STUDY OF PATHOGENIC FACTORS OF *STAPHYLOCOCCUS AUREUS* FROM CLINICAL CASES OF LIVESTOCK AND POULTRY

*A **THESIS***

submitted by

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for the award of the degree

of

DOCTOR OF PHILOSOPHY

Under the Guidance of

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July 2017

Dedicated

to

my family members...

DECLARATION

I, Mohana Sheela Ganugula, hereby declare that I have personally carried out the work presented in the thesis entitled **"Study of pathogenic factors of *Staphylococcus aureus* from clinical cases of livestock and poultry"** under the guidance and supervision of Dr. S. Krupanidhi Sri Rama, Department of Biotechnology, Vignan's University, Guntur, and Andhra Pradesh. The results embodied in this thesis have not been submitted to any other University or Institute for any other degree or diploma.

Mohana Sheela Ganugula

THESIS CERTIFICATE

This is to certify that the thesis entitled STUDY OF PATHOGENIC FACTORS OF *STAPHYLOCOCCUS AUREUS* FROM CLINICAL CASES OF LIVESTOCK AND POULTRY submitted by GANUGULA MOHANA SHEELA to Vignan's Foundation for Science, Technology and Research University, Vadlamudi, Guntur, Andhra Pradesh, India, for the award of the degree of **Doctor of Philosophy** is a bonafide record of the research work done by her under our supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma.

Place : Guntur

Date : 13th June 2017.

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ABSTRACT

Staphylococcus aureus is a common dairy and poultry pathogen causing mastitis in dairy animals and septic arthritis, subdermal abscesses and gangrenous dermatitis (GD) in poultry. The infected buffaloes and birds acts as reservoirs for variety of diseases in humans. Therefore in the present study we have undertaken isolation, characterization and screening of virulence determinants of *S. aureus* from mastitis in buffaloes and GD in poultry farms in coastal Andhra Pradesh, India. Following isolation, biochemical tests such as Gram's staining, catalase, oxidase, Voges-Proskauer tests and haemolysis on sheep blood agar were carried out. Presumptive isolates were further screened for the presence of species specific gene *staur* 4 and *staur* 6 by PCR. 52 species specific confirmed *S. aureus* isolated from 60 GD samples and 34 species specific confirmed *S. aureus* from 100 mastitic milk samples were screened for the presence of enterotoxin genes. The percentage occurrence of each Staphylococcal Enterotoxins (SEs) *viz.* staphylococcal enterotoxin E (*see*) 3.84 %, enterotoxin G (*seg*) 1.92 %, enterotoxin I (*sei*) 3.85 % and enterotoxin H (*seh*) 0 % and one isolate (1.92 %) harboured both *seg* and *sei* in poultry. Whereas the percentage incidence of SEs in mastitic milk samples was found to be staphylococcal enterotoxin E (*see*) 5.88 %, enterotoxin G (*seg*) 17.64 %, and enterotoxin I (*sei*) 23.52 % and enterotoxin H (*seh*) 17.64 % and four isolates (11.76 %) harboured *seg, seh* and *sei.* In poultry, 24 (46.15 %) species confirmed *S. aureus* isolates and six (17.64 %) in bovine were positive for *IcaD* gene which aids in biofilm production and the same was further confirmed by culturing on Congo red agar (CRA). In poultry, antimicrobial resistance of isolated species confirmed *S. aureus* strains, high resistance against vancomycin (84.62 %), amoxycillin (80.77 %), ceftriaxone + sulbactam (73.08 %), neomycin (84.62 %), enrofloxacin (69.23 %), gatifloxacin (98.08 %) and cefoxitin (90.38 %), moderate resistance to linezolid (48.08 %) and least resistance against amoxycillin + clavulanic acid (30.77 %) were observed. In bovine, the resistance pattern for antibiotics of isolated species confirmed *S. aureus* strains was found to be high resistance was observed against oxacillin (100 %), ceftriaxone + tazobactum (77.42 %), ampicillin (70.97 %), amoxycillin (67.74 %), ceftriaxone + sulbactam (61.29 %), methicillin (48.39 %), gatifloxacin (41.94 %), penicillin (38.71 %), gentamicin (38.71 %), enrofloxacin (29.03 %), neomycin (25.81 %), Streptomycin (12.90 %), linezolid (9.68 %) and ceftriaxone and cefoxitin (9.68

%) and amoxycillin + clavulanic acid (0.00 %) were observed. It was shown that 57.69 %, 40.38 % and 16 % isolates were found positive for *blaZ*, *mecA* and *blaZ+mecA* genes in poultry and in dairy, 41.18 %, 55.88 % and 20.58 % respectively. This study reports the existence of multi drug resistant virulent *S. aureus* strains from GD infected chicken and also in buffaloes infected with mastitis, thus warranting regular monitoring to avoid zoonosis.

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INTRODUCTION

CHAPTER - 1 INTRODUCTION

Agriculture and animal husbandry are the backbones of Indian economy. The Andhra Pradesh state stands 2nd position in egg production (1417.67 crores), 4th position in meat production (5.66 lakh metric tons) and 5th position in milk production (108.17 lakh metric tons) as per the estimates of GoI during the year 2015-16.

This rural based economic sector is being inherently suffered with several setbacks including bacterial invasions. The present employed control measures look to be compromised due to ignorance in handling the situation regarding prevention and control of the spread of particularly endemic and emerging *S. aureus* strains.

Staphylococcus aureus is a gram positive cocci bacterium which can cause broad range of illness in humans and animals. It multiplies in mucous membranes, skin glands and skin. It also causes varied infections such as inflammations of bones, meninges, rashes and septicaemia (Aklilu et al, 2010). The stimulation of intramammary infection involves the production of a variety of pathogenic factors enabling colonisation, adherence and invasion of the mammary cells of the bovine host by the *S. aureus*. Different *S. aureus* strains seem to pocesses pathogenic factors in increasing manner.

Several vital factors like the use of antimicrobials, supervision of the herd and poultry farms and individual characteristics of the host produce continuous selective pressure on *S. aureus* strains. In spite of this pressure, genetic material swap over between the strains and these strains are likely to be better adapted to survive and becomes more pathogenic. During this evolutionary process, *S. aureus* strains that are likely to be more resistant to antibiotics become more virulent and may likely best fit for survival. Detection of these resistant *S. aureus* strains is achievable using the techniques of molecular microbiology. It also causes food poisoning, Staphylococcal Scaled Skin Syndrome (SSSS), post-operative wound infections, inflammation of lungs (pneumonia), and nosocomial bacteremia in humans, mastitis in bovine and bumble foot disease in poultry (Quinn et al, 2000 and Smyth, 2001).

Mastitis in milch animals and Gangrenous dermatitis (GD) in broiler chickens is accountable for considerable financial losses for the global dairy and poultry industries (Sasidhar et al, 2002 and Li et al, 2010). *S. aureus* is a major causative agent in mastitis

and dermatitis. Extensive and unplanned use of antibiotics in human and veterinary medicine is the key reason for emerging of resistant strains of *S. aureus* (Grema et al, 2015). The generally used antibiotics for the cure of infections caused by *S. aureus* were β-lactam antibiotics including methicillin (Rayner and Munckhof, 2005). However, certain strains of *S. aureus* have developed resistance to methicillin by activation of a protein called PBP2a (Penicillin Binding Protein) which results Methicillin Resistant *Staphylococcus aureus* (MRSA). The MRSA can be classified into Livestock Associated MRSA (LA-MRSA), Community Acquired MRSA (CA-MRSA) and Hospital Acquired MRSA (HA-MRSA) (Grema et al, 2015). The infections caused by MRSA have gained importance as the cure options for these strains are inadequate and these strains have high zoonotic potentials to humans. It is observed that there are increased cases of these strains isolation in domestic animals (Devriese et al, 1975; Hartmann et al, 1997; Tomlin et al, 1999; Lee, 2003; Goni et al, 2004; Rich and Roberts, 2004). There can be cross infection of these strains in humans and animals (Strommenger et al, 2006 and Weese et al, 2006). Thus the human beings acquire MRSA strains due to contact with animals infected by these strains (Verkade and Kluytmans, 2014). The MRSA have been evolved as MDR (Multi Drug Resistant), XDR (Extensively Drug Resistant) and PDR (Pan Drug Resistant) (Dardi Charan Kaur and Sadhana Sanjay Chate, 2015). The sad fact all the time we know that MRSA will not respond to first line of antibiotics as an evidence for the law that the simple bacterial genomes evolve faster (David and Daum, 2010). In the heath domain MRSA strains are labelled as "Superbug" (Debasmitha, 2013). Hence, winning approaches to fight *S. aureus* are the need of the hour (Vincze et al, 2014).

In the current circumstances, it is a crucial job to study the incidence of *S. aureus* in diseases related to livestock and poultry. In India, losses due to mastitis in dairy animals are estimated to be about Rs. 6053.21 crores per annum (Dua, 2001). The onslaught of diseases likes mastitis, not only in India but globally, leads to enormous losses to the dairy sector. As a consequence, this big economic sector is suffering several setbacks including bacterial pathogens, requiring scientific interventions urgently needed to curb the menace of such emerging diseases of livestock in the country. One of such disease is mastitis, which have an adverse impact on quality of milk and its wastage. In the World, 158 million water buffaloes are present out of which 153 million buffaloes are in Asia (Anand et al, 2012). Subclinical mastitis is common in India, varying from 10–50 % in cows and 5–20 % in buffaloes than the clinical mastitis (1–10 %) (Joshi and Gokhale, 2006). *Staphylococcus aureus* is most recognized causative agent in sub clinical and

clinical intra mammary infections like mastitis in milch animals. The Indian dairy industry is facing economic losses by mastitis due to its adverse impact on milk production and milk quality of cows and buffaloes (Joshi and Gokhale, 2006, Sasidhar et al, 2002). *S. aureus* presume to be significant as it is accountable for clinical and subclinical mastitis and infection extends in the herd (Anand Kumar, 2009 and Rambabu, 2013). In India, milk production as emerged as 2nd best commodity. The National Commission on Agriculture had placed dairy after agriculture. Hence the dairy industry can act like cushion against crop failures (Guha and Gera, 2012b).

The overview of the mastitis include the buffalo mammary gland are exposed to various pathogenic bacteria during the lactating and non-lactating time. These pathogens are commonly isolated from infected milk by mastitis and are of two types viz., environmental (non contagious) and non environmental (contagious) microorganisms. The non-contagious microorganisms include *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus* species. These bacteria enter the mammary gland through teat canal and establish infection by damaging the mammary epithelial cells resulting in tissue necrosis. As a result, the extracellular fluids enter into the mammary gland and the same released into the milk. Therefore, visible changes occur in udder and milk. The changes include swelling of the udder, reddening of the mammary gland and milk changes to wateriness and clotting can be seen in mastitic milk (Zhao and Lacasse, 2008).

Maintenance of quality milk production by dairy industry in developing countries like India is a major problem and it is due to lack of efficient hygienic techniques for milking, storing of milk and maintaining herd conditions. And this is the reason in many parts a payment based system is in progress based on the quality of the milk. Hence, there is need to prevent and diagnose the mastitis at field level for each dairy holder for the acquisition of good quality milk and reduce the economic losses (Atasever and Erdem, 2010).

In poultry, a disease can be caused due to the interaction between three major factors called host, agents and environment. The factor host include its breed, age, sex and its immune system, agents include viruses, bacteria, parasites and fungi and last factor environment include the feed, air and water quality, sanitation, vaccination and weather. The general signs of diseased birds include poor appetite, depression, huddling, stunting, runting, poor uniformity, ruffled feathers, coughing, sneezing, and occulo-nasal discharge, problem in breathing, blood litter and increased mortality.

Chugh (2007) named *S. aureus* as "modern Ghengis Khan" as it was considered as most disturbing human pathogen. *S. aureus* produces a wide spectrum of surface components (proteins and capsular polysaccharides), exotoxins. It includes alpha, beta, gamma, delta, leukocidin, enterotoxins, exfoliative toxins and tissue toxic syndrome toxin-1. The most toxic toxin among these is alpha toxin as it harms the intramammary tissues causing vasoconstriction, which leads to localized ischemia and cell necrosis whereas beta and gamma toxins may act as tissue irritants. In mammary explants, alpha toxins, beta toxins and leukocidin causes cell damage and decreased secretory activity (Christopher B. Norman, 2004).

Staphylococcal enterotoxins (SEs) are protein exotoxins identified in 1959. They differ in respect of their amino-acid homology, nucleotide, genes location, molecular weight and iso electric point value. The group of enterotoxins also contains pyrogenic toxins, toxic-shock syndrome toxin (TSST-1), exfoliative toxin A (ETA), exfoliative toxin B (ETB) and Streptococcal Scarlet fever toxin. SEs are resistant to heat and proteolytic enzymes like trypsine, pepsin, chymotrypsine, papain and renin, but this resistance depends on the temperature and pH. These *Staphylococcal* enterotoxin-encoding genes are located on the chromosomal DNA, on pathogenicity islands in phages, transposones and plasmids. In humans, Staphylococcal enterotoxins are accountable for food poisoning. These enterotoxins can be isolated from milk samples infected with mastitis (Nawrotek et al, 2005).

The other important virulence factor of *S. aureus* is its ability to form biofilm *in vivo* which greatly influence its pathogenicity (Vasudevan et al, 2003). The first report on microbial biofilm was reported by Antonie van Leeuwenhoek in the year 1600s (Sivan Elias and Ehud Banin, 2012). Generally the two main interactions in Biofilm is a group of microbial cells connected with surface and roofed in an extracellular matrix primarily made up of materials like polysaccharide (Donlan, 2002). Different microorganisms can form different species biofilms. Generally two main interactions occur between different species in a biofilm which include communication through quorum sensing and cooperation/competition for metabolic activities (Sivan Elias and Ehud Banin, 2012). The biofilm producing bacteria exhibit high resistance to disinfectants, antibiotics and host immune system clearance (Donlan, 2002 and Donlan et al, 2002).

The significance of biofilm is well recognized in medical, environmental, and industrial contexts. Several severe infections are reported to be a result of biofilm formation which

leads to chronic diseases in most cases. These infections are persistent and lead to global public health concern. As a result they reduce the effectiveness of treatments and increase morbidity, mortality and health care cost.

Rich evidence is accessible that pathogenicity of *S. aureus* strains varies. Despite the basic and widely used measures to control and prevent *S. aureus* infections, this microorganism can be a source of herd-wide and farm-wide outbreaks and continue for longer periods. It seems liable that the presently working control measures are inadequate regarding prevention and control of the spread of endemic and emerging *S. aureus* strains. Hence, successful approaches to fight *S. aureus* infections are need of the hour. The strategies have to be initiated, coordinated and implemented with combined and integrated efforts of the researchers, medical and the veterinary professionals (Vincze et al, 2014).

In animal husbandry sector an infection can be a single or multiple microbial entities. The data on the extent of prevalence produced by a single organism will determine the pathogenicity, morbidity and mortality induced by that organism. In such cases, the assessment of prevalence and epizootology plays a vital role in disease diagnosis. In the rural environment, where the human beings and livestock coexist in close proximity the affections of these in-contact animals and birds will be a threat to the farmers work efficiency, economic equilibrium and product output. Hence, the identification of etiological organisms in the infection and infestations are very much significant.

The livestock population in Andhra Pradesh include 112.06 lakhs and poultry 817.84 lakhs, out of which East Godavari, West Godavari, Krishna and Guntur districts are major contributors. Milk production in Andhra Pradesh is 90.83 lakh tonnes and egg production is 127269 lakh tonnes out of which East Godavari, West Godavari, Krishna and Guntur districts are major contributors. Therefore these four districts were chosen in the present study to evaluate the pathogenicity of emerging strains of *S. aureus* which would be a helping guide for prophylactic measures to be initiated by veterinarians.

Various infections prevent in animal husbandry sector, entails discriminate use of antibiotics to save the lives of animals and to reduce economic loss to the farmer. Indiscriminate use of antibiotics may lead to reduced responsiveness to the antibiotics by acquisition of resistance which may turn out to a fatal outcome for not only animals but

for humans also. Hence, collecting the data on antimicrobial resistance in a community which infects human beings as well as animals is of paramount importance.

The information in this thesis will be useful for planning more proficient strategies to control and prevent *S. aureus* infections both in bovine and poultry. Hence, present investigation is proposed with the following objectives:

To evaluate incidence of *S. aureus* in the clinical isolates of various disease conditions of bovine and poultry with special emphasis on mastitis in bovines and dermatitis in poultry.

To investigate the antibiotic resistance pattern by

a. ABST against commonly employed antibiotics.

b. Minimum inhibitory concentrations of certain selected antibiotics.

c. Presence of β-lactam resistance genes like *blaZ* and methicillin resistance genes like *mecA.*

To study the pathogenicity of *S. aureus*-

a. Molecular detection of enterotoxin genes producing *S. aureus.*

b. Phenotypic, genotypic and Microtitre Plate Assay (MTP) detection of biofilm producing *S. aureus.*

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2 REVIEW OF LITERATURE

2.1 CHARACTERISTICS OF *STAPHYLOCOCCUS AUREUS*

The genus *Staphylococcus* comprises of different species which have been classified and differentiated on the basis of a variety of phenotypic characteristics such as morphology, and biochem*ica*l reactions. Pigment was the initial criterion used to classify staphylococcal species, and in 1885, Rosenbach recognized members of the genus *Staphylococcus* based on the colour of colonies. *Staphylococci* forming orange-yellow colonies were named *S. aureus* (or *S. pyogenesaureus*) by Rosenbach, while *staphylococci* forming white colonies were named *S. albus* (or *S. pyogenes albus*) (Kloos, 1980).

Another characteristic feature which was described for differentiation between *staphylococci* was the coagulase test which involves the investigation of the ability of *S. aureus* to clot blood plasma (Kloos, 1980) which paved way for the separation of *Staphylococci* into two main groups Coagulase positive *S. aureus* (CPS) and Coagulase negative *S. aureus* (CNS).

Based on different studies carried by different researchers, at present the genus *Staphylococcus* comprises of 37 species and 17 subspecies (Baird-Parker, 1963; Devriese et al, 1985; Kloos et al, 1976; Kloos and Schleifer, 1975a; Schleifer and Kloos, 1975 and Bannerman and Peacock, 2007).

2.2 MORPHOLOGY AND IDENTIFICATION

S. aureus is a gram-positive, catalase-positive, usually oxidase-negative, facultative anaerobic coccus, which belongs to the family of Micrococcaceae and the group of *Staphylococci*. Different phenotypic methods are been proposed to identify *S. aureus* isolates from humans and animals from other species of *Staphylococcus*. These methods include anaerobic fermentation of mannitol, production of coagulase, production of heat stable thermonuclease and production of acetoin from glucose (Roberson et al, 1992; Raus and Love, 1983; Devriese, 1981).

2.3 VIRULENCE FACTORS IN *S. AUREUS*

The genome of *S. aureus* is circular and 2800 kilo base pair size chromosome, prophages, one or more plasmids, transposones, insertion sequences and other incompletely characterized accessory genetic elements (Projan and Novick, 1997).

The conserved portion of the *S. aureus* core genome includes the house keeping genes necessary for cell growth and division. The define DNA fragments in *S. aureus* chromosome which can repl*ica*te on their own are insertion sequences, bacteriophages, pathogenicity islands and staphylococcal cassette chromosomes. The transfer of these elements in and out of *S.aureus* is responsible for the pathogenicity of the strain, since many of these elements encode virulence or antimicrobial resistance genes.

2.4 IMPORTANCE OF *S. AUREUS*

Staphylococcal enterotoxins can cause skin, heart valve, blood and bone infections, which can lead to septic shock and death. These enterotoxins also cause food poisoning (Saiyers and Whitt, 2002) and the superantigens if present in the blood stream can cause toxic shock syndrome (Waldvogel, 2000). Nosocomial (hospital- acquired) infections are generally caused by Staphylococcal related infections.

S. aureus is one of the most common pathogens which cause mastitis in ruminants and gangrenous dermatitis in poultry (Sasidhar et al, 2002 and Li et al, 2010). *S. aureus* is also one of the main causative agents in gangrenous dermatitis in poultry (Sheela and Krupanidhi, 2015).

2.5 SIGNIFICANCE IN ANIMAL HEALTH

In India, losses due to mastitis in dairy animals are estimated to be about Rs. 6053.21 crore per annum (Dua, K., 2001). Approximately 8 % to the GDP of Indian economy is contributed by dairy industry. But the onslaught of diseases likes mastitis, not only in India but globally, leads to enormous losses to the dairy sector. As a consequence, this big economic sector is suffering several setbacks including bacterial pathogens, requiring scientific interventions urgently needed to curb the menace of such emerging diseases of livestock in the country. One of such disease is mastitis, causing serious wastage and undesirable milk quality in dairy development of tropics. Subclinical mastitis is common

in India, varying from 10–50 % in cows and 5–20 % in buffaloes than the clinical mastitis (1–10 %) (Joshi and Gokhale, 2006).

Staphylococcus aureus is one of the most important causative agents in sub clinical and clinical intra mammary infections like mastitis in milch animals. The Indian dairy industry is facing economic losses by mastitis due to its adverse impact on milk production of cows and buffaloes (Joshi, S., and Gokhale, S., 2006 and Sasidhar, Reddy and Sudhakar, 2002). The poultry industry is being affected by various infectious diseases in chicken flocks like septic arthritis, sub dermal abscesses (i.e., bumble foot), and gangrenous dermatitis (Smyth and McNamee, 2008).*S. aureus* also causes tenosynovitis, yolk sac infection, spondylitis, osteomyelitis, staphylococcal sept*ica*emia, endocarditis, granuloma and in 1970s a new form *S. aureus* infection was described in broiler poultry known as bacterial chondronecrosis with osteomyelitis (BCO) (Nairn and Watson, 1972).

