PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME \textit{Mec} (SCCMec)

TYPES OF METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMAN ORIGIN

By

SANJAY NANJIBHAI GHODASARA

(Registration No: J4-01511-2014)
M.V.Sc. (Veterinary Microbiology)

DEPARTMENT OF VETERINARY MICROBIOLOGY
COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY
JUNAGADH AGRICULTURAL UNIVERSITY
JUNAGADH - 362 001

MARCH – 2018
PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME MEC (SCCMEC) TYPES OF METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMAN ORIGIN

A THESIS SUBMITTED TO JUNAGADH AGRICULTURAL UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN VETERINARY MICROBIOLOGY BY

SANJAY NANJIBHAI GHODASARA
(Registration No: J4-01511-2014)
M.V.Sc. (Vet. Microbiology)

DEPARTMENT OF VETERINARY MICROBIOLOGY COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY JUNAGADH AGRICULTURAL UNIVERSITY JUNAGADH - 362 001

MARCH – 2018
Dedicated
To
My Beloved Parents
&
My Family
“PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME MEC (SCCMEC) TYPES OF METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMAN ORIGIN”

ABSTRACT

Key words: Staphylococcal Cassette Chromosome mec (SCCmec), MRS, HA-MRS, CA-MRS, animal, human

The present study was carried out with the objectives of isolation, identification and molecular characterization of Staphylococcal Cassette Chromosome mec (SCCmec) types along with antimicrobial resistance patterns and presence of virulent genes (toxic genes) in methicillin-resistant staphylococci from animals and humans. Out of 202 animals and 100 human nasal swabs, 86 (42.57%) and 62 (62%) isolates were Staphylococcus spp., respectively based on biochemical and molecular based identification. The antibiogram study revealed higher rates of methicillin, gentamicin, ofloxacin and levofloxacin sensitivity to human isolates, whereas higher susceptibility to amikacin and rifampicin followed by oxytetracyclin and chloramphenicol were observed in animal isolates. Out of total 86 and 62 staphylococci isolates, 74 and 50 isolates were Coagulase Negative Staphylococci (CoNS), 12 (from each) were Coagulase Positive Staphylococci (CoPS). Of these total Staphylococci isolated from both the species, 9 and 20 isolates were identified as Methicillin-Resistant Staphylococci (MRS) from animal and humans, respectively. Out of these MRS isolates, 8 and 18 were Methicillin-Resistant Coagulase Negative Staphylococci (MRCoNS), 1 and 2 isolates were Methicillin-Resistant Coagulase Positive Staphylococci (MRCoPS) from animal and humans, respectively. One isolate was identified as Methicillin-Resistant Coagulase Negative Staphylococcus aureus (MRCoNSA) from the animals and 2 isolates were identified as MRSA from humans. Of these 2 isolates, one isolate was Methicillin-Resistant Coagulase Negative Staphylococcus aureus (MRCoNSA) and one isolate was Methicillin-Resistant Coagulase Positive Staphylococcus aureus (MRCoPSA).

The study conducted for presence of virulence genes and their SCCmec typing on total 235 Staphylococcus spp. including departmental isolates which included, 148 Staphylococcus spp. (86 from animal milk/pus samples and 62 from human nasal swabs) from present study and 87 Staphylococcus spp. (47 from animal milk/pus samples and 40 from human nasal swabs) from departmental isolates. Out of these, 16 (12.03%) from animals and 40 (39.21%) from humans were having mecA gene which were classified as Methicillin-resistant staphylococci. Of these, 3 (2.26%) different isolates were found positive for all these virulence genes i.e. PVL, hla and icaA from
animals, whereas 7 (6.86%), 6 (5.88%) and 4 (3.92%) isolates were found positive for PVL, hla and icaA gene from humans, respectively. The SCCmec typing of MRS isolates were studied from 16 animals MRS isolates, of these, 14 isolates having one of the SCCmec types (SCCmec type I, 2; SCCmec type II, 0; SCCmec type III, 1; SCCmec type IV, 5; SCCmec type V, 6), whereas 2 isolates were Untypable. Out of total 40 humans MRS isolates, 28 isolates having one of the SCCmec types (SCCmec type I, 7; SCCmec type II, 0; SCCmec type III, 3; SCCmec type IV, 9; SCCmec type V, 9), whereas 12 isolates were Untypable. Based on SCCmec typing, 18.75% (3/16) and 25% (10/40) isolates were classified as hospital associated methicillin-resistant staphylococci (HA-MRS), whereas 68.75% (11/16) and 45% (18/40) isolates were classified as community associated methicillin resistant staphylococci (CA-MRS) from animal and humans, respectively. The overall percentage of CA-MRS (63.04%) was higher as compare to HA-MRS (28.26%) among both the species.

The detection of SCCmec types IV and V suggested the prevailed of CA-MRSA strains in this geographical area and occurrence of SCCmec I and II alleles indicated a possible transmission of MRSA from human to animals. The prevailed of same SCCmec types among animal and humans attribute to transmission of MRS from animal to human or vice versa indicating potential zoonotic pathogen prevalence in farm and farm workers.
CERTIFICATE-I

This is to certify that the thesis entitled “PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME MEC (SCCmec) TYPES OF METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMAN ORIGIN” submitted by SANJAY NANJIBHAI GHODASARA in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy (Veterinary Science), in the subject of VETERINARY MICROBIOLOGY to Junagadh Agricultural University is a record of bonafied research work carried out by him under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title. The candidate has fulfilled all prescribed requirements. The assistance and help received during the course of investigation have been fully acknowledged. He has successfully completed the comprehensive/preliminary examination held on May 02, 2017 as required under the regulation for post-graduate studies. He has submitted kachcha bound thesis on 01/03/2018.

Place: Junagadh
Date: 01/3/2018

(J. H. PUROHIT)
Major Guide and
Retired professor and Head (AAU)
Department of Veterinary Microbiology
College of Veterinary Science & A.H.
Junagadh Agricultural University
Junagadh
CERTIFICATE-II

Date: / 3/2018

This is to certify that the thesis/project work report entitled “PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF STAPHYLOCOCCAL CASSETTE CHROMOSOME MEC (SCCmec) TYPES OF METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMAN ORIGIN” submitted by SANJAY NANJIBHAI GHODASARA to Junagadh Agricultural University, Junagadh in partial fulfilment of the requirements for award of the degree of DOCTOR OF PHILOSOPHY (Veterinary Science) in the subject of VETERINARY MICROBIOLOGY after recommendation by the external examiners were defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination was satisfactory. We, therefore, forward with recommendation.

(J. S. Patel)
Minor Guide and Professor and Head
Department of Veterinary Medicine
College of Veterinary Science & A.H.
Junagadh Agricultural University
Junagadh

(J. H. Purohit)
Major Guide and
Retired professor and Head (AAU)
Department of Veterinary Microbiology
College of Veterinary Science & A.H.
Junagadh Agricultural University
Junagadh

(B. S. Chandel)
External Examiner and
Professor & Head,
Dept. of Animal Biotechnology
College of Veterinary Science & A.H
S.D.A.U., Sardarkrushinagar

(Ashish Roy)
External Member and
Retired Professor and Head
Dept. of Veterinary Microbiology
College of Veterinary Science & A.H
AAU, Anand

(P.H. Tank)
Principal and Dean
College of Veterinary Science & A.H.
Junagadh Agricultural University
Junagadh

Approved By

(V.P. Chovatia)
Director of Research and Dean, P. G. Studies
Junagadh Agricultural University
Junagadh
ACKNOWLEDGEMENT

I can do all thing through Maa Khodiyar, who strengthens me and this gives me great pleasure in expressing my deep sense of gratitude first to god almighty the giver of life, wisdom and enablement to complete my Ph.D. degree in this prestigious university.

I express my sincere gratitude and thanks to my Major Guide, Dr. J. H. Purohit Retired Professor and Head, Department of Veterinary Microbiology, College of Veterinary Science and A. H., J.A.U., Junagadh for his constant support, constructive criticism, meticulous supervision, endless inspiration, expert guidance, valuable suggestions, unreserved help throughout the course of my research work at this institute. Without doubt, he is the most altruistic person I have come across so far in my life. I accept the holistic contribution of my guide as a lifelong debt and will never forget the golden moments of last many years spent with him.

I am highly grateful to Dr. J. S. Patel, Professor & Head, Department of Veterinary Medicine, College of Veterinary Science & A.H., J.A.U., Junagadh for his valuable suggestions, altruistic help, constant encouragement and keen interest on the progress of my work.

I acknowledge sincere and profound thanks to my committee members Dr. B. S. Mathapati, Assistant Professor, Department of Veterinary Microbiology, College of Veterinary Science and A. H., J.A.U., Junagadh for his excellent cooperation, constant encouragement, technical guidance and invaluable counsel throughout my research work. I am highly thankful to Dr. H. H. Savsani, Associate Professor, Animal nutrition, Dr. V. A. Kalaria, Assistant Professor, Department of Veterinary Pathology and Dr. P. H. Tank, Principal and Dean, College of Veterinary Science and A. H., J.A.U., Junagadh, for their constant attention, valuable guidance and help throughout the period of investigation.

I wish to record my thanks and indebtedness to Dr. A. R. Pathak, Honorable Vice chancellor, J.A.U., Junagadh, Dr. V. P. Chovatia, Director of Research and Dean PG studies, J.A.U., Junagadh and Dr. A. M. Parakhya, Registrar, J.A.U., Junagadh for allowing Ph.D. study and providing me all facility for research work.

I am highly thankful to the Principal and Dean Dr. P. H. Tank, College of Veterinary Science and A. H., J.A.U., Junagadh for providing Research facilities and an opportunity to pursue my higher studies from such an esteemed institute of Gujarat state.

I am highly obliged and grateful to Dr. B. B. Javia, Assistant Professor and Head, Dr. D. B. Barad, Assistant Professor, Department of Veterinary Microbiology, Dr. A. R. Bhadania, Assistant Professor, Dr. D. I. Fefar, Assistant Professor, Department of Veterinary Pathology, Dr. Jignesh vadalia, Assistant Professor, Department of veterinary surgery and Radiology, Dr. N. R. Padalia, Assistant Professor, department of VCC, College of Veterinary Science & A.H. for their valuable technical and moral support. I extend my thanks to Dr. K. S. Murthy, Research Scientist, Cattle Breeding Farm, J.A.U., Junagadh for granting a permission for the sample collection from CBF during my study.
I am highly thankful to Dr. A. R. Ahlawat, Associate Professor & Head, Department of Animal Genetics and Breeding, Dr. R. J. Padodara, Assistant Professor & Head, Department of Veterinary Physiology & Biochemistry, Dr. S. H. Sindhi, Assistant Professor, Dr. J. B. Kathiria, Assistant professor, Department of Veterinary Public Health, Dr. M. D. Odedara, Associate Professor, Department of Livestock Production Management, Dr. H. B. Patel, Assistant Professor and Dr. U. D. Patel, Associate Professor and Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and A. H., J.A.U., Junagadh, for their encouragement and guidance during the study and Research work.

