
Gentamycin	10 µg	85.72	3.57	10.71	92.86	0.00	7.14	87.14	2.85	10.00
Oxacillin	5 µg	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00
Erythromycin	15 µg	100.00	0.00	0.00	100.00	0.00	0.00	100.00	0.00	0.00
Chloramphenicol	30 µg	96.44	1.78	1.78	92.86	0.00	7.14	95.71	1.42	2.85

Table 9: MICs (mg/l) of parent and adopted strains of *S. aureus* to 19 antimicrobial agents that are commercially available for the treatment of bovine S. aureus mastitis

Strain No.	CHL	CIP	CLI	ERY	FOX	FUS	GEN	KAN	LZD	MUP	PEN	RIF	SMX	STR	SYN	TET	TIA	TMP	VAN
A	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	2
Ap10	8	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	1	1	<=2	2
Asp10	8	0.5	<=0.12	<=0.25	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
B	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
Bp10	8	0.5	<=0.12	0.5	2	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	4	2
Bsp10	16	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	128	8	<=0.5	<=0.5	1	<=2	<=1
C	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	<=1
Cp10	8	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
Csp10	8	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	2
D	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
Dp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	2	<=2	<=1
Dsp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	2
E	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
Ep10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	<=1
Esp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	<=1
F	8	**2**	<=0.12	0.5	**>16**	**>4**	<=1	<=4	2	**256**	**2**	**>0.5**	**256**	16	**2**	1	**4**	**>32**	<=1
Fp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	**1**	<=0.016	128	<=4	<=0.5	1	1	<=2	<=1

Fsp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	**0.5**	0.03	128	8	<=0.5	1	2	<=2	<=1
H	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	**>2**	<=0.016	**>512**	8	<=0.5	<=0.5	1	4	<=1
Hp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	**0.5**	**0.03**	<=64	16	<=0.5	<=0.5	1	4	<=1
Hsp10	<=4	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	<=1	<=0.5	**0.25**	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	2
I	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	**1**	<=0.016	128	8	<=0.5	<=0.5	1	<=2	<=1
Ip10	8	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	<=1
Isp10	<=4	<=0.25	<=0.12	<=0.25	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	2
J	8	0.5	<=0.12	0.5	2	<=0.5	<=1	<=4	2	<=0.5	**1**	<=0.016	128	8	<=0.5	<=0.5	1	<=2	<=1
Jp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	2
Jsp10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	8	<=0.5	<=0.5	<=0.5	<=2	2
Fchx p10	8	0.5	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	1	<=0.016	128	<=4	<=0.5	1	1	<=2	<=1
Fchxsp10	8	<=0.25	<=0.12	0.5	4	<=0.5	<=1	<=4	2	<=0.5	0.5	<=0.016	<=64	8	<=0.5	<=0.5	1	<=2	<=1
ATCC25923	16	0.5	<=0.12	1	4	<=0.5	<=1	8	4	<=0.5	**0.5**	<=0.016	<=64	8	<=0.5	1	1	<=2	<=1
DSM 799	<=4	<=0.25	<=0.12	<=0.25	4	<=0.5	<=1	<=4	<=1	<=0.5	<=0.12	<=0.016	<=64	<=4	<=0.5	<=0.5	<=0.5	<=2	<=1

CHL = Chloramphenicol, **CIP** = Ciprofloxacin, **CLI** = Clindamycin, **ERY** = Erythromycin, **FOX** = Cifoxtin, **FUS** = Fusidic acid, **GEN** = Gentamycin, **KAN** = Kanamycin, **LZD** = Linezolid, **MUP** = Mupirocin, **PEN** = Benzyl penicillin, **RIF** = Rifampicin, **SMX** = Sulphamethoxozole, **STR** = Streptomycin, **SYN** = Synercid, **TET** = Tetracycline, **TIA** = Thiamulin, **TMP** = Trimethoprim, **VAN** = Vancomycin

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Selbständigkeitserklärung zur Dissertation

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt und nur die erwähnten Quellen und Hilfen verwendet habe.
Die Arbeit ist erstmalig und nur an der Freien Universität Berlin eingereicht worden

Ayman El Behiry