Gangrenous dermatitis (GD) in commercial broiler chicken is responsible for substantial economic losses for the global poultry industry (Li et al, 2010).The main causative bacteria involved in GD are *Clostridium septicum*, *Clostridium perfringens, Staphylococcus aureus* and *E. Coli* (Ritter, 2006 and Sheela et al, 2013).GD is a bacterial disease in poultry affecting the skin and the subcutaneous tissues of the abdomen. Most affected areas of the skin are the abdomen, head, thigh and wing (Ritter, 2006). In recent years, for the poultry industry gangrenous dermatitis has consistently ranked as a top priority disease according to the U.S. animal health association's committee on transmissible diseases of poultry and other avian species (Mataragas et al., 2008).

2.6 SIGNIFICANCE IN PUBLIC HEALTH

The ability of *S. aureus* to develop or acquire strategies which provide resistance to different antimicrobials is an additional approach in the impressive arsenal of this pathogen. The development of resistance among different bacterial strains is because of misuse or intensive use of antibiotics for treatments (Lingaas, 1998).

Emergence of MRSA (Methicillin Resistant *S. aureus*) and VRSA (Vancomycin Resistant *S. aureus*) have been reported in livestock in past (Hartmann et al, 1997, Tomlin et al, 1999, Lee, 2003, Goni et al, 2004, Rich, and Roberts, 2004, Goldrick, 2002, Guzman-Blanco et al, 2009 and Tiwari and Sen, 2006).

There are three different types of MRSA namely Hospital Acquired MRSA (HA-MRSA), Community Acquired MRSA (CA-MRSA) and Livestock Associated MRSA (LA-

MRSA) (Grema et al, 2015). As the treatment options for this highly zoonotic MRSA are limited, these infections have gained importance. There is a drastic rise in number of reports of MRSA in domestic dairy animals (Hartmann et al, 1997, Tomlin et al, 1999, Lee, 2003, Goni et al, 2004, Rich and Roberts, 2004). Cross-infection of certain strains of MRSA between humans and animals, were also reported (Strommenger et al, 2006).

It was reported in several parts of the world that MRSA causes life threatening sepsis, endocarditis and osteomyelitis in human beings. Animals can thus act as potential source of MRSA infection to in-contact human beings (Verkade and Kluytmans, 2014).Thus the development of multidrug resistance in MRSA possesses a serious public health concern too.

2.7 DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN MICROORGANISMS

In recent time, antibacterial resistance in bacteria from animal origin and its effect on human health drawn much attention worldwide (Aarestrup, 2006).Antibiotic resistant pathogens constitute an important and growing threat to various living organisms. Among varied antimicrobial drugs, β-lactam antibiotics are currently used in veterinary medicine and thus provide opportunity for selection pressure in development of β-lactam resistance (Li et al, 2007).

2.8 DEVELOPMENT OF ANTIBIOTIC RESISTANCE IN *S. AUREUS*

The indiscriminate use of antibiotics can lead to development of resistant strains and result in the increase in the cost of treatment. The production of β–lactamases encoded by the structural *blaZ* gene and by the production of an altered form of penicillin binding protein 2A (PBP-2A) which is encoded by the *mecA* gene is responsible for β-lactam resistance in *Staphylococci* (Fuda et al, 2005).

Recently, another form of MRSA i.e, *mecC* MRSA recognized which encodes a divergent *mec* gene which can colonize and cause disease in humans and a varied range of other host species. Although reports of mecC MRSA are currently rare, they present a potential diagnostic problem where there is reliance on mecA or PBP2a/2′ detection for MRSA diagnosis, and their emergence raises a several questions for future research (Paterson et al, 2014).

2.9 EMERGENCE OF METHICILLIN-RESISTANT *S. AUREUS*

Extensive and inadvertent use of antimicrobials both in human and in veterinary medicine is the key reason for emergence of resistant strains of *S. aureus* (Rabelloet al, 2005). MRSA affects both human and animal populations (Weese, 2010).

2.10 THE RISE OF MRSA

MRSA isolates in livestock has attained particular attention during recent years (Wulf and Voss, 2008). MRSA was reported in both healthy and sick chicken (Lee, 2003 and Lee, 2006). The control of *S. aureus* infections in livestock and poultry is attained by reliable and rapid identif*ica*tion of *S. aureus* colonies from different clinical samples.

The treatment of bovine mastitis cases potentially becoming compl*ica*te due to the presence of MRSA in mastitis milk samples (Vanderhaeghen et al, 2010). There is a potential risk to the cattle, farm workers and the veterinarians who are exposed to MRSA in bovine mastitis cases (Juhász-Kaszanyitzky et al, 2007).

The concern of the appearance and transfer of antimicrobial resistant bacteria or genetic determinants from animals to humans via food chain is increasing (Piddock,1997). India is lacking comprehensive information on the prevalence of antimicrobial resistant in bovine mastitis pathogens in milk.

2.11 IMPORTANCE AND EPIDEMIOLOGY OF MRSA

S. aureus is one of the important pathogens which cause mastitis in cattle. The widespread usage of intramammary antibiotics in cattle which lead to the first isolation of MRSA from animals in milk from mastitic cows (Devriese et al, 1972, Devriese and Hommez, 1975).The methicillin resistant *S. aureus* (MRSA) and multidrug-resistant *S. aureus* (MDRSA) can be exchanged between humans and animals (Price et al, 2012 and Harrison et al, 2013).

The individuals who are in-contact with livestock and poultry associated MRSA can act as nasal carriers which have been observed in Europe, the USA and in Canada (Maya et al, 2015). In a study conducted by Asfour and Darwish (2011) in Egypt, it was found that most of the MDRSA were MRSA.

2.12 IMPORTANCE AND EPIDEMIOLOGY OF VRSA

The first strain of *S. aureus* with reduced susceptibility to vancomycin and teicoplanin was reported from Japan in 1997(Hiramatsu et al, 1997). Shortly after, two additional

cases were reported from United States (Morb Mortal, 1997). However, first clinical isolate of vancomycin resistant *S. aureus* (VRSA) was reported from United States in 2002 (Morb Mortal, 1997).

More recently some workers have reported vancomycin resistant staphylococcal stains from Brazil (Palazzo, 2005 and Bataineh, 2006).Some strains of vancomycin intermediate *S. aureus* (VISA) were reported from India by Ashdulla and his group in 2003. Another group Song et al have also been reported the emergence of heterogeneous VRSA from India and its neighbouring countries (Song et al, 2004).

The current existence of alarming situation to the clinicians is caused by the VRSA in community and in hospitals. The irrational usage of antibiotics is due to its easy availability at the drug store without prescription, injudicious use in hospitals and unrestrained use in agriculture, animal husbandry and fisheries paved way for the development of antibiotic resistance in developing countries like India (Holloway, 2000).

Vancomycin is an antibiotic which falls under the class of glycopeptides which is mainly used to treat life-threatening infections with MRSA. Generally vancomycin resistance is uncommon among gram-positive bacteria but there are few confirmed reports of vancomycin resistance in *S. aureus*, CoNS and *Enterococcus* spp from different parts of the world. The infections caused by MRSA and by other gram-positive are widely treated by vancomycin which has led to the emergence of vancomycin resistance.

2.13 TRANSFER OF MRSA FROM ANIMALS TO MAN

A wide range of infections were caused by MRSA including bacteremia, endocarditis, pneumonia, skin and soft tissue infections. Generally MRSA infections are mainly classified as community acquired MRSA, healthcare associated MRSA and livestock-associated MRSA (Chambers and Deleo, 2009, Fitzgerald, 2012 and Harrison et al, 2013). The zoonotic transmission of *S. aureus* between livestock, companion animals and humans (Loeffler et al, 2011, Lowder et al, 2009, Pantosti, 2012) has been illustrated by the emergence of MRSA ST398 (Price et al, 2012).

To conclude transfer of MRSA from animals to man pose a potential public health concern. The emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is a major public health concern. Recently, MRSA strains with a novel mecA homologue (mecC), which may go undetected by current diagnostic tests, were described in both livestock and humans suggesting potential zoonotic transmission.

Denmark has reported a significant increase in cases of CC130 mecC-MRSA between 2003 and 2011 and two independent human cases of mecC-MRSA infection directly linked to a livestock reservoir have been identified**.**

Despite host adaptation and specialisation of *S.aureus*, some clones of it seem to lack specific host tropism and can simply be transmitted from animals to humans and vice-versa. This gives rise to the question of whether *S. aureus* diseases should be considered as zoonosis or humanoses; nevertheless, this means that *S. aureus* constitutes a hypothesis for studies in the framework of the One Health concept, involving mutual cooperation between experts in human, animal and public health sciences (Peton and Le Loir, 2014**).**

2.14 LABORATORY DIAGNOSIS FOR MRSA

The development of resistance and consequent spread of resistance to vancomycin in *S. aureus* has been supposed to be a fearsome threat to the already challenging therapy of MRSA (Harrison et al, 2013).

2.15 CONVENTIONAL BIOCHEMICAL TESTS AND SELECTIVE MEDIA

Generally Mannitol Salt Agar (MSA) is used in the clinical laboratory to differentiate pathogenic *Staphylococcus* spp. from the non pathogenic Micrococcus spp. The growing bacteria are able to ferment mannitol and produce an acid pH giving rise to yellow colour colonies on the surface of MSA medium (Kateete et al, 2010). The most important biochemical test is coagulase, which differentiate pathogenic *S. aureus* from non pathogenic strains. All pathogenic *S. aureus* strains are coagulase positive. The other important conventional biochemical tests which are generally used to characterize *S. aureus* are catalase positive and oxidase negative (Quinn et al, 2000).

Growth on blood agar medium decides whether the bacterial isolate is haemolytic or non-haemolytic. The haemolytic bacteria are generally pathogenic. Haemolysis of *S. aureus* was tested on 5 % sheep blood agar medium.

2.16 ANTIBIOTIC SUSCEPTIBILITY TEST (ABST)

The study of resistance pattern of any bacterial isolate involves both qualitative and quantitative determination. The qualitative determination of resistance in each *S. aureus* isolate gives the antibiogram and also the incidence of resistance for each isolate to different antibiotics. This is generally carried by the Kirby-Bauer disc diffusion method.

Voluminous reports are available about disc diffusion test results for *S. aureus* isolates. Rambabu, 2013 reported 56.66 %, 53.33 %, 43.33 %, 36.66 % and 33.33 % of *S. aureus* isolates from bubaline mastitis were resistant to antibiotics vancomycin, amoxycillin +sulbactam, oxacillin, methicillin, ceftriaxone + sulbactam, respectively.

The ABST results are suitable for selection of antibiotics but not effective for quantifi*ca*tion of bioactivity of antibiotics (Eloff, 1998 and 2000). To meet the need for a quick and quantitative assay, a serial microtitre plate dilution method was reported by Eloff, (1998) to screen selected panel of antibiotics for antibacterial activity. The quantitative determination of resistance involves the finding out minimum inhibitory concentrations against selected antibiotics in this case beta lactam and cephalosporin antibiotics were used.

2.17 MOLECULAR TECHNIQUES

Combining the genotyping methods along with phenotypic methods will efficiently help in developing integrated and novel MRSA genotyping strategies for both long and short term investigations. *S. aureus* is an interesting pathogen whose adaptability in terms of infections and hosts makes it a serious threat to food security, animal health, consequently, human and public health (Peton and Le Loir, 2014**).**

2.18 CONTROL AND PREVENTION OF MRSA

The veterinarians have to practice to preserve linezolid and glycopeptides and use only in the cases of MRSA (Sangeetha Joshi, 2013). There are certain new drugs which have been evolved during the last 5 years like linezolid, Quinupristin/dalfopristin, tigecycline, daptomycin, like telavancin and ceftaroline against MRSA (Dardi Charan Kaur and Sadhana Sanjay Chate, 2015).

2.19 ENTEROTOXINS

Enterotoxigenic *Staphylococcus aureus* is one of the major pathogens causing food poisoning worldwide. Because of their biolog*ica*l activities and structural relatedness Staphylococcal enterotoxins (SE) are emetic toxins and are classified as members of the pyrogenic toxin super antigen family (Balaban and Rasooly 2001).

Some *S. aureus* strains has the ability to produce heat stable enterotoxins which is responsible for staphylococcal food poisoning, and ranks as one of the most prevalent

worldwide causes of gastroenteritis (Boerema et al, 2006). Presence of enterotoxigenic *S. aureus* in milk and also in meat leads to a potential health hazard to consumers.

2.20 BIOFILM

One of the major virulence factors of *S. aureus* is its ability to form biofilm invivo which greatly influence its pathogenicity (Vasudevan et al, 2003 and Melchior et al, 2006). Biofilm is defined as a group of microbial cells associated with a surface and enclosed in an extracellular matrix predominantly made up of polysaccharide material (Donlan, 2002). The biofilm formation is considered to be a two step process in which the bacteria first adhere to a surface mediated by a capsular antigen (capsular polysaccharide/adhesion (PS/A)) followed by multipl*ica*tion to form multilayered biofilm, which is associated with production of polysaccharide intercellular adhesion (PIA). Both PIA and PS/A are structurally similar with a common backbone of b-1-6 linked poly glucosamine, but differ in the primary substitutions in their amino groups. The intercellular adhesion (*ica*) locus consisting of the genes *ica* A, D, B and C encodes the proteins mediating the synthesis of PIA and PS/A in staphylococcal species (Mc Kenney et al, 1998 and Cramton et al, 1999). Among the *ica* genes, *ica*A and *ica*C, D have been reported to play a signifcant role in biofilm formation in *S. aureus* and *S. epidermidis*. The *ica*A gene encodes N-acetyl glucosaminyl transferase, the enzyme involved in the synthesis of N-acetylglucosamine oligomers from UDP-N- acetylglucosamine (Arciola et al, 2001a) Bacteria in biofilm exhibit high resistance to antibiotics, disinfectants, as well as to host immune system clearance (Donlan and Costerton, 2002).

The significance of biofilm is well recognized in med*ica*l, environmental, and industrial contexts. Several severe infections are reported to be a result of biofilm formation which leads to chronic diseases in most cases. These infections are persistent and lead to global public health concern. As a result they reduce the effectiveness of treatments and increase morbidity, mortality and health care cost. Biofilm formation in isolates of *S. aureus* from mastitis is also associated with a reduced susceptibility to antibiotics, which is attributed to the decreased diffusion of antibiotics through the biofilm matrix (Amorena et al, 1999).

CHAPTER – 3

EVALUATION OF INCIDENCE OF *S. AUREUS* IN CLINICAL CASES OF LIVESTOC AND POULTRY

CHAPTER – 3 EVALUATION OF INCIDENCE OF *S. AUREUS* IN CLINICAL CASES OF LIVESTOCK AND POULTRY

3.1 INTRODUCTION

S. aureus is a pathogenic endemic and emerging bacterium. It is one of the major causative etiological agents of mastitis in buffaloes and gangrenous dermatitis in broiler chickens (Kumar, 2009 and Sheela et al, 2016). To understand the impact of *S. aureus* in poultry birds and livestock animals one has to begin with the evaluation and incidence of *S. aureus* in clinical cases of livestock and poultry. Hence, in the present investigation the samples were collected from the above said two cases and processed for the isolation of *S. aureus* from the collected samples and followed by biochemical and molecular characterization of *S. aureus* isolates.

3.2 MATERIALS AND METHODS

3.2.1 PLACE, NUMBER AND DISEASES OF SAMPLES INVESTIGATED

A total of 160 samples were collected from different clinical cases in livestock (100) and poultry (60) from costal Andhra Pradesh. Four districts namely Guntur, Krishna, East and West Godavari were included in the study. Swab samples were collected from suspected clinical cases of gangrenous dermatitis disease. Milk samples were collected from buffaloes with clinical mastitis. They were further processed for isolation of *S. aureus*.

3.2.2 GLASSWARE AND CHEMICALS USED

In the present study, glassware from Borosil (India) and Duran (Germany), and routinely used chemicals from Sisco Research Labs (SRL), India were used. Bacteriological media manufactured by Himedia (India) and Sigma Aldrich (India) were used. Master Mix for PCR test from New England Biolabs (NEBL), USA and DNA ladder from Axygen (USA) were used. Plasticware like 2 ml/1.5 ml micro centrifuge tubes, 0.2 ml PCR tubes from Eppendorf (Germany) and PCR micro tips from Labware (USA) were used. Some of the important equipment used in the present study was given in the table 3.1.

Table 3.1 Important equipments used

S. No	Equipment	Details
1	Thermal cycler	Eppendorf, Germany
2	Submarine Gel Electrophoresis	Atto Corporation, Japan
3	Gel documentation system	In Genius bio imaging system, Syngene, UK
4	ELISA	Multiscan ELISA Reader

3.2.3 METHODS OF COLLECTION OF CLINICAL SAMPLES

During the post mortem of the diseased bird, the swabs were collected under sterile conditions and carried to laboratory for further processing. The udder quarters of the buffalo were washed with tap water and dried. Before collecting the sample the first streams of milk were discarded. After that the teat ends were disinfected with cotton swabs soaked in 70 % alcohol and allowed to dry. Approximately 7-10 ml of milk was collected asept*ical*ly into sterile vials. Collected samples were screened by California Mastitis Test (CMT) in case of mastitic milk samples.

The CMT is quick and precise test that assist to determine Somatic Cell Count (SCC). The CMT is conducted to diagnose the presence of subclinical and clinical mastitis as illustrated by Shitandi and Kihumbu, (2004). A squirt of milk from each quarter of the udder was placed in each of four shallow cups in the CMT paddle and an equal amount of the CMT reagent (Appendix) was added. A gentle circular motion was applied in a horizontal plane and the reactivity of the samples was observed. Positive samples showed gel formation within a few seconds. The result was scored based on the gel formation and categorized as negative if there was no gel formation, or positive if there was gel formation ranging from +1 to +3 (Table 3.2). If at least one quarter was positive by the CMT then the buffalo was considered as positive. The mastitic milk samples that showed reactivity in CMT with abnormality in milk were considered as samples from clinical cases, whereas the mastitic samples that have reactivity in CMT but without any visible abnormality in milk were considered as samples from subclinical cases (Rambabu, 2013). The CMT reagent reacts with the white blood cells and the mixture thickens or gels in proportion to the amount of infection present (Akram, 2002 and Khan, 2002).

Table 3.2 Interpretation and scoring of the CMT

CMT Score	Average somatic cell count (cells/ml)	Description of visible reaction
N (negative)	100,000	Mixture remains liquid, with no evidence of thickening
T (trace)	300,000	The slight thickening that forms is seen best by tipping the paddle back and forth and observing the mixture as it flows over the bottom of the cup. Trace reactions tend to disappear with continued rotation of the paddle. To be read at 10 seconds
1 (Weak positive)	900,000	A distinct thickening of the liquid forms, but there is no tendency toward a gel formation. With some milk, the thickening may disappear after prolonged rotation of the paddle (20 seconds or more). To be read at 10 seconds.
2 (Distinct positive)	2,700,000	Mixture thickens immediately, and a gel formation is suggested. As the mixture is swirled, it tends to move in toward the centre, exposing the bottom of the outer edge of the cup. When the motion is stopped, the mixture level out and covers the bottom of the cup. To be read at 10 seconds.
3 (Strong positive)	8,100,000	A gel is formed, which causes the surface of the mixture to become elevated like a partially fried egg. There is usually a central peak that remains projecting above the main mass, even after the rotation of the paddle is stopped

3.2.4 ISOLATION OF *S. AUREUS*

The collected samples were inoculated in Tryptic Soy Broth (TSB) (M/S oxoid, UK) for enriched culture and further streaked on Mannitol Salt Agar (MSA) (M/S oxoid, UK) which is a selective media for *S. aureus*. The inoculated plates were incubated at 37 °C for 24 hrs.

3.2.5 MORPHOLOGICAL CHARACTERIZATION OF *S. AUREUS*

The suspected isolates, which were gram-positive cocci with bunch of grapes appearance were picked up and maintained on MSA slants for further examinations (Kateete et al, 2010). These clinical *S. aureus* isolates were further characterized by cultural and biochemical tests.

3.2.6 CULTURAL CHARACTERIZATION OF *S. AUREUS*

MSA is used for the selective isolation and cultivation of clinical and nonclinical specimens of *S. aureus*. The bacteria, which utilizes mannitol turn the medium yellow those which cannot utilize remains colourless.

3.2.7 BIOCHEM*ICA*L CHARACTERIZATION OF *S. AUREUS*

The suspected isolates were identified using Catalase Test, Spot Oxidase Test, and Voges – Proskauer Test.

3.2.7.1 Catalase test:

Two to three drops of 3 % hydrogen peroxide was taken on glass slide, to test the catalase activity. The positive test ind*ica*tes appearance of bubbles on H_2O_2 drop.

3.2.7.2 SPOT OXIDASE TEST

The dye (N, N, N′, N′- tetramethyl-p-phenylenediamine) solution poured on blotting paper with sterile filter. Using a sterile glass rod, 2-3 colonies (yellow color) from MSA were picked and touched onto the blotting paper gently and observed for development of any dark blue colour (Carter and Cole, 1990).

3.2.7.3 VOGES – PROSKAUER (VP) TEST

S. aureus is cultured in Methyl Red Voges Proskauer (MR-VP) broth (Himedia, india). After 48 hrs of incubation Barritt's reagent A and B added. A positive test is indicated by the development of pink colour (Carter and Cole, 1990).

3.2.8 HAEMOLYSIS ACTIVITY

Haemolytic pattern was tested on 5 % Sheep Blood Agar medium (Grema et al, 2015 and Quinn et al, 2000). These characterizations were further confirmed by molecular detection tools using species-specific primers as described below.