I wish to extend my thanks to Mr. Vikram kodiyatar, Laboratory Technician, Mr. Pratik Morasada, Laboratory Technician, P. G. Academic branch, Mr. Anand Unadkat, Laboratory Technician, Department of AGB, Mr. Chirag Parmar, Laboratory technician Department of Veterinary Microbiology, and Mr. Nitunj Chavada, Livestock inspector, store department, College of Veterinary Science & A.H., J.A.U., Junagadh for their timely help during my study.

I appreciate the co-operation from Dr. Aniruddhaksinh Zala, Post Graduate student, Mr. Divyesh Kamani, Research Associate, Mr. Hardik Gareja for their timely help, support and willing co-operation with good wishes during the entire course of study.

I wish to extend my thanks to my wife, Mrs. Reena Ghodasara, B. Ed. and my sister-in-law, Miss. Jinal patel, DMLT, for their continues encouragement, moral support and technical assistance during the research work.

I appreciate the support from Mr. Ashish Paghadar, Laboratory boy, Department of Veterinary Microbiology and Mr. Jayesh Gadchar, Laboratory boy, Department of LPT, College of Veterinary Science and A. H., J.A.U., Junagadh.

I am also thankful to Dr. R. K. Kathiria, Associate Professor, Office of the Director of Research, J.A.U., Junagadh, Dr. Yogesh Gnelani, Assistant Registrar (Administration), and Dr. D. T. Fefar, Assistant Registrar (Academic), Office of the Registrar, J.A.U., Junagadh. Staff members of Academic Branch of Principal Office and Staff members of Examination Branch of the Registrar Office, J.A.U., Junagadh for their co-operation and help during my studies.

At this inexplicable moment of joy, Words are inadequate in the available lexicon to express my gratitude towards my family. My heart takes breath when I realize the painstaking efforts taken up by my “Family” for making me post graduate of the noblest profession. Words are just not sufficient to express my feeling for their love, blessing, affection and encouragement given to me by my beloved Father Shree Nanjibhai, Mother Prabhaben, Wife Reena and my daughter Kavya for their constant patience and silent support which have truly helped me during the pursuit of my study.

Last but not the least, I shall ever, remain thankful and indebted to all those known and unknown hands who directly or indirectly have been associated with the completion of my work and motivated me to achieve my goal.

Place: JUNAGADH
Date: /03/2018

Sanjay Nanjibhai Ghodasara.
# CONTENTS

<table>
<thead>
<tr>
<th>CHAPTERS</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1.1 Statement of the problem</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1.2 Practical utility of the research problem</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>1.3 Objectives of the study</td>
<td>9</td>
</tr>
<tr>
<td>II</td>
<td>REVIEW OF LITERATURE</td>
<td>10-59</td>
</tr>
<tr>
<td></td>
<td>2.1 Staphylococci as etiological agents for various ailments in animal and humans.</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>2.2 Isolation and identification of <em>Staphylococcus</em> spp. from animal and humans and their biochemical characterization.</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>2.3 Determination of antibiotic resistance in staphylococci by conventional susceptibility testing.</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>2.4 Prevalence and reporting of Methicillin resistance staphylococci among different animal and humans.</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>2.5 Molecular detection of virulence genes (Toxic genes) including Panton-Valentine leukocidin (<em>PVL</em>) gene from MRS organism.</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>2.6 Identification and molecular characterization of Methicillin-resistance staphylococci and SCCmec typing from animal and humans.</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>2.7 Correlation study of SCCmec types with staphylococcal associated virulence gene.</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>2.8 Methicillin-resistant staphylococci and its zoonotic importance.</td>
<td>57</td>
</tr>
<tr>
<td>III</td>
<td>MATERIALS AND METHODS</td>
<td>60-80</td>
</tr>
<tr>
<td></td>
<td>3.1 Location of the study</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3.2 Area of the study</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3.3 Lab wares, media and chemicals for the study</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3.4 Collection of samples from milk, pus/exudate (Abscess) from animal and human nasal swabs</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>3.5 Isolation and identification of staphylococci from animal and human nasal swabs.</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>3.6 <em>In vitro</em> antibiogram pattern of staphylococcus from animal and human beings.</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>3.7 Identification of MRS using specific media</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>3.8 Molecular based evaluation of staphylococcal isolates from animal and humans.</td>
<td>68</td>
</tr>
</tbody>
</table>
3.9 Molecular detection of MRSA from animal and human | 71
3.10 Molecular detection of virulent genes (Toxic genes) from MRS isolates. | 72
3.11 Molecular detection of SCCmec types from methicillin-resistant staphylococci from animal and humans. | 72
3.12 DNA confirmation by agarose gel electrophoresis | 73

**IV RESULTS AND DISCUSSION**

4.1 Isolation and identification of staphylococci from animal and humans and their biochemical characterization. | 84
4.2 Antibiotic susceptibility profile of *Staphylococcus* spp. from animal and human. | 92
4.3 M-PCR for the detection of MRSA from animal and human isolates. | 111
4.4 Molecular detection of virulence genes (Toxic genes) from MRS isolates. | 121
4.5 Molecular characterization of major SCCmec types among Methicillin-resistant staphylococci from animal and humans. | 128
4.6 Correlation study of SCCmec types with virulence genes (Toxic genes). | 134
4.7 Methicillin-resistant staphylococci and its zoonotic and humanosis importance. | 136

**V SUMMARY AND CONCLUSIONS**

138-142

**VI BIBLIOGRAPHY**

I-XXXVI

**VII APPENDICES**

143-146
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE NO.</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Interpretive criteria for susceptibility categorization based on MIC value (mcg/ml) for different antibiotics.</td>
<td>67</td>
</tr>
<tr>
<td>3.2</td>
<td>Quantity and concentration of various components used in PCR.</td>
<td>73</td>
</tr>
<tr>
<td>3.3</td>
<td>Commercially available antibiotics disk with their respective concentration used for staphylococcal isolates.</td>
<td>75</td>
</tr>
<tr>
<td>3.4</td>
<td>Nucleotide sequences of primers used for m-PCR with their product size and reference.</td>
<td>76</td>
</tr>
<tr>
<td>3.5</td>
<td>Nucleotide sequences of primers used for the identification and SCCmec types of MRS.</td>
<td>76</td>
</tr>
<tr>
<td>3.6</td>
<td>Primers used in SCCmec type PCR and the resulting gel band patterns of SCCmec types I-V.</td>
<td>77</td>
</tr>
<tr>
<td>3.7</td>
<td>Nucleotide sequences of primers used for the PCR amplification of the virulence genes of <em>staphylococci</em> spp.</td>
<td>77</td>
</tr>
<tr>
<td>3.8</td>
<td>Thermal cycling condition for different primer pairs in PCR for identification of MRSA organism.</td>
<td>78</td>
</tr>
<tr>
<td>3.9</td>
<td>Thermal cycling condition for different primer pairs in PCR used for SCCmec typing.</td>
<td>78</td>
</tr>
<tr>
<td>3.10</td>
<td>Thermal cycling condition for different primer pairs in PCR for detection of virulence gene.</td>
<td>79</td>
</tr>
<tr>
<td>4.1</td>
<td>Details of the samples from various sources</td>
<td>84</td>
</tr>
<tr>
<td>4.2</td>
<td>Biochemical character of <em>Staphylococcus</em> spp. isolates (Animal and human nasal swabs).</td>
<td>86</td>
</tr>
<tr>
<td>4.3</td>
<td>Coagulase Negative (MRCoNS) and coagulase positive (MRCoPS) MRS isolates from animal ad humans.</td>
<td>87</td>
</tr>
<tr>
<td>4.4</td>
<td>Hemolysis patterns of Methicillin-resistant staphylococci isolates.</td>
<td>87</td>
</tr>
<tr>
<td>4.5</td>
<td>Antibiotic resistance patterns of Methicillin-resistant <em>Staphylococcus</em> spp. from animal and human.</td>
<td>101</td>
</tr>
<tr>
<td>4.6</td>
<td>Antibiotic resistance patterns of <em>Staphylococcus</em> spp. (Including MRS) from animal (n=86) and human (n=62).</td>
<td>102</td>
</tr>
<tr>
<td>4.7</td>
<td>Minimum inhibitory concentrations of selected antibiotics for Methicillin-resistant <em>Staphylococcus</em> spp. from animal isolates.</td>
<td>103</td>
</tr>
<tr>
<td>4.8</td>
<td>Minimum inhibitory concentrations of selected antibiotics for Methicillin-resistant <em>Staphylococcus</em> spp. from human isolates.</td>
<td>104</td>
</tr>
<tr>
<td>4.9</td>
<td>Correlation of antibiotic resistant V/S presence of mecA gene</td>
<td>104</td>
</tr>
<tr>
<td>4.10</td>
<td>Multiple resistance indices of Methicillin-resistant staphylococci from human nasal swabs.</td>
<td>105</td>
</tr>
<tr>
<td>4.11</td>
<td>Multiple resistance indices of Methicillin-resistant staphylococci from animal isolates.</td>
<td>106</td>
</tr>
<tr>
<td>4.12</td>
<td>Culture and PCR based identification of staphylococci from different sources of animal.</td>
<td>115</td>
</tr>
<tr>
<td>4.13</td>
<td>Cultural and PCR based identification of staphylococci from human nasal swabs.</td>
<td>117</td>
</tr>
<tr>
<td>4.14</td>
<td>Composition of reaction mixture for multiplex PCR with simple Master Mix.</td>
<td>119</td>
</tr>
<tr>
<td>4.15</td>
<td>Multiple virulence genes (Toxin genes) in animal and human MRS isolates.</td>
<td>125</td>
</tr>
<tr>
<td>4.16</td>
<td>Prevalence of SCC\textit{mec} types among Methicillin-resistant staphylococci and associated virulence genes among animals and human isolates.</td>
<td>126</td>
</tr>
<tr>
<td>4.17</td>
<td>Occurrence and correlation between SCC\textit{mec} type isolates based on HA-MRS and CA-MRS among animal and human MRS isolates.</td>
<td>129</td>
</tr>
</tbody>
</table>
## LIST OF FIGURE

<table>
<thead>
<tr>
<th>FIGURE NO.</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Research Design for isolation, identification and molecular characterization of MRSA isolates for SCCmec typing.</td>
<td>80</td>
</tr>
<tr>
<td>4.2</td>
<td>Antimicrobial resistance patterns of Methicillin-resistant staphylococcus from animal and humans.</td>
<td>102</td>
</tr>
<tr>
<td>4.3</td>
<td>Antimicrobial resistance patterns of <em>Staphylococcus</em> spp. with different antibiotics in animal and human isolates.</td>
<td>103</td>
</tr>
<tr>
<td>PLATE NO.</td>
<td>TITLE</td>
<td>PAGE NO.</td>
</tr>
<tr>
<td>-----------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>4.1</td>
<td>Colonies of <em>Staphylococcus</em> spp. on Nutrient agar (NA)</td>
<td>107</td>
</tr>
<tr>
<td>4.2</td>
<td>Gram positive cocci in bunch shown distinguishing gram staining pattern of <em>Staphylococcus aureus</em>.</td>
<td>107</td>
</tr>
<tr>
<td>4.3</td>
<td>Mannitol fermentation of <em>Staphylococcus</em> spp. on mannitol salt agar (24 hours at 37°C).</td>
<td>108</td>
</tr>
<tr>
<td>4.4</td>
<td>Methicillin-resistant <em>Staphylococcus</em> spp. colonies on selective chromogenic media (48-72 hours at 37°C).</td>
<td>108</td>
</tr>
<tr>
<td>4.5</td>
<td>Tube coagulase test (1) and Slide coagulation test (2) using rabbit plasma.</td>
<td>109</td>
</tr>
<tr>
<td>4.6</td>
<td>Antibiotic sensitivity test (disk diffusion method) with different antibiotics.</td>
<td>109</td>
</tr>
<tr>
<td>4.7</td>
<td>Determination of minimum inhibitory concentration using E-test.</td>
<td>110</td>
</tr>
<tr>
<td>4.8</td>
<td>Hemolysis patterns of <em>Staphylococcus</em> spp. on sheep blood agar (48 hours at 37°C).</td>
<td>110</td>
</tr>
<tr>
<td>4.9</td>
<td>PCR amplicons of Methicillin-resistant coagulase negative/positive <em>Staphylococcus aureus</em> by m-PCR (16S rRNA gene = 370bp, <em>MecA</em> = 454bp, <em>Coa</em> = 280bp and <em>Nuc</em> = 117bp) from animal isolates.</td>
<td>120</td>
</tr>
<tr>
<td>4.10</td>
<td>PCR amplicon of Methicillin-resistant coagulase negative/positive <em>Staphylococcus aureus</em> by mPCR (16S rRNA gene = 370bp, <em>MecA</em> = 454bp, <em>Coa</em> = 280bp and <em>Nuc</em> = 117bp) from human isolates.</td>
<td>121</td>
</tr>
<tr>
<td>4.11</td>
<td>PCR amplicons of (<em>icaA</em>, <em>hla</em> and <em>PVL</em>) of virulence genes among Methicillin-resistant staphylococci from animal and humans.</td>
<td>126-127</td>
</tr>
<tr>
<td>4.12</td>
<td>SCC<em>mec</em> type specific PCR amplification products (SCC<em>mec</em> type I-V) from animal MRS isolates.</td>
<td>132</td>
</tr>
<tr>
<td>4.13</td>
<td>SCC<em>mec</em> type specific PCR amplification products (SCC<em>mec</em> type I-V) from human MRS isolates.</td>
<td>133</td>
</tr>
</tbody>
</table>
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>/</td>
<td>Per</td>
</tr>
<tr>
<td>%</td>
<td>Per cent</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BTB</td>
<td>Bromothymol blue</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion</td>
</tr>
<tr>
<td>CA-MRSA</td>
<td>Community Acquire Methicillin Resistance <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>ºC</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>ccr</td>
<td>Cassette chromosome recombinase</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>CoNS</td>
<td>Coagulase Negative <em>Staphylococcus</em></td>
</tr>
<tr>
<td>CoPS</td>
<td>Coagulase-Positive <em>Staphylococcus</em></td>
</tr>
<tr>
<td>CoPMRS</td>
<td>Coagulase Positive Methicillin Resistance <em>Staphylococci</em></td>
</tr>
<tr>
<td>CoNMRS</td>
<td>Coagulase Negative Methicillin Resistance <em>Staphylococci</em></td>
</tr>
<tr>
<td>DW</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-deoxyribonucleoside-5 triphosphate</td>
</tr>
<tr>
<td>e.g.</td>
<td>For Example</td>
</tr>
<tr>
<td>et al.</td>
<td><em>et alia</em></td>
</tr>
<tr>
<td>F/R</td>
<td>Forward / Reverse</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>Fig.</td>
<td>Figure</td>
</tr>
<tr>
<td>ºF</td>
<td>Degree Fahrenheit</td>
</tr>
<tr>
<td>gm(s)</td>
<td>Gram(s)</td>
</tr>
<tr>
<td>HA-MRSA</td>
<td>Hospital Acquired Methicillin Resistance <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>hr(s)</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est (that is)</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>mA</td>
<td>Milli ampere</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>v/v</td>
<td>volume / volume</td>
</tr>
<tr>
<td>víz.</td>
<td>Videlicet (namely)</td>
</tr>
<tr>
<td>w/v</td>
<td>weight / volume</td>
</tr>
</tbody>
</table>
CHAPTER-I
INTRODUCTION

*Staphylococcus aureus* is one of the most extensively studied bacteria of genus *Staphylococcus*. *Staphylococcus aureus* is both commensal and pathogen. It is found as a commensal associated with skin, skin glands and mucous membranes and predominately associated with bovine and ovine mastitis. All pathogenic *S. aureus* produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin is used to distinguish *S. aureus* from others members of the genus, which are collectively designated as coagulase-negative staphylococci (CNS) (Ryan and Ray, 2004). CNS also referred to as “*Environmental Staphs*” during recent years, coagulase-negative staphylococci (CNS) have become the most common bovine mastitis isolates in many countries and are regarded as emerging mastitis pathogens (Dutta, 2009; Jakee *et al.*, 2013).

Amongst the Staphylococci group, *Staphylococcus aureus* strains are more dangerous to dairy animals and are of greatest attention for scientific community worldwide as they possess properties *viz.*, capabilities to produce enzymes, toxins and intrinsic virulence factors which cause invasion & tissue damage to mammary gland, it is having ability to survive in the keratin of the teat canal of healthy cow and capability to resist phagocytosis (protein-A). Apart from this there are low level of complements and opsonizing antibodies specific to *Staphylococcus aureus* in milk (Green and Bradley, 2004) and most importantly having an ability to resist antibiotic therapy due to production of beta lactamases a group of enzymes that inactivate penicillin and closely related antibiotics. Staphylococci is one of the leading causes of bacterial infections in developed countries and is responsible for a wide spectrum of diseases, ranging from minor skin infections to fatal necrotizing pneumonia. Since the introduction of penicillin into medical treatment in early 1940s, the resistance for beta-lactamase as started to develop. It was a result of the acquisition of a plasmid, coding for penicillinase, a penicillin-hydrolysing enzyme, which is able to cleave the beta-lactam ring and thus inactivate antibiotic molecule. Penicillin resistant strains soon spread not only in healthcare facilities, but also in the community. To overcome
infections caused by beta-lactamase-producing Staphylococci, narrow spectrum semi-synthetic penicillin (methicillin) was introduced.

However, soon after that, in 1961, first Methicillin-resistant *Staphylococcus aureus* (MRSA) strain was identified. Initially, MRSA strains were encountered only in the hospitals, but in the late 1990s first virulent community-acquired MRSA (CA-MRSA) clones, characterized by the presence of the toxin Panton-Valentine Leukocidin (*PVL*), appeared rapidly and unexpectedly. They quickly spread worldwide, initially only in the community, but later on also in the healthcare facilities, displacing in some countries typical HA-MRSA. For this reason, nowadays, distinction between CA-MRSA and mostly multi resistant HA-MRSA become challenging (Chambers and Deleo, 2009; Deurenberg and Stobberingh, 2008).

Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged globally as a significant nosocomial pathogen of public health concern and it is constantly evolving to resist the antibiotics both in human and veterinary medicine. In recent years, the so-called livestock-associated MRSA has become an additional focus. MRSA isolates are frequently multidrug resistant (MDR), which can result in higher costs, longer treatment times, and higher rates of horizontal transfer of resistance to other pathogens (Noskin *et al*., 2005; Cosgrove, 2006).

MRSA from bovine sources was first reported in 1975 (Devriese and Hommez, 1975), although it has occasionally been reported since then the detailed study of MRSA in bovine mastitis is lacking (Huber *et al*., 2010; Fessler *et al*., 2010; Vanderhaeghen *et al*., 2010a). The presence of MRSA in bovine milk and dairy environments poses potential risk to farm workers, veterinarians, consumers and other farm animals (Lee, 2003) which can have adverse and deleterious public health concerns.

The resistance of *S. aureus* to methicillin is caused by the presence of the *mecA* gene, encoding for an additional 78-kDa penicillin binding protein 2a, (PBP2a orPBP2'). Compared to other PBP, PBP2a has a low affinity for all beta-lactam antibiotics. As a result of that, even in the presence of a beta-lactam antibiotic, the peptidoglycan layer biosynthesis is not disrupted and the bacterium can survive (Berger-Bachi and Rohrer, 2002; Deurenberg and Stobberingh, 2008). The *mecA* gene is located within a *mec* operon together with its regulatory genes: *mecI* and *mecR1*.
(Berger-Bachi and Rohrer, 2002). The mec operon is carried by staphylococcal cassette chromosome mec (SCCmec). The origin of SCCmec is still unknown, but it is proposed that it was acquired by *S. aureus* from *S. sciuri* and that the mecA-positive coagulase-negative staphylococci (CoNS), especially *S. epidermidis*, may be a potential reservoir for the SCCmec element (Mongkolrattanothai et al., 2003). On the other hand, it is suggested that the main source of SCCmec could be MRSA itself (Aires de Sousa and de Lencastre, 2003). There are also suggestions of possible acquisition of the mecA region from *S. fleurettii*, which is a commensal bacterium of animals (Tsubakishita et al., 2010). SCCmec typing, which classifies SCCmec elements on the basis of their structural differences, is applied in epidemiological studies to distinguish MRSA strains or to define an MRSA clone in combination with the genotype of Methicillin-susceptible *S. aureus* (MSSA) strain in which a SCCmec element has integrated.

**SCCmec element composition**

The mecA gene is usually carried on a mobile genetic element called staphylococcus cassette chromosome mec (SCCmec), which contains two main part: the ccr gene complex (ccr) and mec gene complex (mec). The ccr gene complex comprises ccr gene (s), ccrA and ccrB, or ccrC, which encode site-specific recombinases responsible for mobility of SCCmec, and surrounding open reading frames (ORFs). The mec gene complex comprises the mecA gene, its regulatory gene, and insertion sequence upstream and downstream of the mecA gene (Katayama et al., 2001). The composition of almost all SCCmec elements identified so far in *S. aureus* can be presented as (orfX) J3-mec-J2-ccr-J1 (Chongtrakool et al., 2006; Hiramatsu et al., 2002). The exception constitute SCCmec VII and a newly described SCCmec IX, with the ccr gene complex positioned between J3 and J2 regions and the mec gene complex between J2 and J1 regions (Berglund et al., 2008).