3.2.9 MOLECULAR CHARACTERIZATION OF *S. AUREUS*

3.2.9.1 DNA EXTRACTION

The TSB was inoculated with provisionally confirmed *S. aureus* and incubated for 18 hrs at 37 ℃. Out of which, 2 ml of enriched culture was taken and centrifuged at 5,000 rpm for 10 min to pellet the bacterial cell mass. Further, the pellet was washed in phosphate buffer saline (PBS) at 5,000 rpm for 10 minutes followed by washing with TKM-1 solution. The obtained pellet was resuspended in TKM-2 solution and incubated at 37 ℃ for 15 minutes where the cell lysis was achieved. This step was followed by adding 10 % SDS and mixing gently. Subsequently 6M NaCl was added and mixed well and centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected and mixed with absolute alcohol of double the volume of supernatant and centrifuged at 10,000 rpm for 5 minutes. Finally, the pellet obtained was washed twice with 70 % ethanol. The DNA pellet thus obtained was resuspended in 40 µl of sterile Milli-Q water and kept at -80 ℃ for further use in PCR (Aravindakshan et al, 1997) with suitable modifi*ca*tions (Kumar, 2009).

3.2.9.2 MOLECULAR DETECTION OF *S. AUREUS* BY PCR.

S. aureus was detected by species specific primers by PCR in Master cycle rep gradient thermal cycler (Eppendorff, Germany). The forward and reverse primer sequences were 5'ACGGAGTTACAAAGGACGAC 3' and 5'AGCTCAGCCTTAACGAGTAC 3' respectively (Straub et al, 1999) synthesized at Bioserve Biotechnologies (India) Pvt. Limited, India. PCR was run for 35 cycles, initial denaturation at 94 ℃ for 2 min and final elongation at 72 ℃ for 10 min. The PCR conditions are given in the below table 3.3.

Table 3.3 Oligonucleotide primers and PCR conditions for detection of *S. aureus* isolates.

Gene	Primers	Base pairs	Sequence (5'- 3')	Denaturation	Annealing	Extension
S. aureus 23s rRNA	*Staur 4*	1250bp	ACGGAGTTACA AAGGACGAC	94 °C/ 45 sec	64 °C / 60 sec	72 °C / 2min
	Staur 6		AGCTCAGCCTTA ACGAGTAC (Straub et al, 1999 and Riffon et al, 2001)			

F=forward primer, R=reverse primer

3.2.9.3 PCR AMPLIFICATION

The reaction was performed in a 25 µl reaction mixture comprising of 12.5 µl master mix (New England Biolabs, UK), 1.0 µl of forward and reverse primer, 4.0 µl of DNA template and finally 6.5 µl of distilled water. The master mix contained 10 mM TrisHCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 50 units/ml *Taq* DNA Polymerase, 0.2 mM of each dNTP and 0.2 µM of each primer.

The PCR amplicons were analyzed by electrophoresis on a 1.7 % agarose gel stained with 0.5 µg of ethidium bromide / ml in Tris Borate EDTA (TBE) buffer. Electrophoresis were carried out at 90 V for 120 min in submarine gel electrophoresis unit (Atto Corporation, Japan) and the PCR products were visualized in InGenius Gel Documentation System, (Syngene, U.K) along with a ProxiO 100bp DNA ladder (BioLit, SRL, India).

3.3 RESULTS

3.3.1 Clinical Samples

100 mastitis milk samples and 60 swabs from gangrenous dermatitis in poultry were collected and tested for the presence of *S. aureus*.

3.3.2 CMT with mastitis milk samples of buffaloes

Out of 100 milk samples 40 cases were clinical mastitis whereas 60 were subclinical mastitis after testing by California Mastitis Test (Fig 3.1).

Figure 3.1 The CMT test performed in a paddle.

3.3.3 Isolation of *S. aureus*

Out of 100 samples collected from milk samples, 69 (69.0 %) isolates were mannitol fermenting when cultured on Mannitol Salt Agar (MSA) medium and all the isolates were gram positive cocci. From all the 60 GD cases, 52 were gram positive cocci (Figure 3.2) and mannitol fermenting (Figure 3.3).

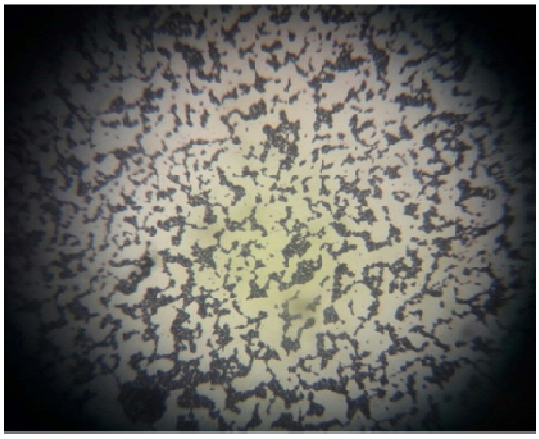

Figure.3.2 Gram's staining- violet coloration of *S. aureus*. Spherical cocci magnified at 100X in Olympus microscope are shown.

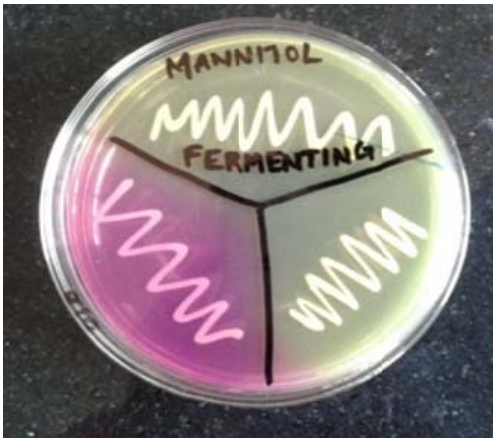

Figure 3.3 Mannitol Salt Agar medium fermented by *S. aureus* isolates resulted in the formation of yellow colour colonies that implies pH change.

3.3.4 Biochemical characterization of *S.aureus*

All the 69 isolates from bovine and 52 isolates were catalase (Figure 3.4), Vogus Proskauer and coagulase positive (Figure 3.5) whereas oxidase negative.

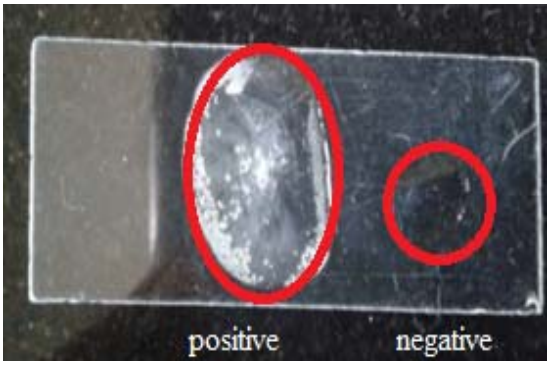

Figure 3.4 Catalase Test to evaluate the pathogenicity of *S. aureus*. The positive test indicates appearance of bubbles on H_2O_2 drop.

Figure 3.5 Coagulase test. The *S. aureus* colony inoculated in rabbit plasma, thick consistency represented positive and colourless tube represents negative for the test.

3.3.5 Haemolysis

Out of 69 provisionally confirmed *S.aureus* isolates from bovine, 20 isolates were α haemolytic, 10 isolates were β haemolytic and 5 isolates shown both α and β haemolysis on 5 % sheep blood agar medium. Remaining 34 isolates were non-haemolytic. And out of 52 isolates from poultry, 45 isolates provisionally confirmed *S.aureus* showed haemolysis on 5 % sheep blood agar medium (Figures 3.6 and 3.7).

Figure 3.6. Complete haemolysis on 5 % sheep Blood Agar Medium (BAM) represented by complete colourless zones surrounding *S. aureus* colony.

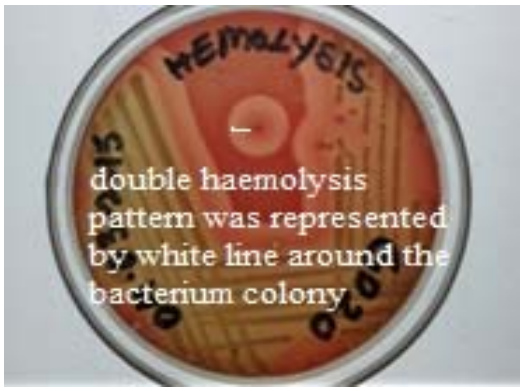

Figure 3.7 Incomplete haemolysis on 5 % Sheep Blood Agar Medium (BAM) the pale red and colourless zone surrounding the *S. aureus* colony is represented by an arrow.

3.3.6 Detection of *S. aureus* by PCR

Out of 69 isolates 34 from bovine and out of 52 from poultry all the 52 (100 %) shown positive in PCR test with species specific primers (Figures 3.8 to 3.15). These confirmed *S.aureus* isolates were further tested to study the antimicrobial resistance pattern to different antimicrobial agents.

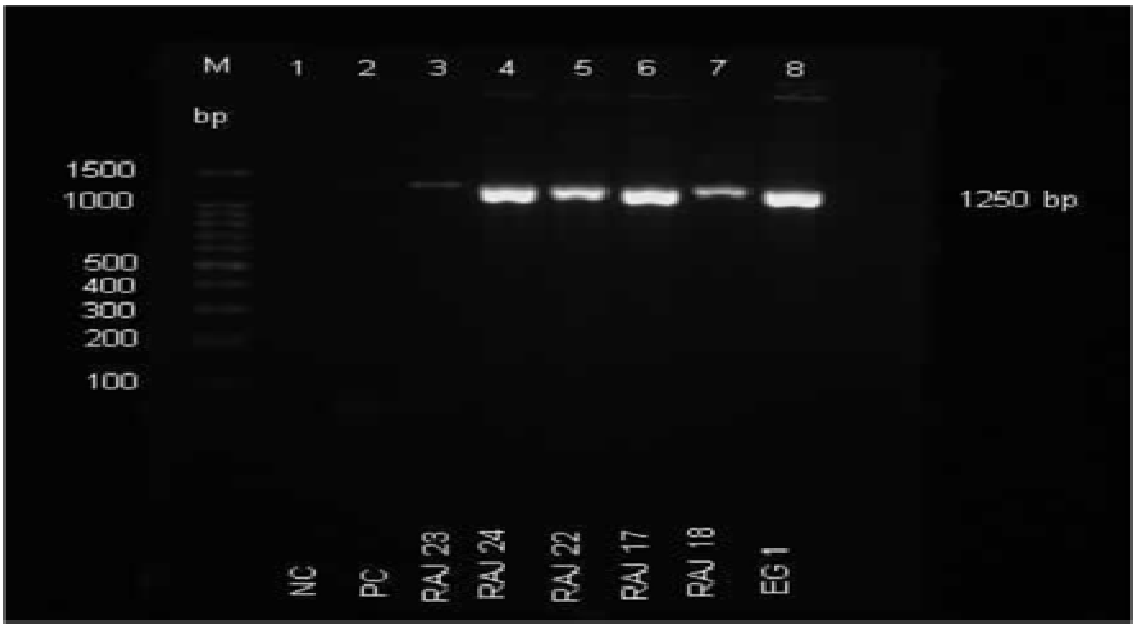

Figure 3.8 Agarose gel electrophoresis patterns of *staur* 4 and *staur* 6 genes to confirm *S. aureus*. Lane M- Marker, Lanes: 1 and 2 negative isolates and Lanes: 3-8 positive for *S. aureus* isolates. The sample names were given in lower side of the figure. Raj- Rajahmundry and EG- East Godavari.

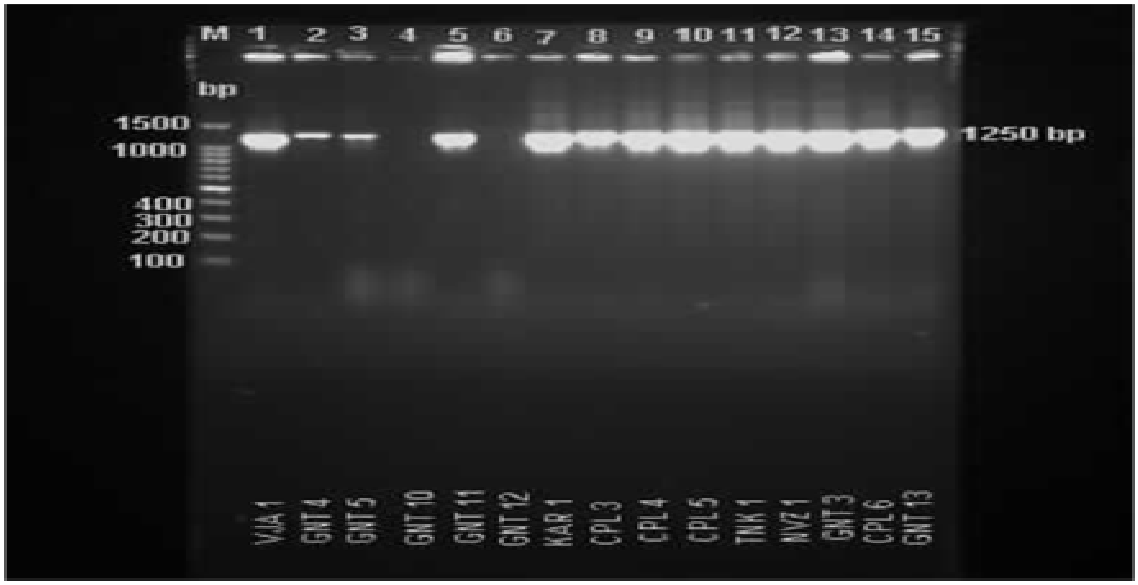

Figure 3.9 Agarose gel electrophoresis patterns of *staur* 4 and *staur* 6 genes to confirm *S. aureus*. Lane M- Marker, Lanes: 4 and 6 negative isolates and Lanes: 1-3, 5 and 7-15 were positive for *S. aureus* isolates. The sample names were given in lower side of the

figure. VJA-Vijayawada, GNT-Guntur, KAR-Kesarapalli, CPL- Cherlapalli, TNK-Tanuku, NVZ-Nuzvid.

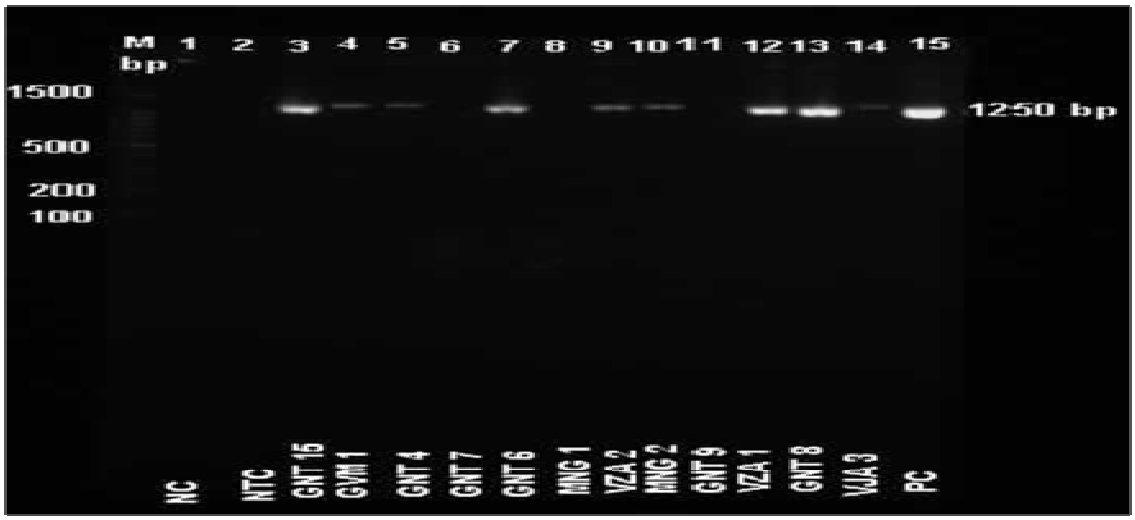

Figure 3.10 Agarose gel electrophoresis patterns of *staur* 4 and *staur* 6 genes to confirm *S. aureus*. Lane M- Marker, Lanes: 1, 2, 6, 8and 11 were negative isolates and Lanes: 3-5, 7, 9, 10, 12-15 were positive for *S. aureus* isolates. The sample names were given in lower side of the figure.VJA-Vijayawada, GNT-Guntur, KAR-Kesarapalli, CPL-Cherlapalli, TNK- Tanuku, NVZ-Nuzvid, NC- Negative control, NTC- Non template control, PC- Positive control, GNT-Guntur, MNG-Mangalagiri, VJA-Vijayawada.

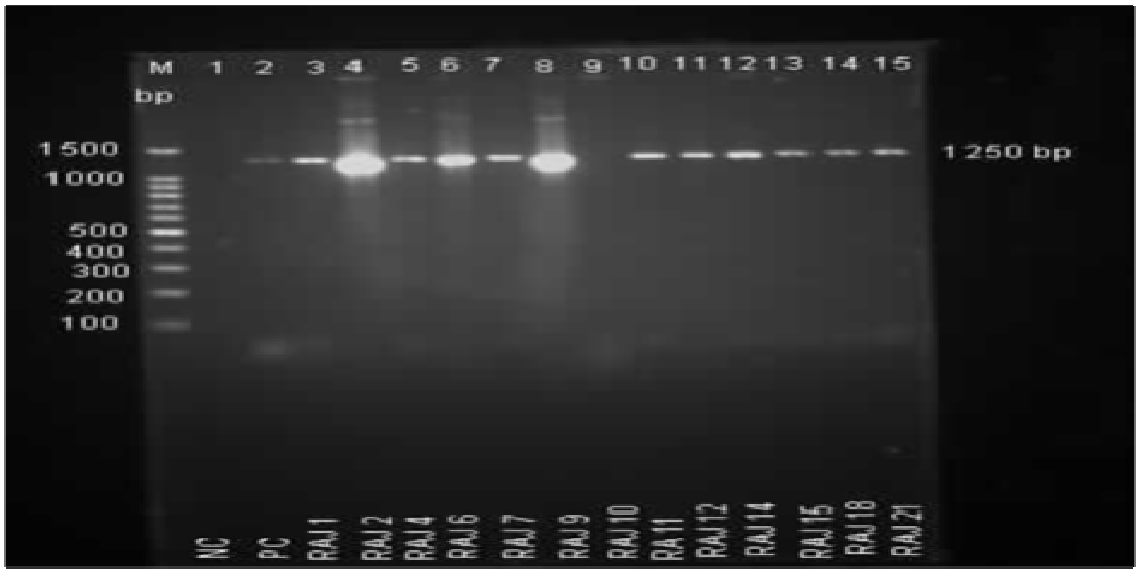

Figure 3.11 Agarose gel electrophoresis pattern of *staur* 4 and *staur* 6 genes to confirm *S. aureus* isolates. Lane: M- Marker, Lanes: 2-8 and 10-15 positive for *S. aureus* isolates and Lanes: 1 and 9 negative for *S. aureus* isolates. The sample names were given in lower side of the figure. NC- Negative Control; PC-Positive Control; RAJ- Rajahmundry.

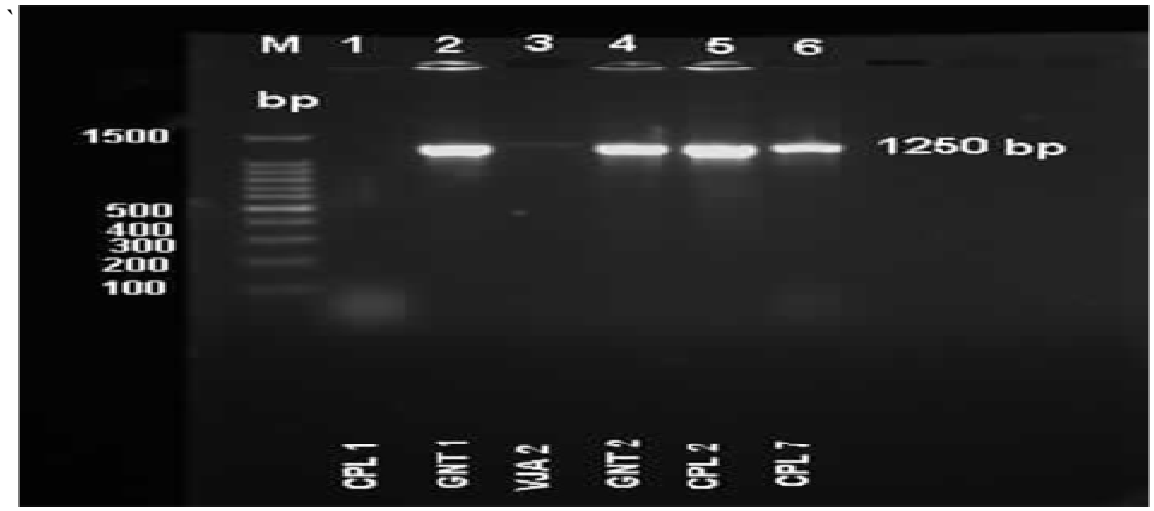

Figure 3.12 Agarose gel electrophoresis pattern of *staur* 4 and *staur* 6 genes to confirm *S. aureus*. Lane: M- Marker, Lanes: 2-6 positive for *S. aureus* isolates and Lane 1 negative for *S. aureus* isolate. The sample names were given in lower side of the figure.

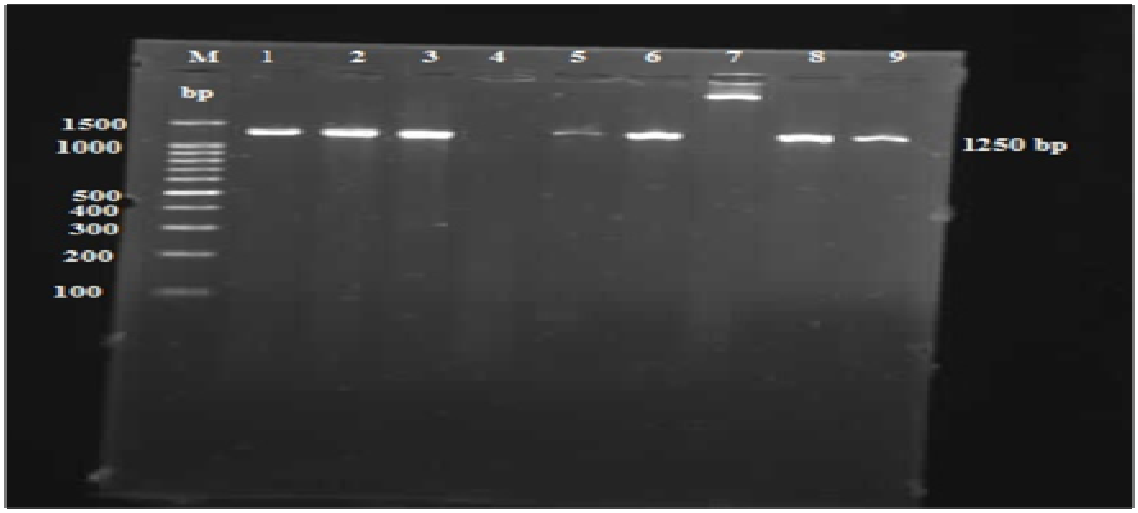

Figure 3.13 Agarose gel electrophoresis pattern of *staur* 4 and *staur* 6 genes to confirm *S. aureus* isolates. Lane: M- Marker, Lanes: 1-3, 5, 6, 8 and 9 positive for *S. aureus* isolates and Lanes: 4 and 6 negative for *S. aureus* isolates.

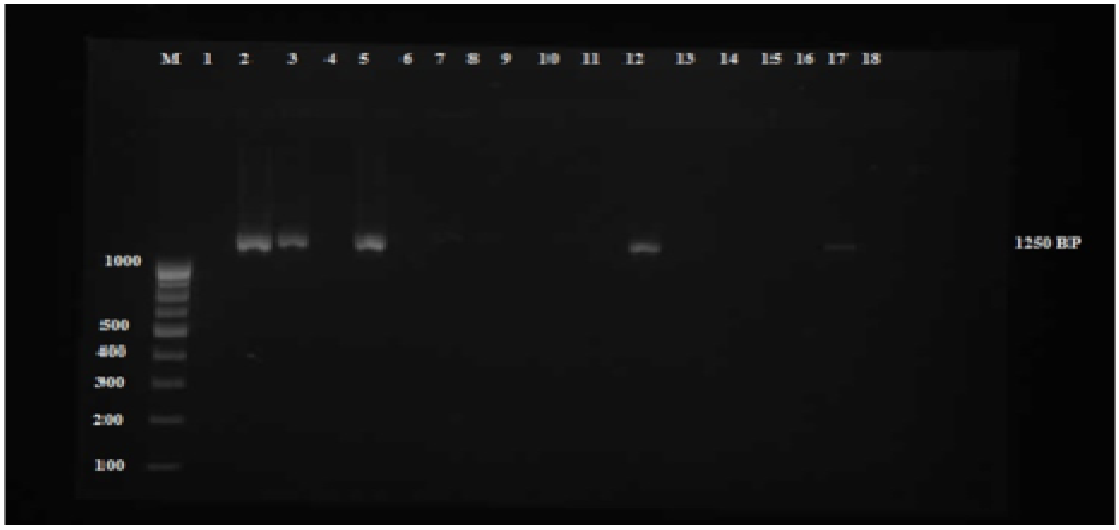

Figure 3.14 Agarose gel electrophoresis pattern of *staur* 4 and *staur* 6 genes to confirm *S. aureus*. Lane: M- Marker, Lanes: 2, 3, 5, 12 and 17 positive for *S. aureus* isolates and Lanes: 1, 4, 6-11 and 13-16 and 18 negative for *S. aureus* isolates.

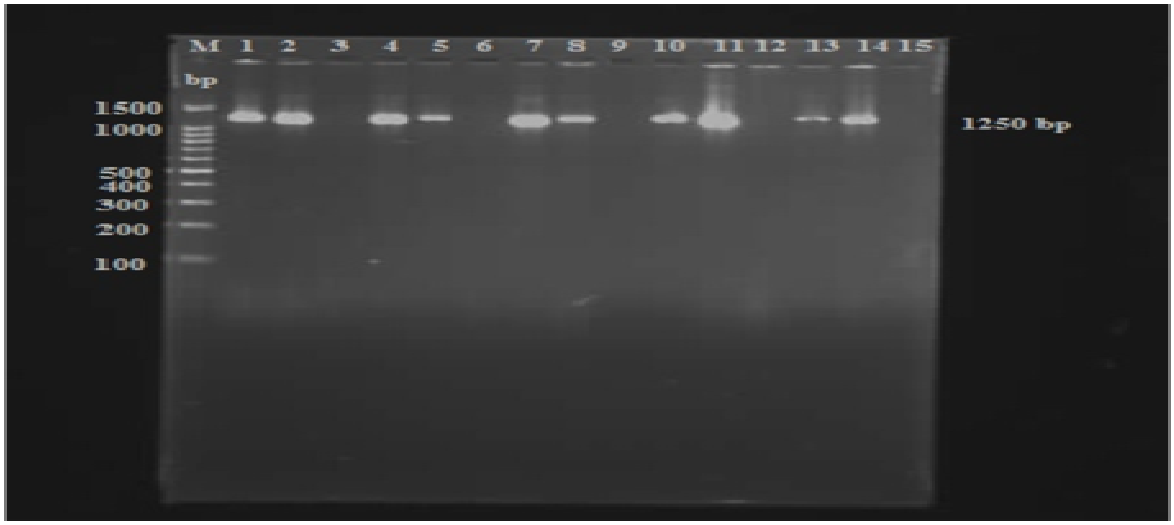

Figure 3.15 Agarose gel electrophoresis pattern showing PCR products for *S. aureus* isolates. Lane: M- Marker, Lanes 1, 2, 4, 5, 6, 7, 8, 10, 11, 13 and 14 positive for *S. aureus* isolates and Lanes: 3, 6, 9, 12 and 15 negative for *S. aureus* isolates

3.3.7 Discussion

In bovine, 69 isolates were provisionally confirmed as *S. aureus* whereas 34 isolates were molecularly confirmed. In poultry, 52 isolates were biochemically confirmed out of which all the isolates were molecularly confirmed as *S. aureus* (Figure 3.16). Chavan and his

group in Hissar reported prevalence of *S. aureus* in Mastitis as 29.33 % coagulase negative and 38.66 % of coagulase positive *S. aureus*. Sharma *et al*, and Roychoudhury and Dutta have also reported many positive cases of *S. aureus* in mastitis. Many researchers have found that *S. aureus* to be more widespread than other species of the same genus in mastitis (Char et al, 1983, Saini et al, 1994, Aromenteros et al, 2006, Unnerstad, 2009). But in coastal Andhra Pradesh where dairy industry is one the major source of income for farmers very few reports were available.

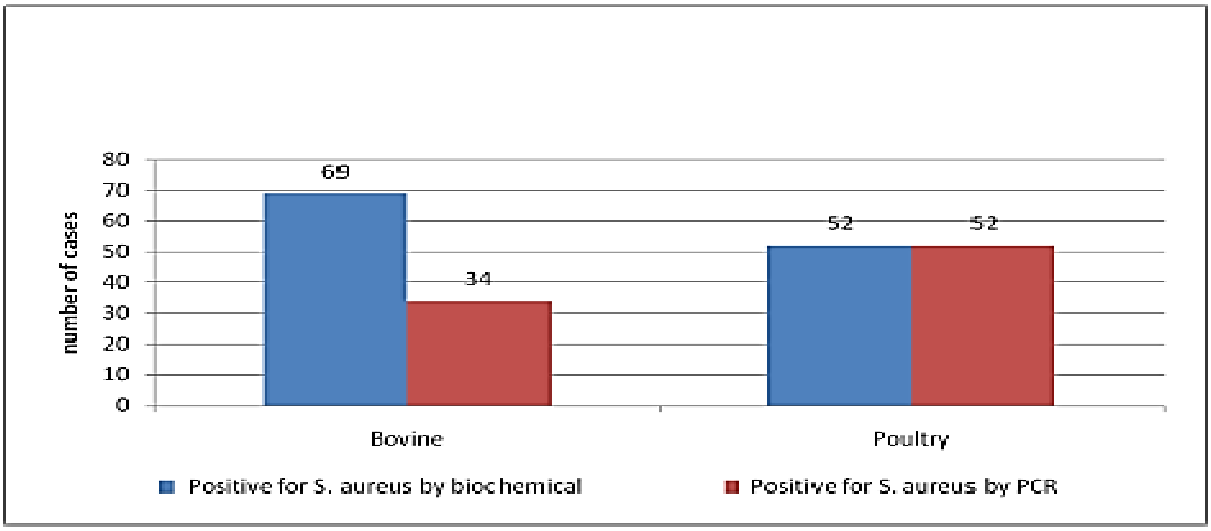

Figure 3.16 The bars showing the iincidence of *S. aureus* isolates in bovine and poultry obtained through the tests adopted.

CHAPTER – 4

INVESTIGATION OF ANTIBIOTIC RESISTANCE PATTERN IN *S. AUREUS* ISOLATES

CHAPTER – 4 INVESTIGATION OF ANTIBIOTIC RESISTANCE PATTERN IN *S. AUREUS* ISOLATES

4.1 INTRODUCTION

S. aureus quickly develops resistance to multiple antibiotics like β – lactam antibiotics, methicillin, vancomycin etc (Majumder et al, 2001). In most of the veterinary medicine antibiotics are used for therapeutic purpose in order to increase the efficiency of feed intake and growth promoters in cattle and poultry (Joshi and Prasad, 2014). Due to the endemic, emergence, acquiring multiple antibiotic resistances and its zoonotic potential *S. aureus* developed as a potent bacterial pathogen for both veterinary animals and humans (Hardy, 2002, Normanno et al, 2007). MRSA has been isolated from cattle, dogs, cats, pigs, horses and poultry worldwide (Leonard and Markey, 2008). In humans these resistant *S. aureus* strains can cause life threatening diseases like sepsis, endocarditis and osteomyelitis (Cuoto et al and Cox et al, 1995). It also causes some toxin-induced diseases such as staphylococcal scalded-skin syndrome, gastroenteritis and toxic shock syndrome (Lowy, 1998 and Waldvogel, 1995). The first MRSA was reported in 1960 and soon after it has been reported in many parts of the World (Jevans, 1961). Now this MRSA became endemic in India representing its incidence in western (25 %) and southern (50 %) parts of India (Patel et al, 2010 and Gopalakrishnan R, Sureshkumar, 2010). In the present investigation, Sensitivity of *S. aureus* isolates to seventeen antibiotics was assessed by Kirby-Bauer disc diffusion method and Minimum Inhibitory Concentration (MIC) assays with the selected antibiotics.

4.2 MATERIALS AND METHODS

4.2.1 STUDY OF ANTIBIOTIC RESISTANCE PATTERN TO SELECTED ANTIBIOTICS

4.2.1.1 Preparation of 0.5 McFarland standards

McFarland standard (0.5) was prepared by adding 0.5 mL of 0.048 M $BaCl_2$ (1.17 % w/v $BaCl_2.2H_2O$) to 99.5 mL of 0.18 % M H_2SO_4 (1 % w/v) with constant stirring. Test culture was compared with 0.5 McFarland standards to get standard inoculums size of 1.0 to 2.0 × 10^8 cfu/ml (CLSI, 2012).

4.2.1.2 QUALITATIVE

S. aureus isolates were inoculated into nutrient broth (Oxoid) at 37 ℃ for 24 h before testing. The turbidity of the growing culture was set to correspond with that of a barium sulphate (0.5 Mc Farland) standard. Subsequently 0.1 ml of the nutrient broth culture was inoculated onto Mueller Hinton agar plates (90 mm diameter disposable petri dishes) and spread over the surface of agar with sterile cotton swabs. Eighteen antimicrobial disks (Himedia, India) were then placed on the surface of each plate with the help of antibiotic disk dispenser and incubated at 37 ℃ for 18 hrs. The antimicrobial agents which were used: amoxicillin (30µg), amoxicillin + clavulanic acid (30/15µg), ceftriaxone (30 µg), ceftriaxone + tazobactam (30/10µg), ceftriaxone + sulbactam (30/15µg), oxacillin (1µg), penicillin (10µg), ampicillin (10µg), methicillin (5µg), vancomycin (30µg), streptomycin (25µg), gentamicin (10µg), neomycin (30 µg), gatifloxacin (5µg), linezolid (30µg), enrofloxacin (10µg) and cefoxitin (10µg). The disc diffusion assay and zone interpretation of each antimicrobial agent was done according to Clinical and Laboratory Standards Institute, 2012 (CLSI, 2012) (Figure 4.1).

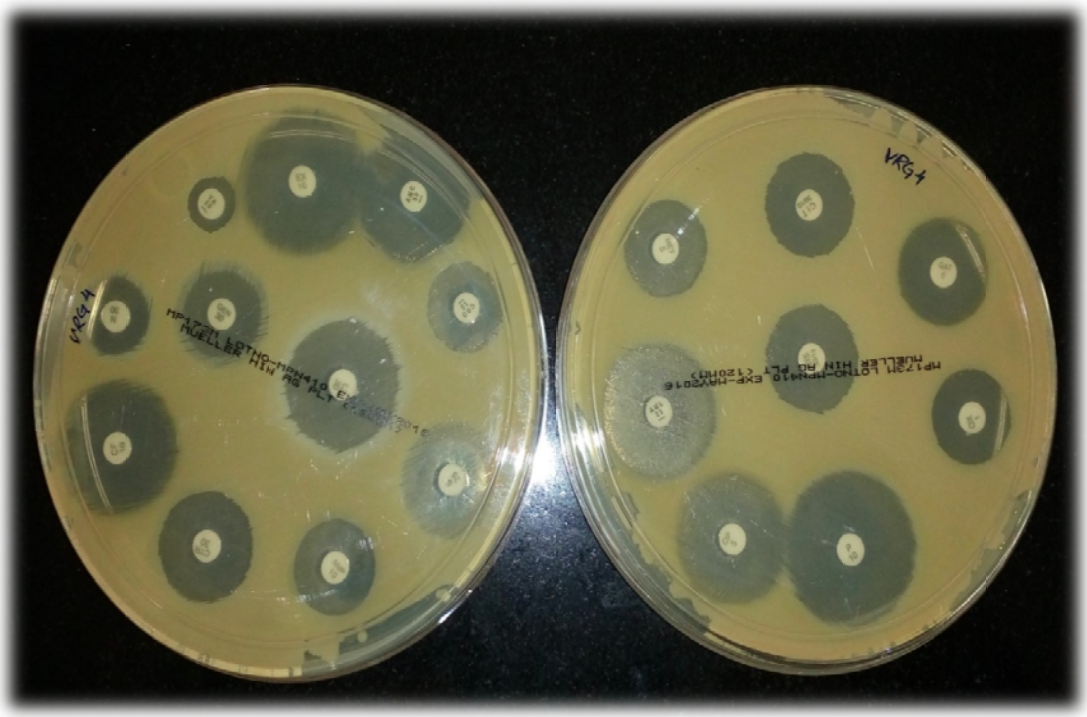

Figure 4.1 ABST by Disc Diffusion Assay on Muller Hington Agar Plates (Himedia, India Pvt Ltd).

4.2.1.3 QUANTITATIVE

Quantitative determination of resistance includes finding out minimum inhibitory concentrations against β-lactam and cephalosporin antibiotics. The antibiotics include Penicillin (SRL, India), Oxacillin (Sigma Aldrich), Ceftriaxone (INTAS), Amoxycillin (SRL, India) and Amoxycillin+Clavulanic Acid (GlaxoSmithKline). This method allows the determination of the MIC of each antibiotic against Staphylococcal species by measuring reduction of tetrazolium violet.

4.3 RESULTS

4.3.1 Antibiotic Susceptibility Test by Kirby-Bauer disc diffusion method

In bovine, the percentage resistance pattern to different antibiotics was described in the table 4.1 and figure 4.2.

Table 4.1 Percentage resistance of *S. aureus* isolates to different antibiotics in bovine.

Amoxycillin 30mcg	Amoxicillin + Clav acid	Ceftriaxone 30 mcg	Ceftriaxone +Tazobactum 30/10	Ceftriaxone + Sulbactam 30/15 mcg	Oxacillin 5mcg	Vancomycin 30mcg	Strptomycin25mcg	Gentamicin 10mcg	Neomycin 30mcg	Enrofloxacin 10mcg	Penicillin 10 mcg	Ampicillin 10 mcg	Methicillin 5 mcg	Gatifloxacin 5 mcg	Linezolid 30 mcg	Cefoxitin 10 mcg
67.74	0.00	9.68	77.42	61.29	100.00	45.16	12.90	38.71	25.81	29.03	38.71	70.97	48.39	41.94	9.68	3.23

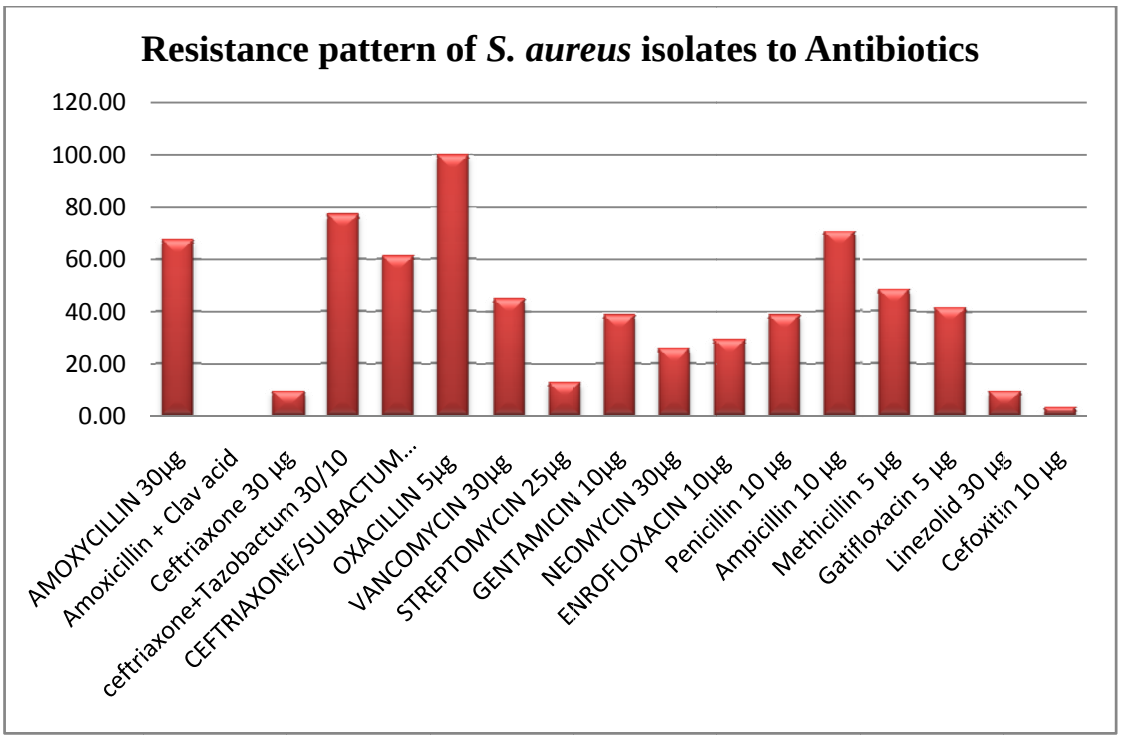

Figure 4.2 Antibiotic sensitivity pattern of isolated *S. aureus* in bovine

In poultry, all the *S. aureus* isolates were studied for their resistance pattern by disc diffusion method and the results are shown in figure 4.3. Highest resistance was recorded for ceftriaxone + tazobactam, amoxicillin and penicillin (100 %) followed by gatifloxacin (98.08 %), ceftriaxone (92.31 %), cefoxitin (90.38 %), ampicillin (88.46 %), vancomycin, streptomycin and neomycin (84.62 %), gentamicin (82.69 %), amoxicillin (80.77 %),methicillin and ceftriaxone + sulbactam (73.08 %), enrofloxacin (69.23 %), oxacillin (67.31 %), linezolid (48.08 %) and amoxicillin + clavulanic acid (30.77 %).