**Nomenclature and types of SCCmec elements**

The first SCCmec element was identified in Japanese *S. aureus* strain, N315 in 1999 and shortly after two additional SCCmec from different MRSA strains were determined (Ito et al., 1999; Ito et al., 2001). Based on detailed structural analysis these three SCCmec elements were classified as types I to III (Ito et al., 2001). In time, both new types of SCCmec, such as SCCmec IV (Ma et al., 2002), SCCmec V (Ito et al., 2004), SCCmec VI (Oliveira et al., 2006), SCCmec VII (Berglund et al., 2008),
SCCmec VIII (Zhang et al., 2009), SCCmec IX, SCCmec X (McCarthy and Lindsay, 2010), SCCmec XI and many new variants of already known SCCmec types have been reported (Shukla et al., 2004; Stephens et al., 2007). The classification of SCCmec element into the types (SCCmec typing), should be based on the combination of the type of ccr gene complex and the class of the mec gene complex present in the cassette, while variants within SCCmec types (SCCmec subtyping) should be defined by differences in their J regions, as it was proposed earlier by Hiramatsu group (Chongtrakool et al., 2006; IWG-SCC, 2009). Accordingly, SCCmec type I, was described additionally as 1B, what indicates the SCCmec element harbouring the type 1 ccr and a class B mec gene complexes. To date, eleven SCCmec types have been defined. The other known SCCmec types are designated type I (1B), type II (2A), type III (3A), type IV (2B), type V (5C2), type VI (4B), type VII (5C1), type VIII (4A), type IX (1C2), type X (7C1) and type XI (8E). There are two systems for naming SCCmec. The first is by Roman numerals (I–XI) given to SCCmec in the order of appearance. This nomenclature has been used widely (Ito et al., 2003). The second naming system is described by Arabic numerals for ccr types (1, 2, 3,…) and an upper case letter for mec class (A, B, C,…) (Chongtrakool et al., 2006)

Methicillin-resistant Staphylococcus aureus (MRSA), a pathogen responsible for many nosocomial infections, was first reported in 1960 (Jevons, 1961). Over the past few decades it has emerged in the community as well, and it is currently considered a threat to public health (Lindsay and Holden, 2004). MRSA needs to be identified below the species level by rapid and reliable typing methods. Staphylococcal cassette chromosome mec (SCCmec) typing accompanied with overall genotyping has already provided strong evidence for the independent origins of health-care associated MRSA and community-acquired MRSA (Naimi et al., 2003). To date, eleven different types of SCCmec (I–XI) have been defined on the basis of the combination of ccr and mec complexes, but only type I–V are globally distributed, while others appear to exist as local strains in the country of origin (Oliveira et al., 2006; Zhang et al., 2009). The regions located between these complexes are called J (joining) regions and in every SCCmec element there are three J regions (J1–J3) (Ito et al., 2003). Polymorphism in J regions (mainly J1) have been described and used for the definition of SCCmec type IV subtypes (Chongtrakool et al., 2006). SCCmec type IV has eight individual subtypes, which are characteristic for some of the notorious nosocomial MRSA clones such as
EMRSA-15 in European countries, and that are starting to emerge in Asian countries (Ehsanollah et al., 2010).

**Public health significance of MRSA**

Resistance to methicillin and other beta-lactam antibiotics is caused by the *mecA* gene, which is situated on a mobile genetic element, the Staphylococcal Cassette Chromosome mec (SCCmec). While hospital acquired HA-MRSA strains are typically multi-resistant, community associated CA-MRSA strains are by large more susceptible to many antibiotics. More recently multi-resistant livestock associated LA-MRSA strains have been recovered from bovine mastitis, but these strains are only prevalent in certain high-risk groups of workers in direct contact with live animals (Judit Szabo, 2014).

Five main SCCmec types have been identified globally. SCCmec types I, II and III are mainly associated with healthcare associated MRSA (HA-MRSA) strains. SCCmec type IV is associated with community associated MRSA (CA-MRSA) strains, as well as with the Paediatric clone MRSA strains. SCCmec type V is associated with CA-MRSA strains and has only been identified in Australia and New Zealand. Numerous variants of each type have also been described (Katayama et al., 2000 and Shore et al., 2005).

HA-MRSA first appeared in the United States in 1968. There are three types of staphylococcal cassette chromosomal *mec* in HA-MRSA: Types I, II and III (Mongkolrattanothai et al., 2003). Type I contains no additional resistance determinants, but Types II and III contain resistance determinants in addition to *mecA*, which are larger elements and may not be suited to community-acquired MRSA (CA-MRSA) strains; these additional genetic elements account for the antimicrobial resistance to numerous antibiotics in addition to the β-lactam agents (Kluytmans-Vandenbergh and Kluytmans, 2006). The three SCCmec types contained in HA-MRSA have an identical chromosomal integration site and cassette chromosome recombinase genes, which are responsible for horizontal transfer of SCCmec (Daum et al., 2002). Thus, HA-MRSA is resistant to many different antibiotics and has a selective advantage as they are spread among patients by hands of personnel and contaminated environmental surfaces. The presence of underlying diseases and multiple types of
instrumentation and procedures predisposes patients to colonization and infection by the multiple drug resistant strains of HA-MRSA.

CA-MRSA strains are usually resistant to β-lactams but susceptible to other antimicrobials like trimethoprim-sulfamethoxazole, clindamycin and tetracyclines and carry mostly staphylococcal cassette chromosome mec (SCCmec) Type IV (Naimi et al., 2003). CA-MRSA strains are also more likely to possess unique combinations of virulence factors and seem to be genetically different from HA-MRSA (Fey et al., 2003). CA-MRSA strains carry genes of Panton Valentine Leukocidin (PVL), belongs to a family of similar bi-component leukocidal toxins produced by staphylococci, with one component belonging to the F class and to the S class (Szmigielski et al., 1999).

**Livestock-associated MRSA**

A third emerging branch of *S. aureus* has been identified in association with livestock animals. The isolation of MRSA from animals was first reported in 1972. This was following the detection of MRSA in milk from mastitic cows (Devriese et al., 1972) but was at that time most likely associated with human to animal transmission of an MRSA strain acquired by the farmer during hospitalization. Since then livestock-associated MRSA (LA-MRSA) has been identified in various animals in several European countries, the US and Asia (Smith and Pearson, 2010). Population genetic studies have identified certain genotypes to be associated with specific host species. Multilocus sequence type ST71, ST97, ST126, ST133 and ST151 are often found among ruminants, and are the major course of bovine mastitis, whereas ST5 is often associated with poultry and ST9,ST433, ST398 are often found in pigs (Holmes and Zadoks, 2011; Moodley, et al., 2012).

LA-MRSA represents a concern in both human and animal health. Healthy colonized animals may act as MRSA reservoirs and source of transmission to other animals. In livestock it usually causes mastitis and soft tissue infection which may enter the food chain subsequently affecting the public health and economy. On the other hand, persons in direct contact with MRSA colonized livestock are at high risk of becoming colonized with LA-MRSA. These in turn, may become the source of transmission to other humans consequently spreading the infection in the community at large. In the process, genetically diverse CA-MRSA may evolve. When patients colonized with these clones are admitted in health care centres, infection may spread to
other patients and health care workers resulting in serious public health consequences (Mehndiratta and Bhalla, 2014). Recent studies have shown that use of antibiotics as prophylactic agents and growth promoters in these animals has resulted in the emergence of drug resistant micro-organisms including MRSA, causing serious and difficult to treat infections in animals as well as in humans (WHO, 1997; Khachatourians, 1998).

For SCCmec type determination different PCR methods have been described; however, they do not always generate concordant data (Boye et al., 2007; Kondo et al., 2007; Shore et al., 2005). Moreover with the available methods more than one multiplex PCR is required for identifying all SCCmec types, which again needs lots of optimization and is also time consuming. The availability of an easy and fast assay for the routine monitoring of SCCmec types in the hospital or community would be more advantageous. Therefore, simplified PCR assay with a combination of established PCR primer sets for the rapid and easy detection of globally distributed SCCmec types can be used.

The worldwide increase in the number of infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) has emphasized the need for fast and reliable identification and typing methods. In addition to genotyping, characterization of the staphylococcal cassette chromosome (SCCmec) type has led to better discrimination of hospital acquired MRSA (HA-MRSA) and community acquired MRSA (CA-MRSA) (Chongtrakool et al., 2006). The multiplex PCR for discrimination of SCCmec types I–IV developed by Oliveira and de Lencastre (Oliveira and Lencastre, 2002) has been used widely, but was designed primarily to characterize HA-MRSA carrying SCCmec types I–III. Currently, an increase in CA-MRSA is occurring worldwide, caused mainly by CA-MRSA belonging to the same multilocus sequence type lineage as HA-MRSA and carrying the smaller SCCmec types IV or V (Aires de Sousa and de Lencastre, 2003). In order to discriminate the classic HA-MRSA with SCCmec types I–III from CA MRSA with SCCmec types IV and V (Boye et al., 2007).

1.1 Statement of the problem

Identification and early diagnosis of pathogenic Methicillin-resistant staphylococcus (MRS) strains at the herd level is possible by the use of different molecular microbiology tools. The available data is limited regarding MRSA strains.
and very scanty of information available for subtyping of MRSA SCCmec elements (I to XI) in animal and human cases under subtropical conditions including India. The SCCmec types I, II, and III are found predominantly in Hospital Acquired Methicillin-resistant Staphylococci (HA-MRS) isolates. The SCCmec type IV and V are typically found in Community Acquired Methicillin-resistant Staphylococci (CA-MRS) strains and lack other multidrug resistance genes (Unal, 2006). While SCCmec element typing has become essential for the characterization of MRS clones in epidemiological studies. Therefore, the present work focused on identification of MRS and detect various SCCmec types using molecular tools from the animal and human origin.

Until recently the most useful technique was the PFGE, which has high discriminatory index, but technically difficult and slow, the portability is limited, and multiple nomenclature and misclassification of some lineages also can occur. The spa typing might be a useful tool for epidemiological studies of MRS, due to its rapidity, accessibility, high throughput, and standardized nomenclature. The MLST has high discriminatory power, define score genetic population and also has portability and standard nomenclature, but the weaknesses of this method are the low throughput and high cost. The DNA microarray hybridization test is the most modern technique, which able to detect of presence of a huge number of genomic loci, but due to the high cost and complexity, this is not preferred in routine practice. The mec (SCCmec) typing has standard nomenclature which able to distinguish between the HA-MRSA and CA-MRSA strains.

Considering all available information of these molecular techniques, the most recommended methods are the spa and staphylococcal cassette chromosome mec (SCCmec) typing. Both are informative enough to define particular strain characteristics and utilize standardized nomenclatures, making them applicable globally (Judit Szabo, 2014). There for SCCmec typing method is adopted for the typing of staphylococci in present work.