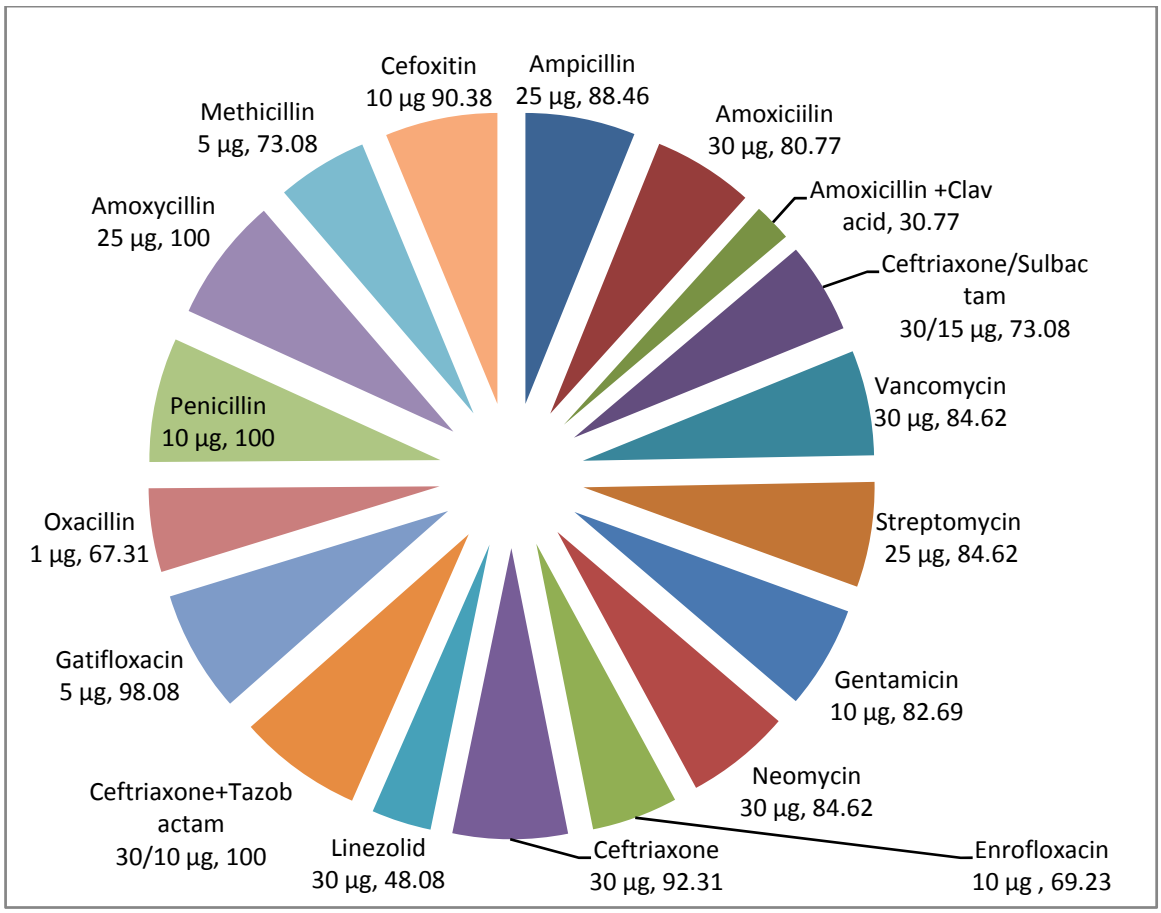

Figure 4.3 Antibiotic resistance patterns in *S. aureus* isolates from poultry.

4.3.2 Minimum Inhibitory concentrations of antibiotics in *S. aureus* for different antibiotics:

Antibiotics resistance pattern was performed to selected antibiotics like penicillin, oxacillin, ceftriaxone, amoxycillin and amoxycillin+clavulanate by quantitative method MIC. The concentration of the antibiotics was 10 mg/ml and the MIC values were tabulated in the tables 4.2 and 4.3 respectively.

4.4 Discussion

In bovine, very high resistance was observed against oxacillin (100 %), Cerftriaxone + tazobactam (77.42 %), ampicillin (70 %), amoxycillin (67.74 %), Ceftiaxone + sulbactam (61.29 %), methicillin (48.39 %), vancomycin (45.16 %), gatifloxacin (41.94 %), gentamicin (38.71 %), enrofloxacin (29.03 %), neomycin (25.81 %) and least resistance was observed against amoxycillin + clavulanic acid (0.0 %), cefoxitin (3.23 %), ceftriaxone and linezolid (9.68 %). Whereas in poultry the condition is different and matter of concern as highest resistance was recorded for penicillin,ceftriaxone + tazobactam, and amoxicillin), followed by gatifloxacin (98.08 %), ceftriaxone (92.31 %),

cefoxitin (90.38 %), ampicillin (88.46 %), vancomycin, streptomycin and neomycin (84.62 %), gentamicin (82.69 %), amoxicillin (80.77 %),methicillin and ceftriaxone + sulbactam (73.08 %), enrofloxacin (69.23 %), oxacillin (67.31 %), linezolid (48.08 %) and amoxicillin + clavulanic acid (30.77 %). The antibiotic sensitivity pattern of *S. aureus* isolates from referral hospitals against penicillin it was recorded as 53.36 %, gentamicin as 55.6 % and erythromycin it was recorded as 31.83 % in Assam (Majumder et al, 2001). Most significant part of the study is the bacteria are showing resistance to most recent antibiotic linezolid. Interestingly vancomycin resistance was also high in this region which is a dangerous alarm for public health concern. In the present investigation it was proved that high resistance was observed in *S. aureus* isolates from poultry than in bovine. There is a high risk of transfer of these resistant strains to humans and cause a wide range of dreadful infections.

Table 4.2 Minimum inhibitory concentrations of antibiotics used against *S. aureus* isolates in Bovine by microtitre plate method. Drug concentration: 10 mg / ml. MIC values: mg / ml

S.No	Sample name	Penicillin	Oxacillin	Ceftriaxone	Amoxycillin	Amoxycillin+ clavulanate
1	MNG 2	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
2	RAJ 4	9.77	78.12	625	< 4.88	< 4.88
3	RAJ 7	156.25	39.06	156.25	< 4.88	156.25
4	RAJ 9	156.25	39.06	156.25	< 4.88	156.25
5	RAJ 12	156.25	39.06	156.25	< 4.88	156.25
6	RAJ 14	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
7	RAJ 15	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
8	RAJ 22	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
9	PRK 22	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
10	PRK 23	9.77	78.12	39.06	19.53	9.77
11	PRK 24	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
12	PRK 25	9.77	78.12	39.06	19.53	9.77
13	PRK 26	9.77	78.12	39.06	19.53	9.77
14	PRK 31	< 4.88	< 4.88	< 4.88	< 4.88	9.53
15	PRK 32	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
16	PRK 33	9.77	78.12	39.06	19.53	9.77
17	PRK 34	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
18	PRK 36	9.77	78.12	39.06	19.53	9.77
19	PRK 37	9.77	78.12	39.06	19.53	9.77
20	RAJ 2	< 4.88	< 4.88	19.53	< 4.88	< 4.88
21	RAJ 6	< 4.88	< 4.88	9.77	< 4.88	< 4.88
22	RAJ 11	156.25	39.06	156.25	< 4.88	156.25
23	GVM 1	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
24	RAJ 1	156.25	39.06	0.156	< 4.88	156.25
25	RAJ 21	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
26	RAJ 23	156.25	39.06	156.25	< 4.88	156.25
27	RAJ 24	< 4.88	< 4.88	9.77	< 4.88	< 4.88
28	PRK 48	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
29	PRK 60	9.77	78.12	39.06	19.53	9.77
30	GVM 2	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
31	ELR 2	78.12	2500	312.5	9.77	39.06
32	ELR 3	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
33	ELR 4	< 4.88	< 4.88	19.53	< 4.88	< 4.88
34	ELR 1	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88

Table 4.3 Minimum inhibitory concentrations of antibiotics used against *S. aureus* isolates in poultry by microtitre plate method. Drug concentration: 10 mg / ml. MIC values: in mg / ml

S.No	Sample name	Penicillin	Oxacillin	Ceftriaxone	Amoxycillin	Amoxycillin+ clavulanate
35	CPL 2	< 4.88	< 4.88	156.25	< 4.88	< 4.88
36	CPL 3	0.625	0.098	0.625	5	2.5
37	CPL 4	< 4.88	< 4.88	156.25	< 4.88	< 4.88
38	CPL 5	< 4.88	< 4.88	0.009	< 4.88	< 4.88
39	CPL 6	< 4.88	< 4.88	< 4.88	< 4.88	19.53
40	CPL 7	19.53	< 4.88	9.77	19.53	9.77
41	GNT 1	0.0195	0.04	0.156	0.3125	5
42	GNT 11	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
43	GNT 13	0.009	0.3125	0.3125	1.25	0.009
44	GNT 2	< 4.88	< 4.88	< 4.88	0.04	0.195
45	GNT 3	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
46	GNT 4	156.25	<4.88	19.53	<4.88	<4.88
47	GNT 5	<4.88	<4.88	<4.88	<4.88	<4.88
48	KAR 1	< 4.88	< 4.88	9.77	< 4.88	< 4.88
49	NVZ 1	< 4.88	312.5	19.53	< 4.88	< 4.88
50	TNK 1	0.0195	0.04	0.156	0.3125	5000
51	VJA 1	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
52	VJA 2	< 4.88	0.0195	0.009	< 4.88	< 4.88
53	GNT 14	< 4.88	19.53	19.53	< 4.88	< 4.88
54	GNT 8	< 4.88	19.53	19.53	< 4.88	< 4.88
55	GNT 17	0.009	0.3125	0.3125	1.25	0.009
56	EG1	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
57	VZA6	< 4.88	39.06	2500	< 4.88	< 4.88
58	GNT 7	< 4.88	19.53	39.06	< 4.88	< 4.88
59	GNT18	9.77	< 4.88	39.06	9.77	9.77
60	VZA2	< 4.88	< 4.88	< 4.88	< 4.88	< 4.88
61	TNK 2	< 4.88	39.06	78.12	< 4.88	78.12
62	VJA6	< 4.88	39.06	19.53	< 4.88	< 4.88
63	VZA1	< 4.88	< 4.88	9.77	< 4.88	< 4.88
64	EG6	0.0195	0.3125	1.25	0.009	< 4.88
65	NVZ 2	0.04	<4.88	<4.88	0.04	0.0195
66	VZA 3	<4.88	0.098	2.5	<4.88	<4.88
67	VZA 5	0.009	0.3125	1.25	<4.88	<4.88
68	GNT 21	0.04	0.0195	0.156	<4.88	0.009

CHAPTER – 5

MOLECULAR DETECTION OF ANTIBIOTIC RESISTANT GENES IN *S. AUREUS* ISOLATES

CHAPTER – 5 MOLECULAR DETECTION OF ANTIBIOTIC RESISTANT GENES IN *S. AUREUS* ISOLATES

5.1 INTRODUCTION

MRSA strains which are associated with livestock and poultry may believed to be zoonotically significant because of its capability to inhabit a broad variety of hosts (Paterson et al, 2012). MRSA strains from bovine and humans were to be transmitted between humans and cattle by using various phenotypic and genotypic approaches (Hata et al, 2010, Juhasz-Kaszanyitzky, 2007, Lee, 2003). The cattle infected with MRSA can act as reservoir and soon after transmitted to other humans and animals causing various dreadful infections (AVMA, 2014; Spoor et al, 2013). Hence, the people who are in close proximity because of their occupational necessity viz. farmers, veterinarians, workers at slaughterhouses have the high risk of getting infections caused by these strains (Paterson et al, 2012, Juhasz- Kaszanyitzky, 2007). There are certain reports as evidence for transmission of MRSA of animal origin to persons who are veterinarians (Wulf et al, 2008; Hanselman et al., 2006; O'Mahony, 2005). Another study which has been reported by Graveland and his group described colonization of MRSA in 32 % of people who were in close contact with calf (Graveland et al, 2008) and in 32 % of hospitalized people who had contact with pigs and veal calves (van Rijen et al, 2008). Although, it have been reported two way transmission of MRSA (AVMA, 2014, Price et al, 2012, Juhasz-Kaszanyitzky, 2007) that is transmission from animal to human occurs via direct contact, through handling of infected animal's product (Nunang and Young, 2007) or by environmental contamination but, the transmission from human to animal is still ambiguous (Weese, 2010). Therefore, to understand the resistance impact of these emerging resistant strains there is a need to understand the genetic makeup of these strains. The two antibiotic resistant genes *mecA* and *blaZ* imparting methicillin resistance and β-lactam antibiotics resistance, responsible for the Multi Drug Resistance (MDR) of these *S. aureus* strains.

5.2 MATERIALS AND METHODS

5.2.1 DETECTION OF ANTIBIOTIC RESISTANCE GENES IN *S. aureus*

Antibiotic resistance genes were detected by performing the PCR assays.

5.2.1.1 METHICILLIN RESISTANT GENE (*mecA*)

The genes include *mecA* responsible for methicillin resistance. The PCR conditions and the primer sequences were given in the table 3. PCR was run for 35 cycles started with initial denaturation at 94°C for 5 minutes, denaturation at 94 °C for 45 seconds, annealing at 50 °C for 30 seconds, elongation at 72 °C for 30 seconds and final elongation was set at 72 °C for 10 minutes for *mecA* oligonucleotide primer sets.

5.2.1.2 Beta- lactamase gene (*blaZ*)

The genes include *blaZ* gene responsible for penicillin resistance. The PCR conditions and the primer sequences were given in the table 3. PCR was run for 35 cycles with initial denaturation at 94 °C for 4 min, denaturation at 94 °C for 1 minute, annealing at 50 °C for 60 seconds, elongation at 72 °C for 60 seconds and final elongation was set at 72 °C for 10 minutes for *blaZ* oligonucleotide primer sets.

Table 5.1 Oligonucleotide primers and PCR conditions for detection of antibiotic resistant genes viz., *blaZ* and *mecA* in *S. aureus* isolates. F=forward primer, R=reverse primer

Gene	Primers	Base pairs	Sequence (5'- 3')	Denaturation	Annealing	Extension
blaZ	*blaZ-F*	517 bp	AAGAGATTTGCCTATGCTTC	94°C / 4min	94°C /60sec	55 °C / 60sec
	blaZ-R		GCTTGACCACTTTTATCAGC (*Vesterholm-Nielsen* et al., *1999*			
mecA	*MecA-F*	162 bp	TCCAGATTACAACTTCACCAGG	94 °C / 45sec	50 °C / 30sec	72 °C / 30sec
	MecA-R		CCACTTCATATCTTGTAACG *(Stegger* et al., *2012*)			

PCR was run for 35 cycles with initial denaturation at 94 °C for 4 min and final elongation at 72 °C for 10 min for *blaZ* oligonucleotide primer sets and for 35 cycles with initial denaturation at 94 °C for 5 min and final elongation at 72 °C for 10 min for *mecA* oligonucleotide primer sets.

5.3 RESULTS

5.3.1 Molecular detection of antibiotic resistance genes in *S. aureus*

The etiology of antibiotic resistance in *S.aureus* isolates was explored by detecting the genes *blaZ* gene which produces the enzyme β-lactamase which will inactivate β-lactam antibiotic by split-opening the β-lactam ring in them and *mecA* gene which affords resistance against methicillin by production of altered penicillin binding protein (PBP2') which does not have affinity for β-lactam antibiotic.

5.3.1.1 Presence of *mecA* and *blaZ* in *S. aureus* isolates

Reactivity of oligonucleotide primers specific to antibiotic resistant genes *mecA* and *blaZ* with *S. aureus* isolates in PCR were shown in the figures 21-29. In bovine, total of 34 isolates were screened for the presence of antibiotic resistance genes namely, *blaZ* and *mecA*. Out of which in 14 (45.16 %) isolates *blaZ* gene was present and in 19 (61.29 %) isolates *mecA* gene was present. Besides, in 7 (22.58 %) isolates both *blaZ* and *mecA* genes were present.

5.4 Discussion

MRSA infections are not only important in human medicine but also in veterinary medicine (Lee, 2003, Voss et al., 2005, Baptise et al. 2005, Smith et al., 2008, Khanna et al, 2008). Devriese and his group (1972) isolated MRSA in a dairy cow suffering with mastitis and Stefani and his group (2012) isolated from pigs. Not only in animals, the epidemiological survey conducted by Paterson and his colleagues in UK (2012) reported spread of MRSA in hospitals individuals particularly who are in contact with frequent animal contact. Many recent reports were reported that MRSA can be transmitted from animals to humans and humans to animals (Umaru et al, 2011). And in countries like Belgium, Denmark and Netherland it was reported that the prevalence of MRSA was high (Köck et al, 2009a, Köck et al, 2009b). In a study conducted by Fabler et al, 2010 reported that 25 MRSA isolates harbored *mecA* gene whereas in the present study 19 isolates pocesses *mecA* gene in bovine and 21 *S. aureus* isolates pocesses the same gene in poultry. The present investigation on development of pandrug resistance is also on par with the earlier findings of Kaur and Chate (2015) who reported the fear of development of pan drug resistance in MRSA isolates. In a study conducted by Jamali and his group reported the prevalence of *blaZ* gene in *S. aureus* isolates was 46 % from raw milk and dairy products. In the present investigation 44 *S. aureus* isolates pocesses *blaZ* gene

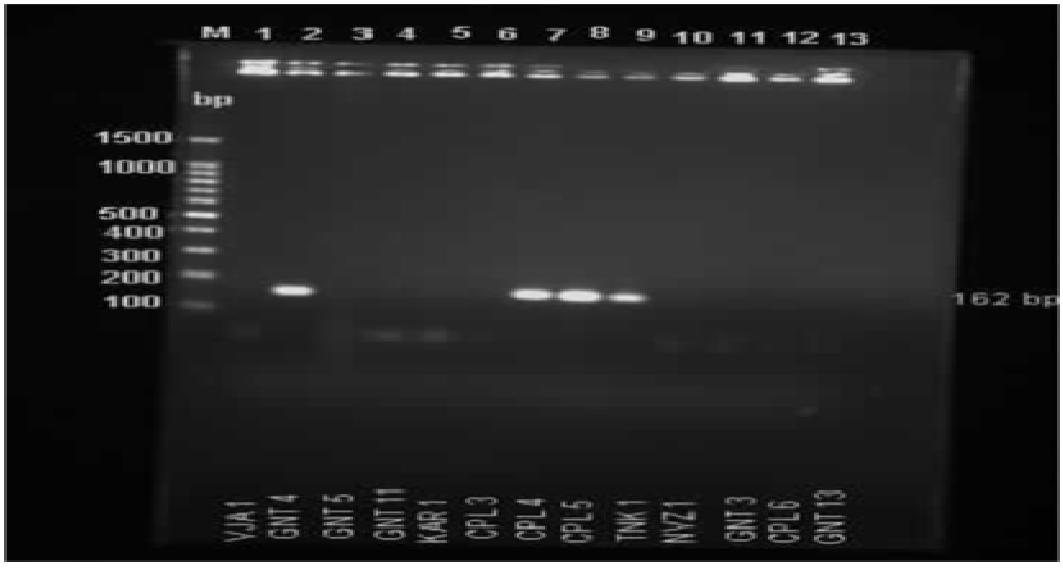

Figure 5.1 Agarose gel electrophoresis pattern showing PCR products for the *mecA* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 2, 7- 9 positive for *mecA* gene whereas 1, 3-6 and 10-13 negative for the *mecA* gene. The sample names were given in lower side of the figure.

Figure 5.2 Agarose gel electrophoresis pattern showing PCR products for the *mecA* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 4 and 11 positive for *mecA* gene whereas 1- 3, 5-10, 12 and 13 negative for the *mecA* gene. The sample names were given in lower side of the figure.

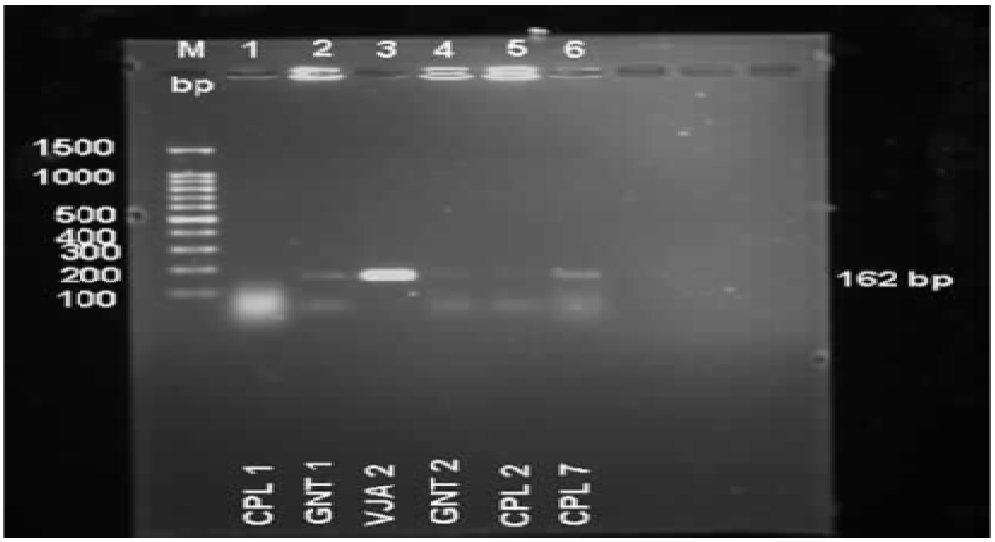

Figure 5.3 Agarose gel electrophoresis pattern showing PCR products for the *mecA* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 2-6 were positive for *mecA* gene whereas 1 negative for the *mecA* gene. The sample names were given in lower side of the figure.

Figure 5.4 Agarose gel electrophoresis pattern showing PCR products for the *mecA* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1, 4, 7- 15 positive for *mecA* gene whereas 2, 3, 5 and 6 negative for the *mecA* gene. The sample names were given in lower side of the figure.

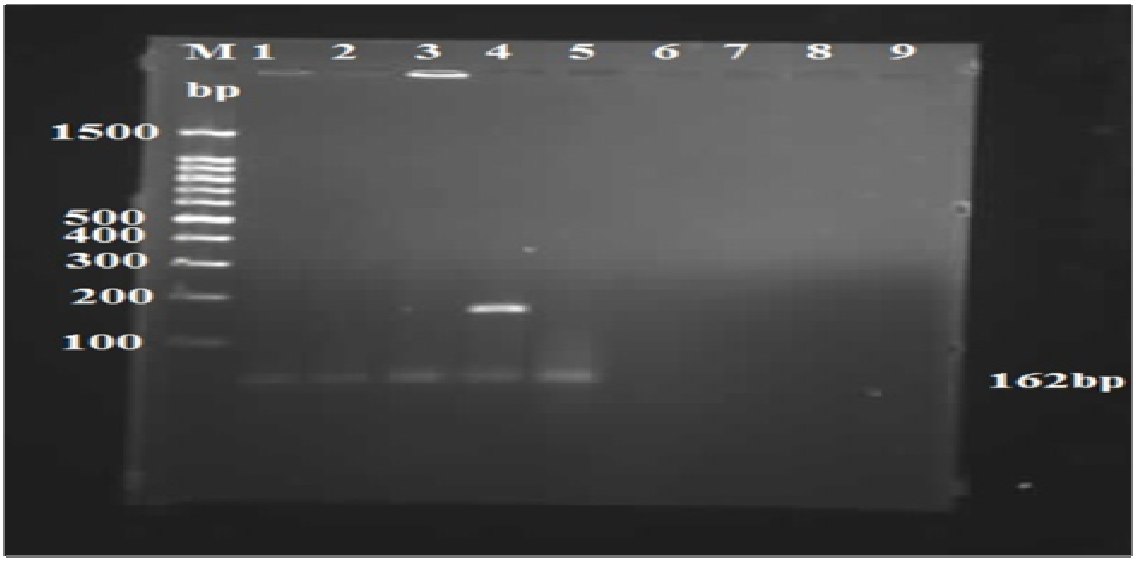

Figure 5.5 Agarose gel electrophoresis pattern showing PCR products for the *mecA* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 4 positive for *mecA* gene whereas 1- 3, 5-9 negative for the *mecA* gene.