Simultaneously, study aimed to ascertain the prevalence of the different agr groups (accessory gene regulator locus) and to evaluate the occurrence of encoding genes for leucocidin (PVL) intracellular adhesins (icaA) and alpha hemolysin (hla) activity in Staphylococci isolates from milk and other clinical condition from animal and human origin.
1.2. **Practical utility of the research problem**

The literature reviewed that there are lots of reports on staphylococcal cassette chromosome *mec* (SCCmec) types in Methicillin-resistance Staphylococci of human origin across the globe including India, but in compare to human, few reports on SCCmec typing have been published from animals across the globe and still very few from India. The identification and molecular SCCmec typing and epidemiological studies have been conducted from pig and human, but scanty of references available regarding animal and human SCCmec types, epidemiological studies and their relationship in the world and in India.

The research work gave overall picture of staphylococcus SCCmec types and virulence factors and toxin genes prevalent in this region in animals and closely associated human beings to this animals, that had facilitate the study of epidemiological correlation between human and animals [(hospital acquired methicillin resistant staphylococci (HA-MRS) or community acquire methicillin resistant staphylococci (CA-MRS)] which helps to adopt effective control strategy. Along with the study of virulence genes (toxic genes) prevalence in the population, there correlation with the SCCmec types had been established during this work. The complete spectrum of antibiotic resistance in staphylococcus has been deciphered insight into pathogenic potentials. The research work had given highlight of overall evolution of staphylococcus as pathogen through the SCCmec typing.

1.3 **Objectives of the study**

1. Isolation and identification of Staphylococci from animal and human origin & their biochemical characterization.

2. To determine antibiotic resistance in Staphylococci by conventional susceptibility testing.

3. Molecular detection of virulence genes including Panton-Valentine leukocidin (*PVL*) gene from Methicillin-resistant Staphylococci (MRS) organisms.

4. Molecular characterization of major types of Staphylococcal Cassette Chromosome (SCCmec) in Methicillin-resistant Staphylococci (MRS) from human and animal origin.
Staphylococcus aureus is well-known epidemic nosocomial pathogen causing considerable human morbidity and mortality worldwide, and it is a leading cause of infections of some economically important livestock species and, as a prominent bacterial cause of contagious bovine mastitis, a major economic burden for the dairy cattle industry (Fitzgerald, 2012a). S. aureus is considered as a significant pathogen with related virulence factors such as slime factor (biofilms), Panton-Valentine leukocidin (PVL), and some enzymes (proteases, lipases, and elastase), which facilitates destruction of host tissues and metastases to other sites (Gordon and Lowry, 2008), treatment of S. aureus infections included semisynthetic penicillin drugs, such as methicillin.

MRSA is primarily mediated by the mecA gene carried on a mobile genetic element (MGE), the Staphylococcal Cassette Chromosome mec (SCCmec), till date, eleven different types of SCCmec (I–XI) have been defined on the basis of the combination of ccr and mec complexes, but only type I–V are globally distributed, while others appear to exist as local strains in the country of origin (Zhang et al., 2009). SCCmec elements have been identified within Staphylococcus sciuri (Juuti et al., 2005), Staphylococcus hominis (Katayama et al., 2003), Staphylococcus epidermidis (Wisplinghoff et al., 2003), Staphylococcus haemolyticus (Hanssen and Sollid, 2007) and S. aureus (Ito and Hiramatsu, 1998). The mecA gene codes for the modified penicillin-binding protein 2a (PBP 2a or PBP 2’). PBP2a is positioned in the bacterial cell wall and has a low binding affinity for β-lactams. MRSA has become apparent as a major cause of Hospital-Associated (HA) and Community-Associated (CA) infections and also isolated from milk (livestock associated) (Devriese et al., 1975; David and Daum, 2010.).

Methicillin-Resistant S. aureus (MRSA) lineages are causes of health care and community associated infections, which are a major burden of disease on a global scale (Stone, 2009) In the last decade, MRSA sequence type 398 (ST398) has found an ecological niche in the pig, cattle, and poultry industries, although other MRSA lineages(e.g., ST1, ST5, ST9, ST97, ST130, and ST433) have been identified in farmed
animals worldwide (Guardabassi et al., 2013) All these lineages are currently termed “Livestock-Associated MRSA” (LA-MRSA).

During the last 15 years, the prevalence of MRSA infections in the community has been increasing. Recently, MRSA has been emerging in livestock. Since 2005, a novel MRSA isolate called clonal complex (CC) 398 has been isolated from pigs and people associated with pig farming in Europe (Voss et al., 2005). Consequently, pigs were assumed to be a possible reservoir of community-associated MRSA. However, CC398 LA-MRSA strains have been found frequently in Europe, and non-CC398 MRSA isolates are predominant in Asia. In particular, the sequence type (ST) 9 MRSA (CC9) strains were mostly reported in association with pig farming (Neela et al., 2009). SCCmec IX-ST9-t337 MRSA isolates from pork and pigs were also reported in Thailand (Vestergaard et al., 2012). CC97 is one of the major S. aureus clonal complexes in bovines, and recently, a livestock origin of the human pandemic CC97MRSA strains has been demonstrated, resulting in two emerging human epidemic CC97 community-acquired/associated MRSA (CA-MRSA) clones (Spoor et al., 2013).

LA-MRSA has emerged in farm animals mainly bovine, swine, companion animals and persons in contact with these animals. Human infections associated with LA-MRSA have also been reported from several parts of the world (Van Cleef et al., 2011). Animal to human and vice versa of resistant strains can have a potential effect on public health if these strains enter into the community and health-care settings (Fitzgerald, 2012b). Transmission of bacterial species between humans and livestock is increasingly being detected in farmers and farm workers in Europe and much of the industrialized world (Chambers and Deleo, 2009). Despite the fact that S. aureus is commonly associated with bovine mastitis, MRSA isolates have been infrequently recorded with the disease (Paterson et al., 2014). There have been a few reports of MRSA colonization and infections in dairy cattle since the very first evidence of MRSA in mastitis in 1972. Recently, a highly divergent mecA gene (now named mecC) in SCCmec type XI and which was found in bovine mastitis S. aureus (Paterson et al., 2014).

The prevalence of MRSA carrier in humans significantly correlated with the percentage of positive cows in the farm, the number of livestock units, and the presence.
of positive bulk tank milk samples. Spohr et al. (2011) found that milk samples of 5.1-16.7% of dairy cows were positive for MRSA, considering that the milking hygiene score correlated with the somatic cell count, which is increased in the presence of mastitis, it is evident that the improvement of hygiene practices might reduce the risk for MRSA to spread on dairy farms. A high rate of animal-to-human transmission of CC398 has been reported in pig farming, as well as a significant difference in MRSA prevalence between farmers and their families (Crombe et al., 2012). In addition to classical risk factors for MRSA carriage, Harbarth et al. (2006) suggested to include the evaluation of contact with livestock as an additional risk factor to the admission screening schedule for hospitals, to identify subjects at higher risk for LA-MRSA colonization, who may be responsible for MRSA CC398 introduction in the nosocomial setting and may favor antimicrobial resistance.

The literature reviewed shows that, there are lots of reports on staphylococcal cassette chromosome mec types in Methicillin-resistance Staphylococcus aureus of human origin across the globe including India, there exist a paucity of information and few reports on SCCmec typing have been published from animals across the globe and very few cases have been reported in India. The identification and molecular SCCmec typing and epidemiological studies have been conducted from pig and human, but scanty of references are available regarding animal and human SCCmec type relationship in the India and world. However, here an attempt has been made to overview the related literature available on the topic under study. A brief account of such literature related to the problem under study has been reviewed and presented under the following major headings:

2.1 Isolation and identification of Staphylococci from animal and human origin & their biochemical characterization.

2.2 To determine antibiotic resistance in Staphylococci by conventional susceptibility testing.

2.3 Molecular detection of virulence genes including Panton-Valentine leukocidin (PVL) gene from Methicillin-resistant Staphylococci (MRS) organisms.

2.4 Molecular characterization of major types of Staphylococcal Cassette Chromosome (SCCmec) in Methicillin-resistant Staphylococci (MRS) from human and animal and origin.
2.1 STAPHYLOCOCCI AS AN ETIOLOGICAL AGENT FOR VARIOUS AILMENTS IN ANIMAL AND HUMANS

The major pathogens involved in mastitis are Staphylococcus aureus, Streptococcus agalactiae, Corynebacterium bovis and Mycoplasma spp. In India, Staphylococcus spp. has been reported to be the main etiological agents of mastitis in cattle and buffaloes with the prevalence rate of 74.71% (Singh et al., 1982), 42.10% (Mishra et al., 1993), 60.32% (Shukla, 1998), 74.04% (Patel et al., 2000), 52.48% (Ghosh and Sharda, 2003), 39.53% (Vishwakarma, 2008), 59.37% (Sahoo et al., 2009), 66% (Hetal, 2016) and 33.34% (Bhagat et al., 2017) among all etiologies.

Infections caused by S. aureus were characterized originally by Sir Alexander Ogston in 1880 (Cheng et al., 2011) and were first reported to cause mastitis in cattle in 1890 (Jones, 1918). In India, Staphylococcus spp. has been reported to be the main etiological agents of mastitis in cattle and buffaloes. It is one of the most frequently isolated contagious pathogens as compared to other microbes in bovine mastitis worldwide (El-Sayed et al., 2006; Moon et al., 2007a). The prevalence of S. aureus in clinical mastitis has been reported varying from 3.3% to 40% (Ferguson et al., 2007; Ericsson et al., 2009). In sub-clinical mastitis, the proportion of S. aureus isolates has been studied in some countries and regions, and reported ranging between 3.2% and 63% (Moon et al., 2007b; Ericsson et al., 2009). Another report has revealed that 3% of total animals are infected with S. aureus (Schukken et al., 2009). However, S. aureus represents 10 to 12% of all clinical mastitis infections (Tenhagen et al., 2009). Worldwide proportion of cows with S. aureus intra mammary infection have been reported (Myllys et al., 1998; Dua, 2001) 21% in Great Britain (Wilson and Richards, 1980), 10% in Finland (Myllys et al., 1998), 11% in Norway (Bakken, 1981), 10% in Denmark (Aarestrup et al., 1995), 9% in California (Gonzalez et al., 1988) and 5% in Vermont (Goldberg, et al., 1991; Hogan, 1997), 10% in USA and India.

Ferriro et al. (1985) screened 4268 quarters of 1067 lactating cows and found that 1046 (24.50 per cent) quarters produced suspect milk and obtained 896 cultures mainly from quarters with subclinical mastitis. The predominant isolates were S. aureus being isolated from 152 cultures (16.96 per cent) and S. epidermidis from 119 cultures (13.25 per cent). For Streptococcus species, the distribution was 108 (12.05 per cent) S. agalactiae, 79 (8.82 per cent) S. uberis and 46 (5.14 per cent) S. dysgalactiae.
Further, they also isolated 12 yeasts (majority *Candida albicans*), 12 *Nocardia* spp. and three mycelial fungi.

Verma (1988) examined 136 cows for subclinical mastitis and found that 42.1 percent of animals were positive. Among isolates obtained, *S. aureus* were predominated (34 of 61 samples), other organisms isolated were *Streptococcus* spp. (4), *E. coli* (7), *Klebsiella* spp. (2), *Corynebacterium* spp. (6), *Proteus mirabilis* (2) and the fungi *Aspergillus fumigatus*, *Geotrichum* and *Saccharomyces* species.

Kaya et al. (1998) examined 141 milk samples collected from cows with clinical mastitis for pathogenic bacteria. They isolated *S. aureus* (57 per cent), *Streptococcus* spp. (8 per cent), *E. coli* (5 per cent), *Lactobacillus* species (5 per cent), *Klebsiella pneumoniae* (5 per cent), *C. pyogenes* (4 per cent) and three per cent of *Pseudomonas aeruginosa* isolates.

Das and Kanna (1994) characterized *S. aureus* strains from food handlers. The number of the coagulase-positive isolates were 14 (56%) and 12 (48%) from 25 nasal and 25 hand swabs respectively. The overall percentage of food handlers carrying *S. aureus* was 52%. The result indicated that human food handlers could be a potential source of enterotoxigenic *S. aureus* and responsible for food poisoning.

Datta and Rangenkar (2001) tested 173 milk samples from 45 cows for mastitis, of which 46 samples from 29 cows (64.4%) were found positive on california mastitis test and cultural examination. Results revealed the predominance of *Staphylococcus* spp. (60.9%), followed by *Streptococcus* spp. (23.9%), *Pseudomonas* spp. (6.5%) and *Bacillus* spp. (2.25%).

Balakrishnan et al. (2004) obtained 40 bacterial isolates from 65 milk samples. The spectrum comprised of *S. aureus* (35%), *E. coli* (27.5%), *S. agalactiae* (17.5%), *P. aeruginosa* (12.5%), *S. dysgalactiae* (2.5%), *Pasteurella haemolytica* (2.5%) and *Actinobacillus capsulatus* (2.5%).

Megra et al. (2006) investigated 200 human specimens for bacteriological examination from nasal cavity, tonsil, trachea and the lung (50 each), amongst them 154 (77%) contained bacteria. Bacterial species identified were coagulase-negative *Staphylococcus* (22.8%), *Staphylococcus aureus* (17.2%), *Pasteurella multocida* (11.9%), *Corynebacterium pseudotuberculosis* (8.8%), *Bacillus* species (7.4%), *Actinomyces pyogenes* (6.7%), *E. coli* (6.0%), and *Micrococcus* species (1.0%).
Karsten et al. (2006) studied 218 nasal swabs and found at least one Staphylococcal species was to be colonized in 92.8% of the cases. Overall, 52 S. aureus isolates encompassing 47 MSSA and 5 MRSA isolates (exhibiting different spa types) and 311 isolates of CNS (S. epidermidis, n = 219; S. haemolyticus, n = 32; S. warneri, n = 15; S. hominis subsp. hominis).

El-Jakee et al. (2008) investigated a total of 409 samples to detect the occurrence of Staphylococci among the diseased animals and human, the highest isolation rate was observed in human samples (36%) followed by dog (28%), bovine (24.8%), ration (14.7%) and chicken (12%) samples. A total of 78 S. aureus isolates secured from different animals and human origins were characterized and identified using the most important conventional biochemical tests as anaerobic glucose fermentation, catalase, coagulase, acetone production, novobiocin sensitivity and mannitol fermentation.

Alli et al. (2008) reported that S. aureus was the most frequently recovered bacterial species accounting for 49.53% of all the isolates from mastitis in buffaloes followed by S. agalactiae (23.83%), Staphylococcus hyicus (8.88%), Bacillus spp. (3.74%), Staphylococcus hominis (1.40%), E. coli (1.40%), Staphylococcus xylosus and Corynebacterium spp. (0.93% each).

Alzohairy (2011) studied the frequency of MRSA among animals. A total of 400 samples were collected from camels, sheep, cows, and goats. From 334 Staphylococci recovered, 158 (47.3%) were coagulase-positive Staphylococcus, among them 90 (57%) were MRSA and 68 (43%) were Methicillin-sensitive Staphylococcus aureus (MSSA). The remaining strains 176 (52.7%) were coagulase-negative staphylococcus, including 32 (18.2%) were Methicillin-resistant coagulase-negative staphylococcus and 144 (81.8%) were methicillin-sensitive coagulase-negative staphylococcus. High rate of MRSA and MR-CNS were isolated from camel and sheep while lower rates were observed in goat and cow. Multi drug resistance (MDR) rate among MRSA and MR-CNS isolates was high.

Marcela et al., (2011) examined human nasal and throat swabs of the farm staff and nasal swabs of animals. In total 1729 samples were examined and 634 strains were isolated by means of the cultivation method and used in this study. Generic identification of the staphylococci isolates was performed by biochemical tests and all
S. aureus and CNS isolates were checked by the PCR method for the presence of mecA gene which was responsible for Methicillin-resistance. S. aureus was the most frequently identified species from the samples tested (n = 557; 32.2%), followed by S. haemolyticus (n = 32; 1.9%), S. chromogenes (n = 24; 1.4%), S. epidermidis (n = 20; 1.2%), and S. caprae (n = 1; 0.16%). Among the resistant Staphylococci (n = 49), S. aureus (n = 25; 51%) was found the most frequently, followed by S. epidermidis (n = 17; 34.7%), S. chromogenes (n = 6; 12.2%), and S. haemolyticus (n = 1; 2%).

Hanning et al. (2012) characterized S. aureus from whole retail poultry carcasses and compare the isolates to S. aureus isolates from humans. A total of 25 S. aureus isolates were collected from 222 whole poultry carcasses. A total of 17 S. aureus isolates obtained from healthy humans were included and characterized in the same way as the poultry isolates. Staphylococcus spp. were recovered from all poultry carcasses. Only 25 poultry carcasses (11.2%) were contaminated with S. aureus. These results indicate a low prevalence of S. aureus present in poultry, and the isolates were not phenotypically similar to human isolates. The low number of S. aureus isolates from this study indicated that chicken carcasses would appear to not be a significant source of this bacterium.

Abera et al. (2013) studied total of 66 lactating cows and 264 quarters based on clinical and California Mastitis Test (CMT). Out of collected and cultured samples, 70(63%) samples were positive for aerobic and one facultative an aerobic bacteria. The following bacteria were isolated: S. aureus (35.71%), coagulase negative Staphylococcus (15.71%), Streptococcus spp. (11.42%), Staphylococcus intermedius (7.14%), E. coli (5.71%), P. haemolytica (7.14%), P. aureuginosa (4.28%), Bacillus spp. (5.71%) and micrococcus spp. (7.14%).

Sharma et al. (2015) studied the prevalence of S. aureus among dairy cattle. A total of 80 milk samples were collected from clinical and subclinical cases of mastitis from cows (40) and buffalos (40). The incidence of S. aureus was higher (50.00%) in clinical mastitis in comparison to that of subclinical mastitis (17.50%). The results revealed that the incidence of S. aureus in clinical as well as sub-clinical mastitis was higher in cattle in comparison to buffaloes.

Garipcin and seker (2015) investigated, nasal carriage rates of Methicillin-resistant Staphylococcus aureus (MRSA) in cattle and humans in close contact with
cattle. The nasal swab samples were collected from 35 different private farms (150 humans and 250 cattle) in a fyonkarahisar province of Turkey. The nasal carriage rates of MRSA in humans and cattle were determined as 8.7% (13/150) and 1.2% (3/250), respectively.

Hetal (2016) observed the prevalence rate of \textit{Staphylococcus} spp. is highest in milk samples (66%) and human nasal swabs (70%) among all other isolates.

Sun \textit{et al.} (2017) studied presence of MRSA and Methicillin-susceptible \textit{S. aureus} (MSSA) from work related injuries, relevant health events such as skin and soft tissue infections and nasal swabs of veterinarians working on pig farms and they were found a prevalence of \textit{S. aureus} was 64%, and MRSA was 9.5%.

2.2 ISOLATION AND IDENTIFICATION OF \textit{STAPHYLOCOCCUS} SPP. FROM ANIMAL AND HUMANS AND THEIR BIOCHEMICAL CHARACTERIZATION.

2.2.1 Mannitol fermentation

Member of the genus \textit{staphylococcus} are gram positive cocci (0.5-1.5um in diameter) that occur singly, in pairs, tetrads, short chain (three or four cells) and irregular grape-like cluster. They are non-motile, non-spore forming and usually non-capsulated or have limited capsule formation, most species are catalase positive and oxidase negative (Quinn \textit{et al.}, 1994). They can tolerate salt concentration up to 7.5% in culture media, mannitol fermentation (Baired-Parker, 1963). \textit{Staphylococci} can withstand the osmotic pressure created by 7.5% NaCl, while this concentration will inhibit the growth of most other gram-positive and gram-negative bacteria as reported by Koch in 1942. The results were confirmed and improved by Chapman, (1945) by the addition of this salt concentration to Phenol Red Mannitol Agar, as \textit{Staphylococcus aureus} usually ferments mannitol. Non-pathogenic staphylococci usually show less luxuriant growth on this medium after the incubation period so it is important criteria to identify staphylococci.

Many authors have described use of mannitol fermentation criteria with higher salt concentration in medium for the selective isolation of staphylococci. Vogel and Johnson (1960) reported that mannitol fermentation reaction of coagulase-positive staphylococci is indicated by the development of yellow zones surrounding the colonies. This is due to a pH change which results in the phenol red indicator turning
from red to yellow. Baird-Parker (1963) considered mannitol fermentation as one of the useful characteristic for identification of *Staphylococcus aureus*. Later on Merchant and Packer (1967) and Morse (1980) have described mannitol fermentation as an important test for the identification of *Staphylococcus aureus*. Varadaraj and Rangnathan (1985) reported mannitol fermentation as well glucose utilization as important indicators of enterotoxigenicity of Staphylococci.

In India, Purohit (1990) isolated *Staphylococcus aureus* by using mannitol salt agar as a primary medium. Pandya (1991) observed hundred per cent correlation between mannitol fermentation and coagulase production (using rabbit plasma) in *Staphylococcus aureus* isolates. Later on Bhandari (2007) studied on *Staphylococcus aureus* isolated from clinical and sub clinical mastitis in bovines and found 74.41 per cent isolates were positive for mannitol fermentation using mannitol salt agar. Patel (2007) reported that all the 80 (100 %) *Staphylococcus aureus* isolates showed mannitol fermentation on mannitol salt agar. Nimavat (2015) studied on *S. aureus* isolated from clinical and sub clinical mastitis in bovines and found 53.22% isolates were positive for mannitol fermentation using mannitol salt agar. Hetal (2016) studied on *Staphylococcus spp.* isolates from milk samples and human nasal swabs and found 41.75% isolates were positive for mannitol fermentation using mannitol salt agar.