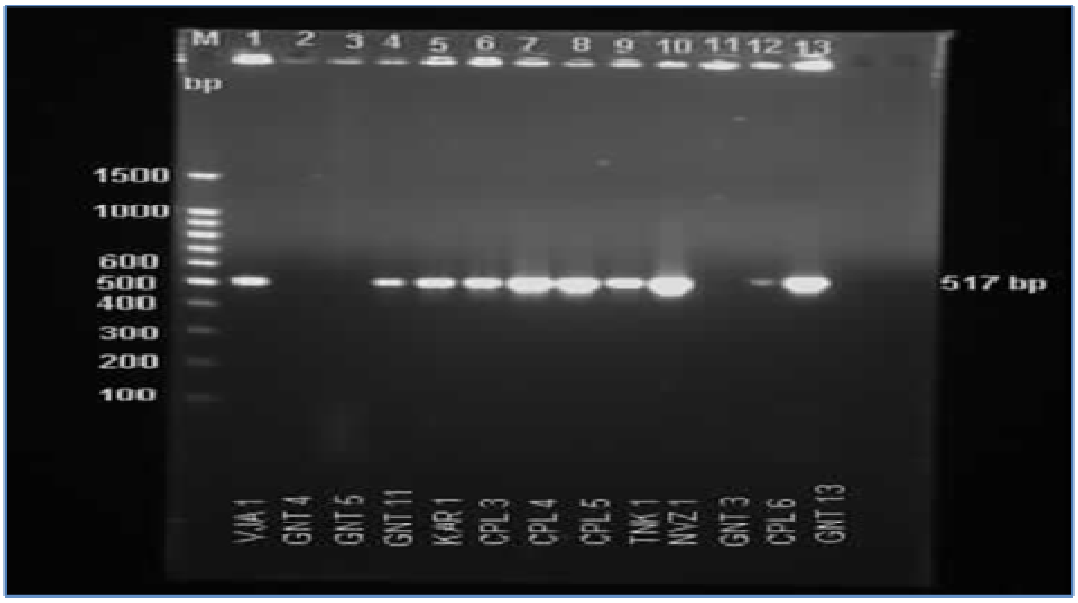

Figure 5.6 Agarose gel electrophoresis pattern showing PCR amplifi*ca*tion products for the *blaZ* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1, 4- 10 positive for *blaZ* gene whereas 2, 3 and 11 are negative for the *blaZ* gene. The sample names were given in lower side of the figure.

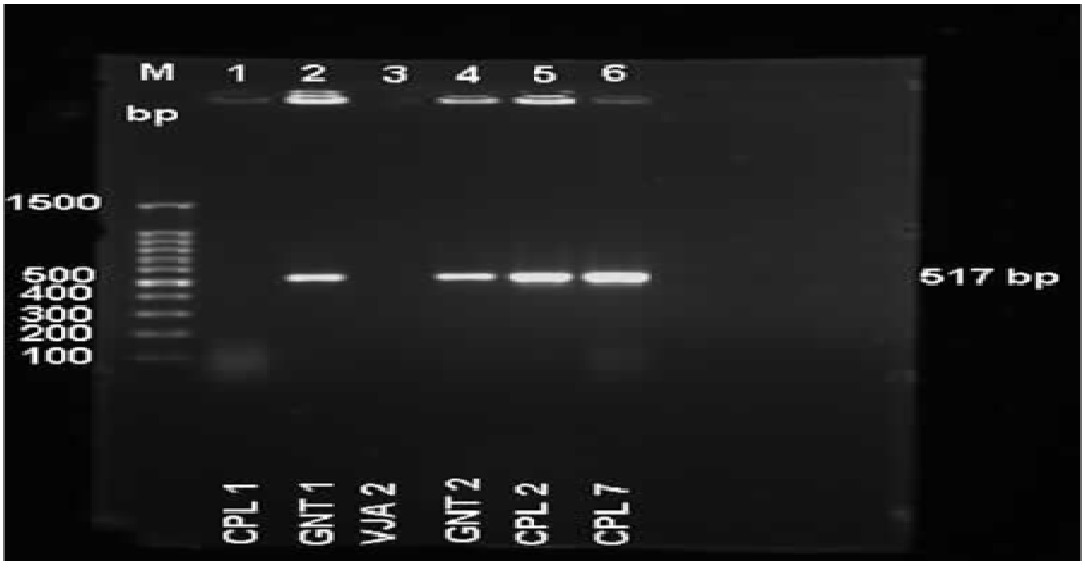

Figure 5.7 Agarose gel electrophoresis pattern showing PCR products for the *blaZ* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 2, 4-6 positive for *blaZ* gene whereas 1 and 3 negative for the *blaZ* gene. The sample names were given in lower side of the figure.

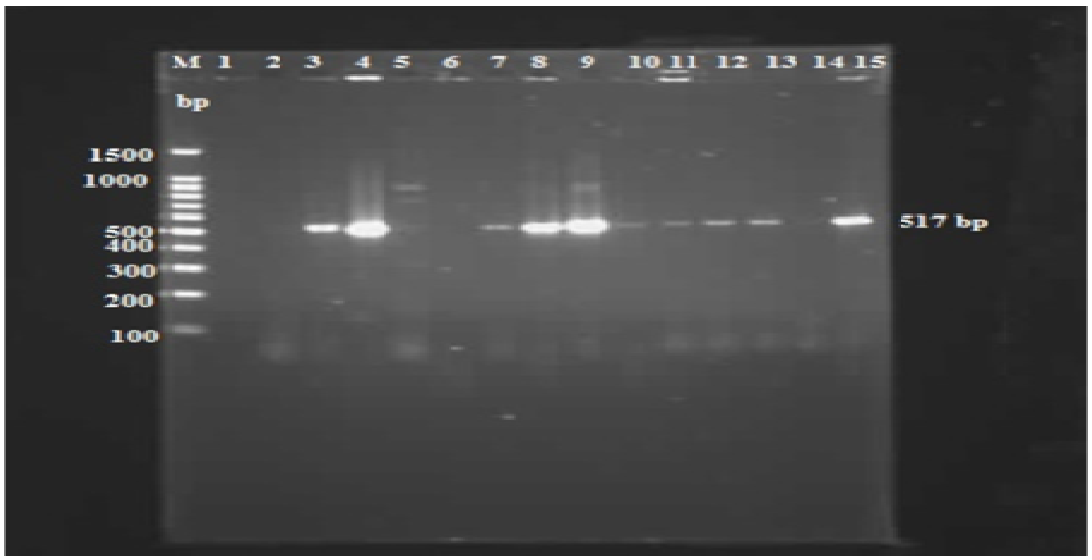

Figure 5.8 Agarose gel electrophoresis pattern showing PCR products for the *blaZ* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 3, 7-13 and 15 positive for *blaZ* gene whereas 1, 2, 5, 6 and 14 negative for the *blaZ* gene.

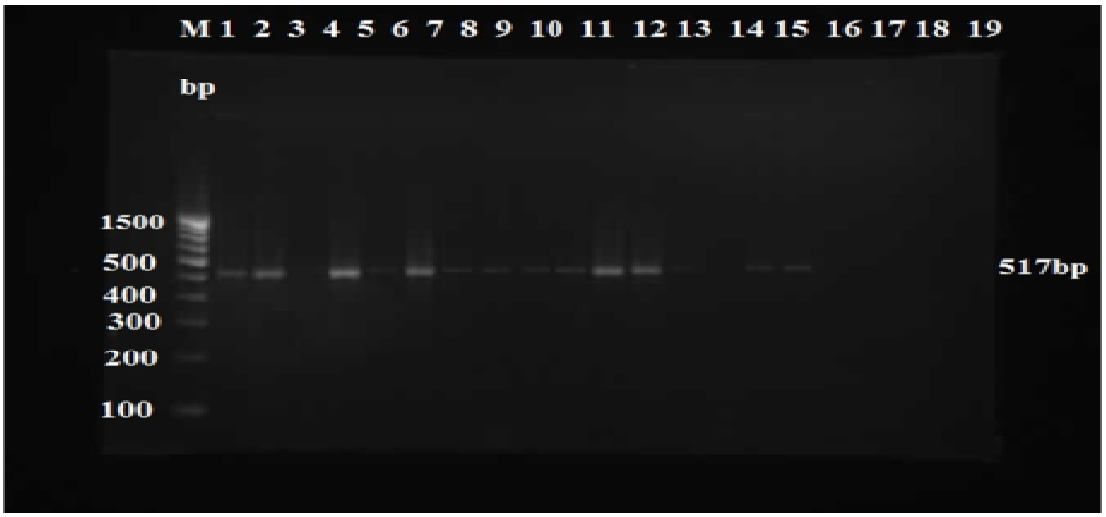

Figure 5.9 Agarose gel electrophoresis pattern showing PCR products for the *blaZ* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1, 2, 4-13, 15 and 16 positive for *blaZ* gene whereas 3, 14 and 17-19 negative for the *blaZ* gene.

In poultry, out of 52 *S. aureus* isolates, 21 (40.38 %) isolates are positive for the presence of *mecA* gene responsible for methicillin and 30 (57.69 %) isolates are positive for the presence of *blaZ* gene. Whereas 16 (30.76 %) isolates are positive for the presence of both *mecA* and *blaZ* genes. The results give a clear picture that prevalence of MRSA in

this region is increasing alarmingly. It was observed that 30 % of the *S. aureus* isolates in poultry and 22 % in bovine as they possess both *mecA* and *blaZ* these isolates are multi drug resistant.

In an overall view, 46.51 % incidence of *mecA* gene and 51.16 % incidence of *blaZ* genes was observed both in bovine and poultry. Incidence of both *mecA* and *blaZ* was 26.74 % (table 5.2).

Table 5.2 Prevalence of antibiotic resistant genes in *S. aureus* isolates.

Test / Gene	**BOVINE**		**POULTRY**		**TOTAL**	
	No	%	No	%	No	%
Positive for *S. aureus* by PCR	34	34	52	86	86	53.75
Number of isolates positive for *mecA* gene	19	55.88	21	40.38	40	46.51
Number of isolates positive for *blaZ* gene	14	41.18	30	57.69	44	51.16
Number of isolates positive for both *mecA* and *blaZ* genes	7	20.58	16	30.76	23	26.74

CHAPTER – 6

GENOTYPIC DETECTION OF PATHOGENIC FACTORS IN *S. AUREUS*

CHAPTER – 6 GENOTYPIC DETECTION OF PATHOGENIC FACTORS IN *S. AUREUS*

6.1 INTRODUCTION

S. aureus is proved to be a successful invading bacterial pathogen in livestock, poultry and in humans due to its ability in producing various virulence factors (Fluit, 2012). The virulence factors include enzymes like catalase, coagulase, nuclease, staphylococcal exotoxins like Staphylococcal enterotoxins, staphylococcal super antigen genes (*see*, *seg-seo* and *seq*), Toxic Shock Syndrome Toxin 1 (TSST-1) and biofilm associated genes like BAP (Biofilm Associated Protein), Inter-Cellular Adhesion A and D (*icaA* and *icaD*) (Fluit, 2012;). Therefore these pathogenic factors aid the *S. aureus* to become endemic and emerging in causing infections and increases the severity in its pathogenicity. And thus it can cause a wide variety of diseases in both humans and animals representing it as a public health concern as well as animal welfare and economic threat to dairy farming.

6.2 Materials and Methods.

6.2.1 Molecular detection of enterotoxin gene in *S. aureus*

The specific genes of enterotoxins were detected by PCR using specific primers of enterotoxins. The following are the genes detected:

1. Staphylococcal enterotoxin e (*see*)

2. Staphylococcal enterotoxin g (*seg*)

3. Staphylococcal enterotoxin h (*seh*) and

4. Staphylococcal enterotoxin i (*sei*)

The PCR conditions and number of cycles were given in the table 4.

Table 6.1 Oligonucleotide primers and conditions used in PCR for detection of *S. aureus* enterotoxin genes.

Gene	Primers	Base pairs (bp)	Primer sequence (5'- 3')	Initial denaturation	Denaturation	Annealing	Extension
See	**See1**	**209**	**AGGTTTTTTCACAGGTCATCC**	**94 °C / 4 min**	**94 °C / 1 min**	**50 °C / 1 min**	**72 °C / 1min**
	See2		**CTTTTTTTTCTTCGGTCAATC**				
Seg	**SegF**	**642**	**AATTATGTGAATGCTCAACCCGATC**	**94 °C / 5 min**	**94 °C / 2 min**	**55°C / 2 min**	**72°C / 1Min**
	SegR		**AAACTTATGGAACAAAAGGTACTAGTTC**				
Seh	**SehF**	**478**	**CAATCACATCATATGCGAAAGCAG**	**94 °C / 5 min**	**94 °C / 2 min**	**55 °C / 2 min**	**72 °C / 1 min**
	SehR		**CATCTACCCAAACATTAGCACC**				
Sei	**SeiF**	**576**	**CTCAAGGTGATATTGGTGTAGG**	**94 °C / 5 min**	**94 °C / 2 min**	**55 °C / 2 min**	**72 °C / 1min**
	SeiR		**AAAAAACTTACAGGCAGTCCATCTC**				

PCR was run for 35 cycles with final elongation at 72 °C for 10 min.

6.2.2 Biofilm:

6.2.2.1 Phenotypic detection of biofilm production

The biofilm production of all *S. aureus* isolates was evaluated by culturing the organism on Congo red agar (CRA) medium. This test was prepared by dissolving the following substance in 1liter of distal water (brain heart infusion broth, 37 g; sucrose 50 g; agar10 g; Congo red agar, 0.8 g). The medium was autoclaved at 121 °C at 15 bound for 15 minutes. Congo red stain was prepared as a concentrated aqueous solution and autoclaved separately from the other medium constituents, and then added when the agar had cooled to 55 °C. Plates were inoculated and incubated aerob*ica*lly for 24 hrs at 37 °C, followed by storage at room temperature for 48 hrs. A positive result was ind*ica*ted by black colonies with a dry crystalline consistency.

6.2.2.2 MICROTITRE PLATE ASSAY

Quantification of *S. aureus* isolates for biofilm formation was carried out by using the microtitre plate assay described by Dwivedi and Singh, 2016 with modifications. An overnight culture of *S. aureus* was grown in Brain Heart Infusion (BHI) broth (HiMedia Laboratories, India) and incubated for 16-18 hrs at 37°C. The volumes and concentrations added in the wells given in the table 6.2 From the overnight culture 1ml was transferred to 10 ml of each 1 % sucrose, 1.5 % sucrose and 2 % sucrose. The culture was adjusted to 5 MF units. Now from each culture 250 µl of volume was transferred into the microtitre plate wells. The blank wells contain only broth and the plates were incubated at 37 °C for 24 hrs. After 24 hrs of incubation the plankton suspension and the nutrient suspension was aspirated followed by washing with Phosphate Buffered Saline (PBS) (300 µl) three times. The plates were strongly shaken to remove all the non-adherent bacterial cells. The remaining bacterial cells were fixed with 96 % (250 µl) ethanol by allowing it for 15 minutes and plates were emptied and left to dry. Now each of the microtitre plate was stained for 5 minutes with 200 µl of 2 % crystal violet. The excess stain was rinsed off by placing the microtitre plate under running tap water. The stained plates were again dried and the biofilms were visible as purple colour rings in the well as shown in the Figure 4.3.1. In order to quantify the biofilm 200 µl of 33 % (V/V) glacial acetic acid was added to each well and then optical density (OD) was measured by using Multiscan ELISA Reader.

6.2 Standardization of microtitre plate Assay for biofilm quantification in *S. aureus* isolates

MF	A	100 ml of 5 MF +100ml of 1% sucrose	150 ml of 5 MF +150ml of 1% sucrose in TSB	Shift 100 ml + 100ml of 1% sucrose in TSB	Shift 100 ml + 100ml of 1% sucrose in TSB	100 ml of 5 MF +100ml of 1.5% sucrose in TSB	150 ml of 5 MF +150ml of 1.5% sucrose in TSB	Shift 100 ml + 100ml of 1.5% sucrose in TSB	Shift 100 ml + 100ml of 1.5% sucrose in TSB	100 ml of 5 MF +100ml of 2% sucrose in TSB	150 ml of 5 MF +150ml of 2% sucrose in TSB	Shift 100 ml + 100ml of 2% sucrose in TSB	Shift 100 ml + 100ml of 2% sucrose in TSB
Sucrose	B	100 ml of 5 MF+100 ml of 1 % TSB sucrose	100 ml of 5 MF +100 ml of 1.5 % TSB sucrose	100 ml of 5 MF+100 ml of 2 % TSB sucrose	50 ml of 5 MF+150 ml of 1 % TSB sucrose	50 ml of 5 MF+150 ml of 1.5 % TSB sucrose	50 ml of 5 MF+150 ml of 2 % TSB sucrose	25 ml of 5 MF+175 ml of 1 % TSB sucrose	25 ml of 5 MF+175 ml of 1.5 % TSB sucrose	25 ml of 5 MF+175 ml of 2 % TSB sucrose			

In the row A1-A12 different volumes of 5 MF (Mac Farland) was tried. In the row B1-B9 different concentrations of sucrose was added and in the rows C1-C9 different concentrations of antibiotic penicillin was added.

6.2.2.3 GENOTYPIC DETECTION OF BIOFILM PRODUCING GENES

Biofilm formation ability of organisms involves detecting Inter Cellular Adhesion D (*ica D*) in the *S. aureus* isolates. The PCR conditions and the number of cycles were given in the table 6.3.

Table 6.3 Oligonucleotide primers and PCR conditions for detection of biofilm producing gene *icaD* in *S. aureus* isolates.

Gene	**Primers**	**Base pairs**	**Sequence (5'- 3')**	**Denaturation**	**Annealing**	**Extension**
icaD	*icaD-F*	198 bp	ATAGGTCAAGCCCAGACAGAG	94 ^{o}C / 45 sec	49 ^{o}C / 45 sec	72 ^{o}C / 10 min
	icaD – R		GCTTGACCACTTTTATCAGC Vasudevan et al, 2003			

F=forward primer, R=reverse primer

PCR was run for 35 cycles with initial denaturation at 94 °C for one min and final elongation at 72 °C for 10 min for *icaD* oligonucleotide primer sets.

6.3 RESULTS AND DISCUSSION

6.3.1 Molecular detection of enterotoxin genes in *S.aureus*

In bovine, 2 *S.aureus* isolates (5.88 %) possess *see* gene, 6 isolates (17.64 %) possess *seg* and *seh* genes, 8 isolates (23.52 %) were positive for *sei* gene and 4 isolates (11.76 %) were positive for three SEs, *seg*, *seh* and *sei*.

In poultry, 2 isolates (3.84 %) were positive for *see* gene, 1 isolate (1.92 %) positive for *seg* gene and 2 isolates (3.85 %) were positive for *sei* gene. The distribution of SE genes in *S. aureus* isolates in bovine and poultry were tabulated in table 6.3.

Table 6.4 The distribution of SE genes in *S. aureus* isolates in clinical cases of mastitis in bovine and dermatitis in poultry.

	Bovine		**Poultry**		**Total**	
	No	**%**	**No**	**%**	**No.**	**%**
Total no. of clinical isolates	**100**		**60**		**160**	
No. of isolates positive for *S.aureus* by biochemical tests	**69**	**69**	**52**	**86.67**	**121**	**75.62**
No. of isolates positive for *S.aureus* by PCR	**34**	**34**	**52**	**86.67**	**86**	**53.75**
No. of isolates positive for *see* gene	**2**	**5.88**	**2**	**3.84**	**4**	**4.65**
No. of isolates positive for *seg* gene	**6**	**17.64**	**1**	**1.92**	**7**	**8.14**
No. of isolates positive for *seh* gene	**6**	**17.64**	**0**	**0**	**6**	**6.98**
No. of isolates positive for *sei* gene	**8**	**23.52**	**2**	**3.85**	**10**	**11.63**
No. of isolates positive for *seg*, *seh* and *sei*	**4**	**11.76**	**0**	**0**	**4**	**4.65**

The PCR results showed that the primers produced amplicons consistent with their predicted sizes (Figures 6.1 to 6.7). The results proved that *S. aureus* isolates from bovine mastitis cases harboured high percentage of SE genes when compared to poultry.

Another important pathogenic factor in *S. aureus* is its ability to form biofilm. The host immune responses are mostly vain in the case of persistant biofilm infections (Nathan and Mark, 2011). In addition, the penetration of several antibiotics such as cefotaxime, oxacillin and vancomycin had reduced throughout *S. aureus* biofilms (Singh et al, 2010).