Das and Khanna (1995) examined biochemical characterization of *S. aureus* strains isolated from meat, fish and food handlers. The biochemical tests for the coagulase-positive type isolates showed that 48 (73.84%) were phosphate producers, 49 (75.38%), 46 (70.76%) were anaerobically glucose and mannitol fermenters and 59 (90.76%) were acriflavin resistant.

El-Jakee *et al.* (2008) characterized 78 *S. aureus* isolates secured from different animals and human origins using the most important conventional biochemical tests such as anaerobic glucose fermentation, catalase, coagulase, acetone production, novobiocin sensitivity and mannitol fermentation.

Sharma *et al.* (2011) isolated *S. aureus* from the raw milk samples inoculated on mannitol salt agar plates incubated for 24 to48 h at 37°C and observed for bacterial growth. Simultaneously, he observed for catalase, hemolysis and coagulase production.

Tyagi *et al.* (2013) identified 68 isolates of *Staphylococcus aureus*. All the isolates were catalase positive. On sugar fermentation all the isolates were positive for
mannitol fermentation, while 59 (86.76%) and 53 (77.94%) isolates fermented glucose and lactose.

Livia et al. (2013) isolated *Staphylococcus aureus* by culturing on 5% defibrinated sheep blood agar and identified by standard biochemical tests viz., catalase test, coagulase production on rabbit plasma, mannitol fermentation, acetoin production, maltose and trehalose utilization.

Sarkar et al. (2014) reported the identification of the *Staphylococcus aureus* by streaking samples onto mannitol salt agar plates and incubated for 24-48 hours at 37°C. Based on mannitol fermentative colonies she identified 184 *Staphylococcus aureus* from 250 samples including 200 milk and 50 nasal swabs.

Patnaik et al. (2014) cultured milk sample on mannitol salt agar, and isolates were subjected to various biochemical tests, viz., catalase test, coagulase test, hemolysis test, pigment production test, methyl red test, nitrate reduction, oxidase test, gelatin liquefaction test, and sugar fermentation tests for characterization and identification of *Staphylococcus aureus* from subclinical mastitis cases.

Hetal (2016) characterized bovine and human isolates based on biochemical test. Out of total 47 and 44 isolates, 20 and 18 isolates have fermented mannitol and produced yellow colonies from bovine and human samples, respectively whereas, other isolates were non mannitol fermenters and produced white colonies. All 47 isolates of milk and 44 isolates of human were catalase positive and oxidase negative. Out of total 47 and 44 isolates, 12 and 10 isolates were coagulase positive, 35 and 34 isolates were coagulase negative from bovine and human isolates, respectively.

Simultaneously, she identified MRS isolates by streaking on Hi Chrom MeReSa agar which is selective media for detection of MRS. Out of total 50 milk and 50 human samples, 7 and 19 isolates could grow within and produced dark greenish blue colonies, respectively and identified as Methicillin-resistant *Staphylococcus* spp. Of these 7 milk isolates and 19 human isolates, 2 from milk and 8 from human appeared within an incubation period of 48 hours and rest 5 milk and 11 human isolate colonies appeared in the time period of 72 hours.
2.2.2 Coagulase production

The first report of coagulase activity was given by Loeb in 1903 (Loeb, 1903). He reported that staphylococci were capable of influencing the coagulation process of blood. In his experiments, he mixed small volumes of bouillon broth cultures of bacteria with 3 cubic centimeters of goose plasma. After incubation, he noted that “staphylococcus pyogenes aureus” (now known as Staphylococcus aureus) was capable of causing the blood to coagulate, usually within 5 hours. This version of the coagulase test is now referred to as the tube coagulase test. Nine species of bacteria were tested, and while some response was seen with several of the other species, S. aureus produced the strongest and swiftest response. Further experiment using rabbits determined that the organism was able to affect mammalian blood as well. He also observed that a sterilized culture of the organism was not able to coagulate blood. Shortly after that, Much (1908) described an apparently related phenomenon where S. aureus cells immediately clumped when mixed with plasma, which is now called the slide coagulase test or clumping test. We now know that several other species of Staphylococcus are capable of giving a positive coagulase test (Bascomb and Manafi, 1998). There are several reports available in which authors have described coagulase test as indicator for identifying pathogenic staphylococci.

Duthie (1954) stated that coagulase produced by S. aureus might be of two types, one bound to the cell wall, responsible for clotting of plasma in slide test and another liberated as free coagulase on the medium, responsible for clotting of plasma in the tube test. Later on Yadav et al. (1972) tested 104 isolates of S. aureus which clotted rabbit and sheep plasma within 24 hours. Bas et al. (1974) reported that Staphylocoagulase was a single chain protein with a molecular weight of 61,000 could be produced from S. aureus strain in vitro which is responsible for clumping of plasma.

Niazi et al. (1987) tested 45 S. aureus isolates for coagulase test with different plasma viz., human, rabbit, pigeon, sheep, guinea pig and rat. They observed that the rabbit plasma gave the best result followed by pigeon, human, sheep, rat and guinea pig.

Chatterjee et al. (1990) experimented the physiological characters of 82 coagulase-positive and 20 coagulase-negative strains of staphylococci isolated from the milk samples, nasal and abscess swabs of cattle and nasal swab of pigs. Of the
biochemical tests mannitol fermentation and phosphatase test proved to be of value in differentiating coagulase-positive from coagulase negative staphylococci. In the coagulase-positive group, 40 (48.78%) produced golden color colonies, 28 (34.14%) were white and remaining 10 (12.19%) were yellow. Out of 20 coagulase negative strains the respective numbers were 5, 11 and 4.

On mannitol fermentation test, out of 82 coagulase-positive strains, 81 (98.78%) were positive to mannitol and maximum number of strains fermented it within 24 hours. Out of 20 coagulase-negative strains, only 5 (25%) fermented mannitol after 24 hours of incubation. In this study, 95.12% of coagulase-positive strains and only 20% of coagulase negative strains were positive for phosphatase test. On sugar fermentation test, all the carbohydrates were fermented with the production of acid without gas by the majority of both coagulase-positive and negative staphylococci, and so this was not useful for differentiation.

Kamata et al. (1990) reported, coagulase-negative staphylococci (CNS) were identified in 94 out of 172 clinical mastitis cases (54.7%).

Pandya (1991) performed coagulase production test of 128 S. aureus and found that 128 (100%), 121 (94.53%) and 43 (33.59%) isolates were positive with rabbit plasma, human plasma and poultry plasma, respectively.

Mubita et al. (1995) isolated and identified 100 Staphylococci from nasal cavity of apparently healthy dogs. Of these, 96% of the isolates were able to grow in medium containing 7.5% Nacl, 72% isolates were coagulase positive and 30% fermented mannitol.

Das and khanna (1994) conducted a study on the biochemical characterization of S. aureus isolated from food handlers. During the study, they identified 14 (56.00%), 12 (85.70%) and 12 (48%), 11 (91.66%) isolates were coagulase and phosphate positive from nasal and hand swabs, respectively. All the coagulase positive isolates were positive for glucose, mannitol fermentation (anaerobic) and acriflavin resistance characters. The biochemical findings reveal that majorities of the isolates were coagulase-positive and that coagulase test has a definite correlation with other biochemical properties.
Fitzgerald et al. (2000) reported that amongst 144 isolates of *S. aureus* recovered from cows, 107 (74.3%) isolates tested positive for clumping factor in a slide agglutination test.

Qureshi et al. (2002) studied coagulase activity in 30 *S. aureus* isolated from cutaneous wounds and abscesses in camel against plasma from rabbits, cattle, buffaloes, sheep, goats, horses, camels and man at 1, 3 and 5 hours. They found that various isolates coagulate the plasma from rabbits, human, buffaloes, horses, cattle, goat, camel and sheep in decreasing order of superiority.

Ghosh et al. (2003) tested 164 *Staphylococcus* strains for coagulase test, of these 58 (35.4 %) isolates resulted for positive coagulase test.

Refai et al. (2005) obtained 40 *S. aureus* strains from clinical mastitis milk samples, of these 14 (35.00%) and 32 (80.00%) isolates were found positive for clumping factor and tube coagulase respectively.

Cremonesia et al. (2005) isolated 93 *Staphylococcus aureus* strains based on primary streaking on Baird Parker Agar and confirmed as *S. aureus* based on catalase, coagulase, and Dnase test.

Bhanderi (2007) observed that out of 43 isolates only 16 (37.2%) isolates of *S. aureus* were positive for coagulase production with rabbit blood plasma.

Tiwari et al. (2008) evaluated different phenotypic test for coagulase detection. Out of total 288 staphylococci, 237 were coagulase positive. Based on this study they recommended tube coagulase test as highly sensitive test for routine laboratory identification of pathogenic staphylococci.

Kumar et al. (2011a) examined nasal swabs obtained from 84 healthcare workers. The prevalence of nasal colonization with *Staphylococcus aureus*, different biochemical tests were done to isolate *Staphylococcus aureus*. Species confirmation for *Staphylococcus aureus* was done using the tube coagulase test and DNase test.

Tyagi et al. (2013) identified 68 isolates of *Staphylococcus aureus*, out of these, all the isolates were catalase positive while 54 (79.41%) and 56 (82.35%) isolates showed positive slide and tube coagulase test, respectively.

Rusenova et al. (2013) identified 73 Staphylococci based on hemolytic activity and pigment production from 453 individual mastitic milk samples, out of which 60
Review of Literature...

*Staphylococcus aureus* strains were found coagulase positive with tube coagulase test in rabbit plasma.

Sarkar *et al.* (2014) reported identification of pathogenic staphylococci from bovine milk as well as human nasal swabs based on tube coagulase test and observed out of 184 isolates 138 staphylococci have given positive tube coagulation after incubation of 1 hour.

Nimavat (2015) observed that out of 62 isolates only 32 (51.61%) isolates of *S. aureus* were positive for coagulase production with tube coagulase test.

Parth *et al.* (2016) processed 185 milk samples from cows and buffaloes and screen for subclinical mastitis. Of these, 53 bacterial isolates obtained from bovine mastitis were found positive for *Staphylococcus* spp. and found positive to catalase test and 23S rRNA ribotyping tests were analyzed for production virulence factor. Out of 53 isolates, 53 (100%), 49 (92.45%), 29 (54.72%), 44 (83.02%) and 42 (79.25%) isolates were found positive for mannitol fermentation, Coagulase production, lipase activity, DNase activity and slime production respectively.