Staphylococcus aureus is a clinically relevant pathogen due to its antimicrobial resistance and evasion of the host immune system. In conjunction with the multitude and redundancy of its virulence factors in avoiding host responses and influencing disease, S. aureus is able to form intricate micro-colonies termed biofilms. Although neutrophils are

capable of invading the biofilm, the bacterial community is able to thwart this attack and may also skew the immune response to survive attack. Staphylococcus aureus is the etiological agent to a myriad of human acute infections, however, its ability to form biofilm in host emanates into chronic and recalcitrant disease. Current therapies for treating and preventing chronic biofilm-mediated infections are limited to surgical intervention and prolonged antibiotic regiments or addition of antimicrobial compounds to indwelling-medical devices. Vaccination studies have begun to take biofilm development into consideration, and with the combination of genomic and proteomic-based techniques have identified numerous potential vaccination candidates. However, the physiological heterogeneity and subsequent multifarious protein expression throughout the biofilm must be carefully examined for development of an efficacious S. aureus vaccine. Alternatively, modulating the host immune response may prove advantageous in resolving chronic S. aureus infections and warrants further investigation.

Figure 6.1 Agarose gel electrophoresis pattern showing PCR products for the *seg*gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1, 3, 10 and 12 positive for *seg* gene. Lanes: 2, 4-9, 11, 13-15 negative for *seg* gene.

Figure 6.2 Agarose gel electrophoresis pattern showing PCR products for the *sei* gene in *S. aureus* isolates. Lane: Lane M- Marker, Lanes: 8 and 10 positive and Lanes: 1-7, 9, 11-15 negative for *sei* gene.

Figure 6.3 Agarose gel electrophoresis pattern showing PCR products for the *sei* gene in *S. aureus* isolates. Lane: M- Marker, Lane 7 positive. Lanes: 1-6 and 8-15 negative for *sei* gene.

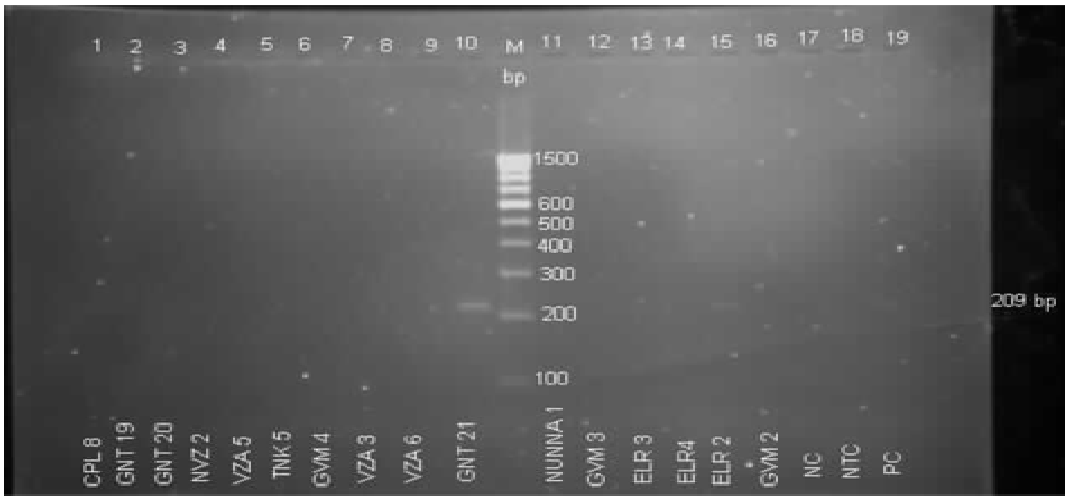

Figure 6.4 Agarose gel electrophoresis pattern showing PCR products for the *see* gene in *S. aureus* isolates. Lane: M- Marker, Lane 10 was positive for *see* gene. Lanes: 1-9 and 11-19 were negative for *see* gene. The sample names were given in lower side of the figure.

Figure 6.5 Agarose gel electrophoresis pattern showing PCR products for the *see* gene in *S. aureus* isolates. Lane: M- Marker, Lane: 9 positive for *see* gene. Lanes: 1-8 and 10-15 negative for *see* gene. The sample names were given in lower side of the figure.

Figure 6.6 Agarose gel electrophoresis pattern showing PCR products for the SE genes in *S. aureus* isolates. Lane: M- Marker, Lane 1 was positive for *seg* gene. Lanes: 4, 6 and 13 positive for *seh* gene. The sample names were given in lower side of the figure.

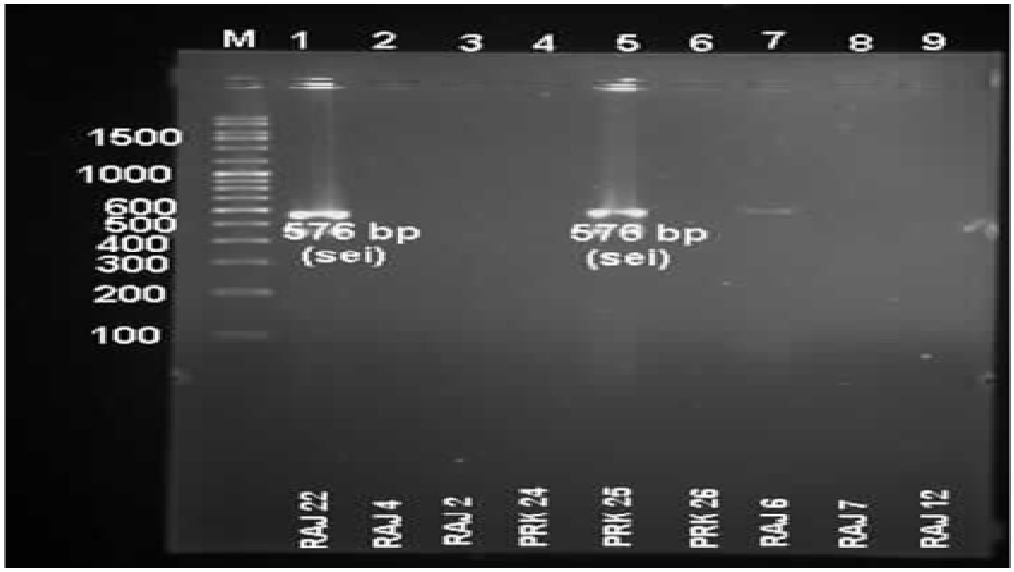

Figure 6.7 Agarose gel electrophoresis pattern showing PCR products for the *see* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1, 5 and7 positive for *sei* gene. Lanes: 2-4, 6, 8, and 9 negative for *sei* gene.

6.3.2 Biofilm:

6.3.2.1 Phenotypic detection of biofilm production

Formation of black colour colonies on Congo Red Agar (CRA) medium plates indicates the production of biofilm in *S.aureus* isolates as shown in the Figure 6.8. The *S.aureus* isolates which produced black colour colonies on CRA medium plates they were further processed for molecular confirmation for the presence of biofilm genes. The percentage positive for species confirmed *S. aureus* isolates on CRA medium was 17.64 % (n=6)in bovine and in poultry 46.15 % (n=24) were positive on CRA medium.

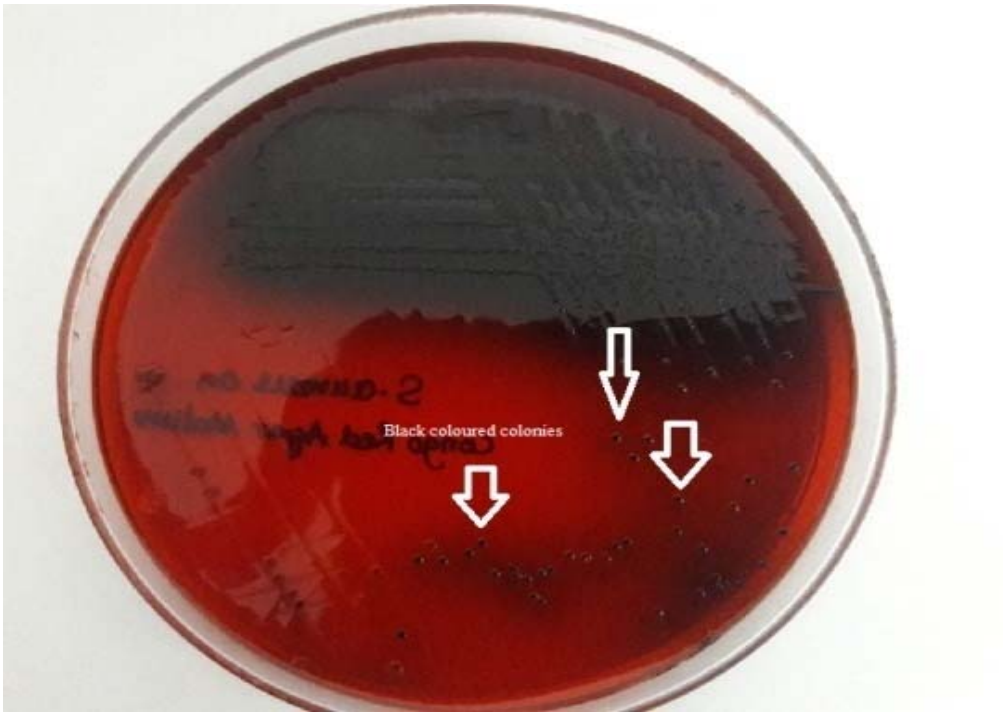

Figure 6.8 Phenotypic detection of biofilm production in *S. aureus* isolates on CRA medium. The black coloured colonies shown by white arrows on the CRA medium.

6.3.2.2 Genotypic detection of biofilm producing *S.aureus* isolates:

The *S. aureus* isolates which reacted with *icaD* specific primers in bovine it was recorded was 17.64 % (n=6) and in poultry 46.15 % (n=24) were positive (Figure 6.9-6.11). The percentage incidence of *icaD* gene in species confirmed *S. aureus* was given in the table 6.4.

Table 6.4. Incidence of bacterial biofilm genes in *S. aureus* isolates

BOVINE			**POULTRY**		
	Incidence			**Incidence**	
Total number of *S. aureus* isolates	*Ica D* (No. of isolates)	*BAP* (No. of isolates)	Total number of *S. aureus* isolates	*IcaD* (No. of isolates)	*BAP* (No. of isolates)
34	**6**	**0**	**52**	**24**	**0**

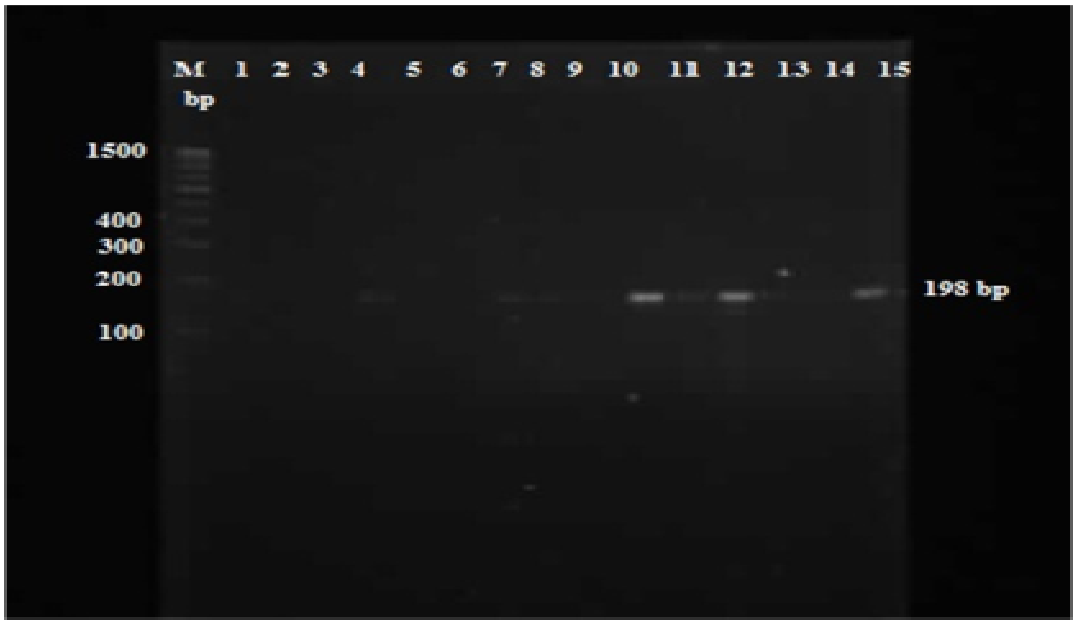

Figure 6.9 Agarose gel electrophoresis pattern showing PCR products for the *icaD* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 4, 8-12 and 15 positive for *icaD* gene. Lanes: 1-3, 5-7 and 15 negative for *icaD* gene

Figure 6.10 Agarose gel electrophoresis pattern showing PCR products for the *icaD* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 7- 9, and 18 positive for *icaD* gene. Lanes: 1-6 and 10-19 were negative for *icaD* gene.

Figure 6.11 Agarose gel electrophoresis pattern showing PCR products for the *icaD* gene in *S. aureus* isolates. Lane: M- Marker, Lanes: 1-3, 5-12, 14 and 15 positive for *icaD* gene. Lanes: 4 and 13 negative for *icaD* gene.

6.3.2.3 Microtitre Plate Assay

The observed maximum absorbance was recorded at 590 nm as 0.463 (Figure 6.12) which ind*ica*ted that these isolates were strong biofilm producers; it was 0.1 to 0.2 as weak biofilm producer and 0.2 to 0.3 as moderate biofilm producer. Out of six *S. aureus* isolates from bovine four isolates were found to be strong biofilm producers and two were moderate biofilm producer. Whereas in poultry, out of 24 *S. aureus* isolates 20 isolates were strong biofilm producer, one isolate was moderate and three isolates were weak biofilm producers.

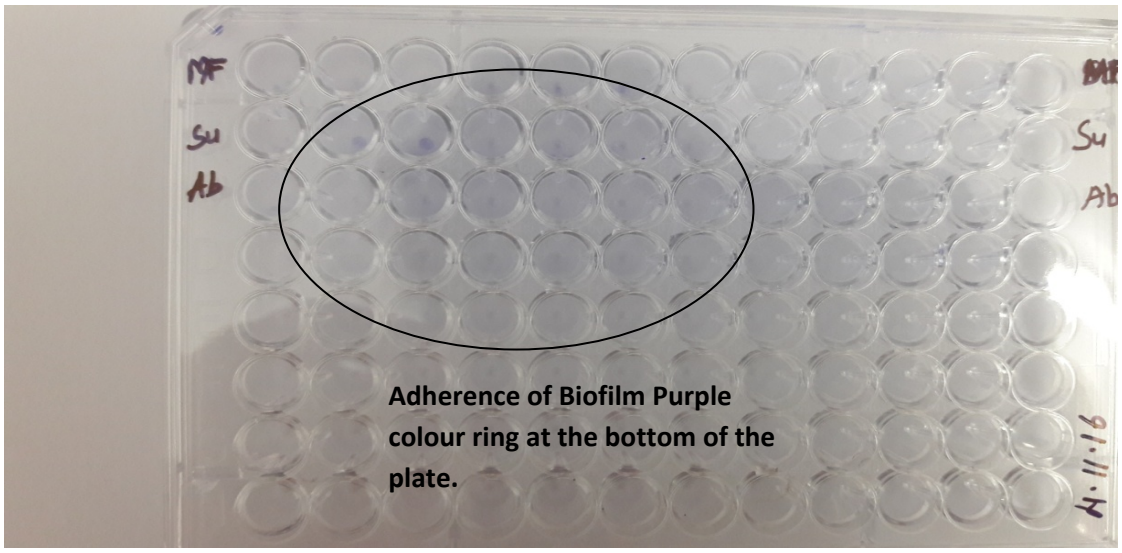

Figure 6.12 Quantification of Bio-film by MTP Method: Maximum absorbance observed at 590 nm indicates that the *S. aureus* isolate as strong bio-film producer

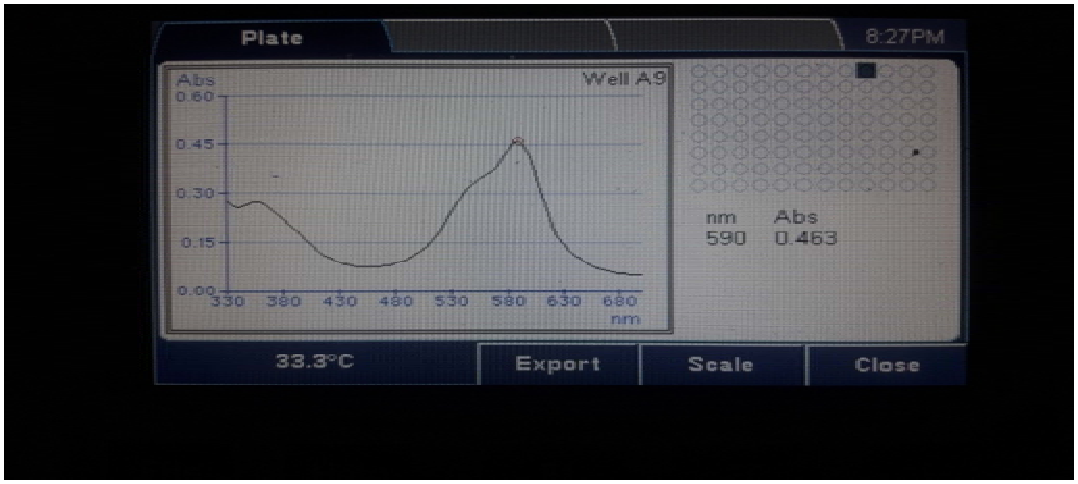

Figure 6.13 Quantification of Bio-film by MTP Method: Maximum absorbance observed at 590 nm indicated that the *S. aureus* isolate as strong bio-film producer.

6.4 DISCUSSION

From the above results we can summarise that the presence of enterotoxin genes in *S. aureus* isolates in bovine is relatively high when compared to *S. aureus* isolates in poultry. In bovine, 5.8 % (see), 17.64 % (*seg* and *seh*), 23.52 % (*sei*) *S. aureus* isolates was reported and in case of poultry *S. aureus* isolates, 3.84 % (see), 1.92 % (seg), 3.85 % (*sei*) and finally seh gene was absent. Katsuhiko Omoe and his group (2001) reported 77.4 % of *S. aureus* isolates harboured SE genes.

The ability of production of biofilm by *S. aureus* isolates increases it pathogenicity and makes the bacterial strain to become resistant to antibiotics. In the present study it has been shown that six isolates out of thirty four (11.11 %) pocesses *icaD* gene whereas in poultry isolates twenty four isolates out of fifty two (46.16 %) harboured *icaD* gene proving that the presence of *icaD* gene is high in poultry.

CHAPTER 7

DISCUSSION

7.1 DISCUSSION

A total of 160 samples were collected from clinical cases of bovine and poultry in four districts of Coastal Andhra Pradesh *viz*., Guntur, Krishna, East and West Godavari. The milk and swab samples which were collected from clinical cases were processed to enrichment for 16-18 hrs in TSB. In microscopic morphology, out of all the enriched cultures 69/100 and 52/60 in dairy and poultry samples respectively showed Gram positive cocci in bunches. When cultured on MSA medium all the 69 and 52 from dairy and poultry samples fermented mannitol and produced yellow colour colonies after 18 – 24 hrs of incubation at 37 °C. And all these samples were found to be catalase positive and oxidase negative. These findings are on par with earlier reports on isolation and characterization of *S. aureus* from clinical cases of mastitis (Carter et al, 1990; Kateete et al, 2010 and Quinn et al, 2012). Depending on the biochem*ica*l characterisation of *S. aureus,* the samples were later confirmed by PCR. There are voluminous reports present where they used PCR as a diagnostic tool to identify *S. aureus* using species specific oligonucleotide primers. Anand Kumar, (2009), Rambabu, (2013) and Sheela et al, (2015) adopted the PCR for detection of *S.aureus* in mastitic milk samples.

The prevalence of *S. aureus* in 100 mastitic milk samples collected from bovine, 34 (34 %) were positive with species specific primers for *S. aureus*. This signifies risk of Staphylococcal mastitis in the dairy animals in coastal Andhra Pradesh. In India, prevalence of *S. aureus* in cattle milk was found 58.3 % Kumar (2009) in Andhra Pradesh, 34.01 % Pradhan et al, (2011), in Nepal it was 29.7 % (Joshi et al, 2014) and in Ethiopia it was 28.1 % (Abera et al, 2012). Another two studies in India revealed an incidence rate of 56 % (Sudhan et al, 2005) and 50 % (Shrestha S. and Bindari, 2012). Alike to our findings, other workers from India like Chavan et al, (2007) in Hissar found prevalence of *S. aureus* in mastitis cases 38.66 % coagulase positive and 29.33 % of coagulase negative *S. aureus*. Sharma et al, 2009 and Roychoudhury and Dutta, 2009) have also reported many positive cases of *S. aureus*. Many workers have found *S. aureus* to be more prevalent than other species of the same genus [31, 32, 33 and 34] in mastitis. However, very few systematic reports were available for bovine mastitis problem in coastal Andhra Pradesh where dairy industry is one the major source of income for farmers. In poultry, 60 samples were collected out of which the prevalence of *S. aureus* was 52 (86.6 %). Whereas in Bangladesh it was reported that 54 (90 %) were positive for

S. aureus in poultry (Yeasneen Ali, 2015). Very little literature was cited for the prevalence of *S. aureus* from GD in poultry. In a nutshell, the prevalence of *S. aureus* was found to be 53.75 % in clinical cases of livestock and poultry which suggested that the control of endemic and emerging *S. aureus* strains in poultry and dairy diseases will require a regular, persistant and urgent concern.

Staphylococcus aureus is the major cause of large-scale morbidity and mortality in both humans and animals (Lowy, 1998). Livestock-associated *S.aureus*, including multidrug-resistant *S.aureus* (MDRSA) and methicillin-resistant *S.aureus* (MRSA), can be exchanged between animals and humans (Price et al, 2012 and Harrison et al, 2013). The nasal carriage has been observed among individuals who are in contact with livestock and poultry throughout Europe, the USA and Canada (Smith and Pearson, 2011). Though human-to- human transmission of livestock-associated strain may occur, they appear to be transmitted less effectively than human-adapted strains (Van Rijen et al, 2014). When compared to intermittent or non-colonisation *S. aureus*, persistent nasal colonisation with *S. aureus* is having an increased risk of infection in the clinical setting (Wertheim et al, 2005).

The presence of LA-MRSA CC398 in the human food chain not only demonstrates the established risk through direct contact with animals it also showsa potential possible further pathway for the transmission of antimicrobial resistance from livestock and poultry to the broader human population (Hadjirin et al, 2015). Inadvertent non-therapeutic use of antibiotics for prophylactic and probiotic purpose, aggravate the risk of development and propagation of antibiotic resistant bacteria (Marshall and Levy, 2011) and studies have proved that antibiotic-resistant bacteria can be transmitted to humans involved in livestock and dairy production management sites (Smith and Pearson, 2011), and from these sites the bacteria are mobilised by means of multiple environmental pathways (Graham et al, 2009; Gibbs et al, 2006 and Rule et al, 2008). Previous studies of persistence have not investigated methicillin-susceptible *S. aureus* (MSSA) or MDRSA, though carriage of these bacteria may have important impl*ica*tions for clinical care and public health (Wertheim et al, 2005).

Infectious diseases of chicken flocks are a major economic burden on the poultry industry. The incidence and prevalence of Staphylococcal dermatitis was reported from long back (Devriese L.A. and Hommez J. (1975) and Lowy, 1998), though currently there are few published reports of incidence from India. The present study ind*ica*ted that the

incidence of *S. aureus* is alarmingly high in diseases of cattle and poultry. Since *S. aureus* is a pandemic organism, the chances of zoonotic transmission to human beings remains to be a potential threat.

In the present study we have attempted to address the antibiotic resistance problem. A total of thirty four and fifty two species specific confirmed *S. aureus* isolates from milk samples and swab samples were tested for antibiotic resistance to obtain crucial information regarding the potential threat of antibacterial resistance in animal diseases viz., mastitis in buffalo and chicken and its possible zoonotic potential. A huge number of isolates were observed to demonstrate resistance to multiple antimicrobials. Frequent and long-term use of a particular antibiotic in a specific region creates a selection pressure in the organisms, resulting in development of resistance in bacteria (23 Sabour et al, 2004 and Moon et al, 2007). In the present investigation, a very high level of resistance was recorded to Oxacillin (100 %), followed by Ceftriaxone + Tazobactam (77.42 %) and Ampicillin (70.97 %). These were followed by Amoxicillin (67.74 %), Ceftriaxone + Sulbactam (61.29 %), Methicillin (83.87 %), Vancomycin (45.16 %), Gatifloxacin (41.94 %), Gentamicin and Penicillin (38.71 %). Maximum susceptibility was shown to Amoxycillin + Clavulanic Acid (0.0 %) followed by Cefoxitin (3.3 %), Ceftriaxone and Linezolid (9.68 %), Streptomycin (12.90 %), Neomycin (25.81 %) and Enrofloxacin (29.03 %). This result is alarming as the organisms are acquiring resistance against commonly employed antimicrobials for Gram positive bacteria used in human beings. The percentage of penicillin-G resistant isolates (38.71 %) in this study was higher than those reported in American herds and European herds (Erskine et al, 2002; Makovec and Ruegg, 2003 and Vintov et al, 2003). There was a higher prevalence of MRSA (48.39 %) as compared with those in similar reports in the literature (Moon et al, 2007 and Van den Eede et al, 2009). Moreover, the multidrug resistance proportion was higher in MRSA than in MSSA isolates for various antimicrobials (Wang et al, 2003 and 23). Unlike the findings of Pankaj et al (2013), in the present study *S. aureus* isolates (9.68 %) were resistant to ceftriaxone. Studies conducted by several workers Sharma et al, 2007, Chavan et al, 2007, Roy Choudhury and Dutta, 2009 and Sharma et al, 2010 have showed increased resistance towards different traditional and newly introduced antimicrobials. In support to these studies, the antibiogram obtained in the current study indicated higher resistance towards newer and older antimicrobials. This proves that *S. aureus* demonstrates a distinctive ability to quickly respond to newer antimicrobials with the development of an appropriate resistance mechanism. The exact mechanism of development of resistance requires a

thorough investigation since it creates an alarming situation of non-responsiveness of antibiotic and transmission of resistance across other genera. Further the positive *S. aureus* isolates were screened for the presence of antibiotic resistant genes like *blaZ* which is responsible for penicillin resistance and *mecA* gene responsible for methicillin resistance. In the present investigation from milk samples the percentage positive for *mecA* was 61.29 %, whereas in the investigation carried by Memon et al, 2013 *mecA* gene was absent in *S. aureus* isolates from bovine mastitis. Lee, 2006 isolated 525 *S. aureus* isolates, out of which 19 (3.61) were positive for *mecA* gene from bovine mastitis cases. In Finland out of 135 isolates only one isolate of *S. aureus* from bovine mastitis was *mecA* positive (Gindonis et al, 2013) and in another study carried in West Bengal only 18.42 % was positive for the presence of *mecA* gene in *S. aureus* from bovine mastitis (Paul, 2015). In Tamilnadu (Gindonis et al, 2013), the percentage positivity for *mecA* was 10.34 %. The present study is clear evidence that is a high risk of emergence of MRSA in India with special reference to Coastal Andhra Pradesh. This may probably due to improper use of antimicrobials by unqualified people, which is prevailing in this area, though we don't have conclusive treatment history of individual animals that are tested. Unlike to the results of Memon et al, 2013 where they got 82 % 0f *blaZ* positive in the present work the percentage positive of *blaZ* gene was 45.16 %, whereas in Tamilnadu it was 10.34 %. Few reports were reported for the presence of MRSA in poultry. Among them, Davy Persoons (2009) and his group reported MRSA in broiler chickens whereas in the present investigation, the observed methicillin resistance was 73.08 % in poultry which was higher than 67.5 % reported by Gundogan and his group. But in Switzerland, no MRSA was reported in poultry (Huber, 2009). Yeasneen Ali (2015) in Bangladesh reported the percentage of *mecA* gene was 21.43 % whereas in the present investigation it was reported that 40.38 % was present and *blaZ* gene it was 57.69 % and 7 *S.aureus* isolates (30.78 %) pocesses both genes in the same isolate. From the results it was clearly proved that *S. aureus* isolates are emerging with greater and unique resistance pattern.

Staphylococcal enterotoxins (SEs) are a huge heterogenic group of protein exotoxins, rather differential in respect of their nucleotide and amino-acid homology, as well as the location of their genes, molecular weight and isoelectric point value. SEs was identified in 1959 as the extracellular proteins produced by some *S. aureus* strains. These enterotoxins are known as the pyrogenic toxins and this group also contains other staphylococcal toxins (staphylococcal toxicshock syndrome toxin-TSST-1, A and B exfoliative toxins and streptococcal scarlet fever toxin). Twenty one serolog*ica*l types of staphylococcal

enterotoxins are distinguished. SEs are thermo-stable proteins, resistant to many proteolytic enzymes (pepsin, trypsin, chymotrypsin, renin and papain), but this resistance depends on the temperature and pH. Staphylococcal enterotoxin-encoding genes are located in the chromosomal DNA and Pathogenicity Island such as in phages, transposons and plasmids. In humans, staphylococcal enterotoxins are responsible for food poisoning, as these enterotoxins are also isolated from milk samples infected with mastitis (Nawrotek et al, 2005). In the present investigation, it was reported that the virulent enterotoxin genes in *S. aureus* isolates from mastitic milk samples were amplified using primers and the results revealed that two isolates (5.88 %) were found positive for *see* gene, six isolates (17.64 %) positive for *seg* gene, eight isolates (23.52 %) were positive for *sei* gene and six isolates were found *seh* positive. Four isolates were found to be positive for genes, *seg*, *seh* and *sei*. And in case of poultry, two isolates (3.84 %) were found positive for *see* gene, one isolate (1.92 %) positive for *seg* gene, two isolates (3.85 %) were positive for *sei* gene and none of the isolates were found *seh* positive. One isolate was found positive for genes, *seg* and *sei*. In poultry isolates, studied by Mostafab Nemati (2013) 57 % of the *S. aureus* isolates harboured *seg* and *sei* and other genes are absent. From the results it was clearly observed that the presence of enterotoxin genes is high in mastitic milk samples than in poultry.

Another major virulence factor of *S. aureus* is its ability to form biofilm *in vivo* which greatly influences its pathogenicity (Vasudevan et al, 2003). Biofilm is defined as a group of microbial cells associated with a surface and enclosed in an extracellular matrix predominantly made up of polysaccharide material (Donlan, 2002). Bacteria in biofilm exhibit high resistance to antibiotics, disinfectants, as well as host immune system clearance (Donlan, 2002 and Donlan et al, 2002). Formation of black coloured colonies on CRA medium plates indicated the attribute of *S. aureus* to synthesize biofilm. In addition all these isolates were found positive for *icaD* specific primers confirming the prevalence of pathological factor, biofilm. These biofilm producing colonies constituted 46.15 % and 17.64 % of the confirmed *S.aureus* isolates in poultry and dairy respectively. Dhanewade et al, (2010) reported that 35.29 % strains of *S. aureus* isolated from bovine mastitis in their study were positive for *icaD* gene. The isolates which were positive for *IcaD* gene were further processed for the quantification of biofilm. It was observed that the maximum absorbance recorded at 590 nm is 0.463 which indicated that the isolates were strong biofilm producers.

Depending upon the type of organisms and use of antimicrobials in a particular region antibiotic resistance patterns vary among different farms, regions, states and countries. Prudent use of antimicrobials in the dairy animal and poultry birds is important, necessary and worthwhile. Therefore, antimicrobial sensitivity test is recommended before institution of treatment, so that injudicious antibiotic usage and thus the development of resistance will be prevented. Moreover, prophylactic management measures against mastitis and GD rather than therapeutic management using antimicrobials has to be encouraged in dairy as well as in poultry industry. The information obtained from the present study will be of helpful to the dairy and poultry industries and veterinarians and zoonotic disease experts which will be useful not only in prioritizing mastitis and GD control efforts but in zoonotic transmission control as well.

CHAPTER - 8

CONCLUSIONS AND SCOPE FOR FUTURE WORK

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8.1 CONCLUSIONS

Out of 100 mastitic milk samples and 60 GD samples from bovine and poultry 69 % (n=100) and 52 % (n=60) were provisionally confirmed due to *S. aureus* by morphological, cultural and by biochemical tests. Whereas, 34/69 and 52/52 samples from bovine and poultry were confirmed as *S. aureus* with species specific primers *staur 4* and *staur 6* in PCR.

With regard to antibiotic sensitivity test in confirmed *S. aureus* isolates by species specific primers in poultry, highest resistance was recorded for ceftriaxone+ tazobactam, amoxicillin (25µg) and penicillin (100 %) followed by gatifloxacin (98.08 %), ceftriaxone (92.31 %), cefoxitin (90.38 %), ampicillin (88.46 %), vancomycin, streptomycin and neomycin (84.62 %), gentamicin (82.69 %), amoxicillin (30 µg) (80.77 %),methicillin and ceftriaxone +sulbactam (73.08 %), enrofloxacin (69.23 %), oxacillin (67.31 %), linezolid (48.08 %) and amoxicillin + clavulanic acid (30.77 %). The order of resistance patterns of *S. aureus* isolates to different antibiotics in mastitic milk samples: oxacillin (100 %), Ceftriaxone + Tazobactam (77.42 %), ampicillin (70.97 %), ceftriaxone + Sulbactam (61.29 %), amoxicillin (67.74 %), methicillin(48.39 %), vancomycin (45.16 %), gatifloxacin (41.94 %), gentamicin (38.71 %), penicillin (38.71 %), neomycin (25.81 %), streptomycin (12.90 %), linezolid (9.68 %), ceftriaxone (3.3 %), cefoxitin (3.3 %) and amoxycillin + clavulanic Acid (0 %). These results showed that *S. aureus* isolates were found resistant to most of the antibiotics. Most signifi*ca*nt part of the study is that *S.aureus* isolates were showing resistance to most recent antibiotic linezolid which is being used currently against *S.aureus* infections. Interestingly, vancomycin resistance was also found high among this endemic isolate which is a dangerous alarm for public health concern.

Out of 34 and 52 confirmed *S. aureus* isolates in bovine and poultry that were tested for the presence of *blaZ* and *mecA* genes 41.17 % (n=14) and 57.69 % (n=30) *S. aureus* isolates were positive for *blaZ* gene, whereas, 19 and 21 *S. aureus* isolates were positive for *mecA* gene in bovine and poultry respectively. However some isolates which were positive for *mecA* were also found to be positive for *blaZ*. These isolates were 20.58 % (n=7) from bovine and 30.76 % (n=16) from poultry which signify that these strains of *S. aureus* were considered to be Multi Drug Resistant *S. aureus* (MDRSA).

Further in relation to the enterotoxins production, a total of four confirmed *S. aureus* isolates harbored *see* gene, seven isolates pocesses *seg* gene, six isolates harbored *she* gene and 10 isolates harbored *sei* gene respectively in both bovine and poultry. This clearly showed that milk and meat may contaminate with SEEs when consumed by humans in a population which may lead to food poisoning.

With regard to biofilm production 17.64 % (n=6) confirmed *S. aureus* isolates and 46.15 % (n=24) isolates were positive for biofilm formation on CRA medium from bovine and poultry and all these *S. aureus* isolates were positive for the presence of *icaD* gene. As per microtitre plate assay (MTP) out of 30 isolates 20 isolates were strong biofilm producers whereas 8 isolates were moderate and 2 isolates were weak biofilm producers.

The present status of emerging and re-emerging *S. aureus* infections in India has been reviewed. The true prevalence of these diseases is not known. Since we exist in a "global village", we cannot afford to be self-satisfied about the remarkable social, public health and economical burden of these diseases. Effective inspection is the key to their early control. There is a need to develop rapid, simple, affordable and specific diagnostic facilities to study the epidemiology at the community level. The approach to fight against these diseases needs a effective risk communication, epidemic preparedness, strong public health structure and rapid response.

8.2 SCOPE FOR FUTURE WORK

1 As the *S. aureus* isolates are endemic and emerging with different virulent approaches we can expand the geographical locations for the study.

2 Even though recently PCR have been described for identification of *S. aureus* by using species specific oligonucleotide primers, still there is a need for specific, rapid and simultaneous detection of virulent genes of *S. aureus* strains for epidemiological and disease diagnostic purposes.

3 The prevalence of virulent gene doesn't mean that it has been expressed. So the study can be extended to evaluate the gene expression of different pathogenic factors by Real Time PCR.

4 When we throw light on the multiple contradictory information, the true relationship between nature of the *Staphylococcal* exotoxins and their pathogenesis stay on to be resolved

5 Though the current research has focused on biofilm formation and its role in host immunity toward infection, development of effective anti-biofilm *S. aureus* therapies are needed.

6 The future scope of work may rise in the direction of development of antibodies targeting each pathogenic factor in emerging and endemic *S. aureus* isolates.

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RESEARCH PAPERS PUBLISHED

1. Mohana Sheela G, Ramani Pushpa R N and Krupanidhi S (2016). Molecular Detection of blaZ and mecA Genes and Study of Antibiotic Resistance Pattern in Clinical Isolates of *Staphylococcus aureus* from Bovine Mastitis in Coastal Andhra Pradesh. Current Trends in Biotechnology and Pharmacy. 10 (4) 298-308.

2. Mohana Sheela G and Krupanidhi S. (2015) Prevalence, biochem*ica*l characterization and molecular detection of *Staphylococcus aureus* in different clinical cases of livestock and poultry in coastal Andhra Pradesh. International Journal of Microbiology Research, 7 (5) 698-7 02.

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M.Sc. -Biotechnology	:	Monissory Mahila Kalsala, Nagarjuna University in 2010.
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RESEARCH EXPERIENCE:

- **PhD Biotechnology (2013-Till date)**. Thesis Title: **"Study of pathogenic factors in *Staphylococcus aureus* from clinical cases in livestock and poultry."** The research work emphasizes on 1. Rapid and accurate detection of *Staphylococcus aureus* for controlling various diseases in live stock and poultry. 2. Incidence of *S.aureus* in dairy herds and commercial flocks might help us to formulate strategies for reducing the spread of infection. 3. Detection of the virulence factors helps to identify the strains of *S. aureus* circulating in coastal Andhra. 4. Study of resistance pattern in *S. aureus* isolates to different antibiotics.

- Worked as a **Senior Research Fellow (2012 March- 2015 March)** in the **NIAB-DBT** research project entitled **"Exploration of antibacterial, immunomodulatory and anti-inflammatory activity of certain polyphenolic compounds and role of NSAIDs on activity of antibiotics in Bubaline Mastitis"** at Department of Veterinary Microbiology, NTR-College of Veterinary Sciences, Gannnavaram.

- **M.Sc., dissertation** on the thesis entitled **"Development of a Bact-ELISA model for sero monitoring in disease diagnosis".**

ACCOLADES

- **Won first prize in working model presentation** at National Seminar on "BioTech-Forays into Environment" organized by Post Graduate Dept of Life-Sciences, **Andhra Loyola College,** Vijayawada, Andhra Pradesh.

- **Won Jr. Scientist Award at International conference** on "Recent advances in biosciences and applications of engineering in production of biopharmaceuticals and 9th annual convention of association of biotechnology and pharmacy." Organized jointly by Dept of Biotechnology**, KL University, Vaddeswaram, Guntur** and Dept. of Biotechnology and Pharmacy, Acharya Nagarjuna University, Guntur, December 2015**.**

- **Presented paper in National Symposium** on "Challenges and advances in disease diagnosis of Livestock, Poultry and Fish, Redefining the role of veterinary pathologists." **At NTR College of Veterinary Sciences, Gannavaram,** December, 2015**.**

- **Presented paper and poster at International conference** on "15th annual convention of Indian Society for Veterinary Pharmacology and Toxicology" at **National Dairy Research Institute, Karnal, Harayana and won best poster award,** January, 2016**.**

- **Presented paper at First A.P. Science Congress, 2015** "Science for Smart Technologies" at **Sri Venkateshwara University, Tirupati,** January, 2016**.**

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TECHNICAL SKILLS:

Immunology: ELISA, Immuno Electrophoresis.

Molecular Biology: Genomic DNA isolation and purification, RNA Isolation, PCR, Chromatography, Protein Estimation Methods, Agarose Gel Electrophoresis, cell lines maintenance, Testing antibacterial activity of antibiotics, plant tissue culture and animal tissue culture.

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PAPERS PUBLISHED:

Mohana Sheela G and Krupanidhi S. (2015) *Prevalence, biochemical characterization* and *molecular detection of Staphylococcus aureus in different clinical cases of livestock* and *poultry in coastal* And*hra Pradesh.* International Joumal of Microbiology Research, ISSN:0975-5276 and EISSN: 0975-9174, VolumeT, Issue5, 2015, pp 698-7 02.G.

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- National Seminar on **"Agricultural Biotechnology"** organized by Department of biotechnology, Jagarlamudi Kuppuswamy choudary college, Guntur, February 2009.

- UGC sponsored National Seminar on **"Emerging Trends in Biosciences",** organized by Montessori Mahila Kalasala, Vijayawada, February 2009.

- National Seminar on **"Role of Biotechnology in Alleviating Environmental Pollution",** organized by Montessori Mahila Kalasala, Vijayawada, February 2009.

- National Seminar on **"Biotech-Forays in to Environment (Biosastra 2K10)",** organized by PG Department of Life Sciences, Andhra Loyola College, Vijayawada, February 2010.

- Workshop on **"Biotechn-Industry Awareness Campaign for B.TECH Students."**at Vignan University, Vadlamudi, Guntur dist, October 2014.

- International conference on **"Recent advances in biosciences and appl*ica*tions of engineering in production of biopharmaceut*ica*ls and 9th annual convention of association of biotechnology and pharmacy."** Organized jointly by Dept of Biotechnology, KL University, Vaddeswaram, Guntur and Dept. of Biotechnology and Pharmacy, Acharya Nagarjuna University, Guntur, December 2015.

- National Symposium on **"Challenges and advances in disease diagnosis of Livestock, Poultry and Fish, Redefining the role of veterinary pathologists."** At NTR College of Veterinary Sciences, Gannavaram, December, 2015.

- International conference on **"15th annual convention of Indian Society for Veterinary Pharmacology and Toxicology"** at National Dairy Research Institute, Karnal, Harayana January, 2016**.**

- First A.P. Science Congress, 2015 **"Science for Smart Technologies"** at Sri Venkateshwara University, Tirupati, January, 2016.

- International Conference on **"Emerging trends in synthesis of nano particles in Agri Biotechnology- Research Commercialization"** organized at Loyola Academy Degree and P.G. College, Alwal, Secunderabad, February, 2016.

- **G. Mohana Sheela**, Vamsi Krishna B, K Ravi, Suresh N Nair, Mohammad I and Krupanidhi S.K. (2017) "*In Silico* and *invitro* antibiofilm activity of selected phytochemicals on the biofilm producing *S. aureus*."on the annual conference of "Indian society of veterinary pharmacology and toxicology and a national symposium on "Animal health and production: Challenges and Opportunities in Veterinary pharmacology and toxicology, held at Navsari Agricultural University, Navsari, Gujarat, INDIA. **(Best poster award).**

PERSONAL PROFILE

Name	:	G. Mohana Sheela.
Father's Name	:	G.V.R. Brahmam.
D.O.B	:	08/08/1986.
Marital Status	:	Married.

I hereby declare that the above provided details are true to the best of my knowledge and belief.

Place: Vijayawada.