### 2.2.3 Hemolysin production

Williams and Harper (1947) observed that *Staphylococcus aureus* strain may produce α or β hemolysins alone or in combinations. They further noted that β lysin was characteristic of animal strains of *Staphylococcus aureus*. Elek and Levy (1950) postulated that the hemolytic pattern of pathogenic staphylococci on blood agar plates could be explained by three hemolysins, α, β, and δ occurring in only one of the seven possible combinations, while fourth hemolysin epsilon was found in some coagulase negative strains. According to them α and δ lysins were the most frequently found to be human strains, whereas β lysin was a characteristics of animal strain and uncommon in human strains. Munch-Peterson and Gardiner (1965) identified 227 isolates of staphylococci on ox blood agar. Of these, 43 isolates (18.9 %) were alpha hemolytic, 54 (23.8 %) beta hemolytic, 17 (7.5 %) alpha-beta type and 56 (24.7 %) non-hemolytic strains of *Staphylococcus aureus*. Jones and Shannon (1972) carried out a survey on an experimental herd for two lactation and reported that beta hemolytic staphylococci were harboured by 80 and 60 per cent of the cows in first and second lactation respectively. Ellitot *et al.* (1976) reported beta hemolytic *Staphylococcus aureus* as common bacteria
isolated from milk of randomly selected healthy cows under national mastitis survey of dairy herds in Australia.

Elias and Kofer (1980) examined the hemolysin production of 300 *Staphylococcus aureus* strains isolated from human post mortem material and bovine mastitis. They found that strains isolated from human material produced most frequently $\alpha + \delta$ and less frequently $\delta$ or $\alpha$ lysins. The bovine strains produced mostly $\beta + \delta$ or $\alpha + \beta + \delta$ lysins, less frequently $\beta$ or $\delta$ lysins. Stearner and Birkbeck (1980) reported that delta hemolysin was cytolysis for a wide range of erythrocyte and most frequently found in human originated *Staphylococcus aureus*.

Hodges *et al.* (1984) examined 831 coagulase positive and 95 coagulase negative staphylococci and found $\alpha$, $\beta$ and $\delta$ hemolysins in various combinations or occurring single in 98.30 per cent of coagulase positive and 60 per cent of coagulase negative staphylococci. Matsunaga *et al.* (1993) examined 58 *Staphylococcus aureus* strains isolated from bovine mastitis milk in Japan for their productivity of virulence-associated factors and they found that 74.1, 65.5 and 12.1 per cent isolates produced alpha, beta and gamma hemolysins, respectively.

Chatterjee *et al.* (1990) studied on 370 milk samples, nasal and abscess swabs of cattle and nasal swabs of pigs, and isolates were differentiated by mannitol fermentation and phosphatase tests. Of these, 92.68% of the coagulase-positive strains were hemolytic and beta toxigenic strains, either alone or in combination with alpha and/or delta hemolysis.

Fitzgerald *et al.* (2000) reported that amongst 144 *Staphylococcus aureus* isolates from cows, over 90 per cent of isolates demonstrated hemolytic activity on sheep or rabbit red blood cells and all isolates harbored the gamma hemolysin ($hlg$) locus. Da Silva *et al.* (2005) isolated staphylococci from 29 goat’s mastitis milk from Brazilian dairy herds. The isolates were analyzed for the production of $\alpha$, $\beta$ and $\gamma$ hemolysin on sheep and horse red blood cells. Of the strains studied, 80 per cent demonstrated hemolytic activity *Staphylococcus aureus* isolates showed hemolytic activity for all hemolysin types studied 76.7 per cent of them produced alpha hemolysin, 74.4 per cent beta hemolysin and 83.7 per cent produced gamma hemolysin.

Ali-Vehmas *et al.* (2001) identified 12 out of 50 strains of *Staphylococcus aureus* recovered from healthy and mastitic bovine milk for beta and delta hemolysins.
They stated that mastitic milk decreases directly the lytic effect of both beta and delta hemolysins of *Staphylococcus aureus* on hemolytic blood agar plates.

Ghosh (2003) tested 58 coagulase positive *Staphylococcus aureus* strains for hemolysin production and reported that 6.9, 15.5, 10.3, 18.9, 12.1 and 13.8 per cent produced the alpha, beta, gamma, alpha-beta, beta-gamma and gamma-alpha hemolysins respectively, while five strains (8.6 %) were found to be non-hemolytic in nature.

Todar (2005) reported alpha-toxin (α-hemolysin) is the best characterized and most potent membrane-damaging toxin of *Staphylococcus aureus*. It is expressed as a monomer that binds to the membrane of susceptible cells. Subunits then oligomerize to form heptameric rings with a central pore through which cellular contents leak. In humans, platelets and monocytes are particularly sensitive to alpha toxin. Susceptible cells have a specific receptor for α-toxin, which allows the toxin to bind causing small pores through which monovalent cations can pass. Beta toxin mainly damages membranes rich in this lipid. The classical test for β-toxin is lyses of sheep erythrocytes. The majority of human isolates of *Staphylococcus aureus* do not express β-toxin. A lysogenic bacteriophage is known to encode the toxin. δ-toxin is a very small peptide toxin produced by most strains of *Staphylococcus aureus*. It is also produced by *S. epidermidis*. The role of δ-toxin in disease is unknown.

Turkyilmaz and Kaya (2006) isolated 180 strains of *Staphylococcus* spp. from bovine mastitis, dog with otitis externa and chickens with various infections. Of which, 29.4 per cent strains were identified as *Staphylococcus aureus*. The rate of positiveness for hemolysis for coagulase positive strains were 58.9 per cent and coagulase negative strains were 28.9 per cent respectively. Bhanderi (2007) reported that majority of *Staphylococcus aureus* isolates exhibited α hemolysin 27 (62.79 %), whereas 7 (16.27 %) isolates showed β hemolysin and 9 (20.93 %) isolates were non-hemolytic on 5 per cent sheep blood agar. During the same year, Patel (2007) reported that out of total 80 (20 isolates each from sheep, goats, cattle and buffaloes) isolates, 19 (23.75 %), 48 (60.00 %), 13 (16.25 %) and 23 (28.75 %) were shown alpha, beta, gamma and alpha-beta hemolysin production on sheep blood agar, respectively.
Tyagi et al. (2013) identified 68 isolates of Staphylococcus aureus, out of these, all the isolates were catalase positive and on sheep blood agar, 48 (17.58%) isolates showed β-hemolysis.

Nimavat (2015) revealed 46 isolates produced partial/complete hemolysis around colony and 17 were non hemolytic on blood agar media. All 62 isolates were catalase positive and oxidase negative.

Parth et al. (2016) processed 185 milk samples from cows and buffaloes and screened for subclinical mastitis. Out these, 53 bacterial isolates obtained from bovine mastitis were found positive for Staphylococcus spp. Out of 53 isolates, alpha, beta, gamma and alpha-beta haemolysin production were observed in 18 (33.96%), 26 (49.06%), 4 (7.55%) and 5 (9.43%) isolates respectively.

2.3 DETERMINATION OF ANTIBIOTIC RESISTANCE IN STAPHYLOCOCCI BY CONVENTIONAL SUSCEPTIBILITY TESTING.

Staphylococcus aureus can cause a wide spectrum of diseases in both humans and animals. In humans, staphylococcal infections are frequently reported and can be contracted inside or outside of a health care setting (Ortega et al., 2010). An important public health issue concerning staphylococcal infections is the development of drug resistance due to the intensive use of antimicrobials in human and veterinary medicine (Shanehbandi et al., 2014). The virulence factors are crucial to establish the infection and cause pathogenesis for S. aureus. Besides pathogenesis, antibiotic-resistance is other crucial aspect as it makes difficult to eradicate this pathogen from herds and protects against drugs therapy. The emergence of antibiotic-resistance has been shown in S. aureus mastitic dairy animals (Hendriksen et al., 2008). These characters narrow down the treatment possibilities, and suggest the need of changes in the spectrum of antibiotics to be used in veterinary hospitals. Different groups of antibiotics are in use for treatment of mammary gland infections. Strains of S. aureus have been reported to show resistance against different antibiotics viz. beta-lactams, aminoglycosides, fluoroquinolones, lincosamides, macrolides and streptogramin that are commonly used by veterinarian (Wang et al., 2008).

Methicillin-Resistant Staphylococcus aureus (MRSA) strains are one of the most important causes of health care–associated infections worldwide. MRSA was first
described in 1961 (Jevons, 1961), soon after the introduction of methicillin. Methicillin-resistance is due to the acquisition of the \textit{mecA} gene, which encodes a penicillin-binding protein (PBP2a) with a low affinity for \beta-lactams. The \textit{mecA} is located on a mobile genetic element called staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}). Currently, 11 main types of SCC\textit{mec} (I through XI) are recognized (IWG-SCC. 2009). SCC\textit{mec} types I, IV, V, VI, and VII usually cause only \beta-lactams antibiotic resistance, but SCC\textit{mec} types II and III harbor resistance to multiple classes of antibiotics because of the additional drug resistance genes integrated into SCC\textit{mec} as plasmids and transposons (Riva \textit{et al.}, 2015).

Das and Khanna (1995) examined a total of 209 CPSA strains against 8 commonly used antibiotics by disc diffusion method on nutrient agar plates. The results revealed that gentamycin, chloramphenicol and erythromycin inhibited 191 (91.38\%), 189 (90.43\%) and 156 (74.64\%) strains while ampicillin, oxytetracycline, streptomycin and penicillin-G inhibited the growth of 103 (49.28\%), 94 (44.97\%) and 90 (43.06\%) strains of \textit{S. aureus} respectively. Resistance to cotrimoxazole was comparatively high (69.86\%). It was evident that gentamicin, chloramphenicol and erythromycin can be of better value in the treatment of \textit{S. aureus} infections.

De Oliveira \textit{et al.} (2000) determined the minimum inhibitory concentrations (MIC) for 811 strains of \textit{S. aureus} isolated from cases of bovine mastitis in 11 countries (Denmark, England, Finland, Germany, Iceland, Ireland, Norway, Sweden, Switzerland, United States, and Zimbabwe). Only 12 strains could be phenotypically classified as MRSA, but they were all \textit{mecA} negative. The MIC determinations were performed by a broth microdilution method that adhered to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). MIC for oxacillin was 1.0\(\mu\)g/mL.

Kabir \textit{et al.} (2005) screened 30 cows in Bangladesh with clinical mastitis and obtained \textit{S. aureus} in 11 cows. \textit{In vitro} drug sensitivity test revealed highest sensitivities to cepharedine, chloramphenicol, ampicillin (100\% each), while erythromycin, gentamicin and penicillin inhibited the growth of 10 (90.91 \%), 9 (81.82 \%) and 8 (72.73 \%) strains of \textit{S. aureus} respectively.

Parmar \textit{et al.} (2006) performed \textit{in vitro} antibiotic sensitivity test for 11 and 3 isolates of \textit{S. aureus} from clinical mastitis and sub clinical mastitis, respectively and
revealed that maximum isolates were sensitive to tetracycline (78.57%), followed by enrofloxacin (57.14%), ampicillin (50.00%), gentamicin (50.00%), cotrimoxazole (42.85%), ciprofloxacin (28.57%), pefloxacin (62.91%), amikacin (18.62%) and furazolidone (21.43%).

Turutoglu et al. (2006) carried out antibiotic susceptibility test on 103 S. aureus and 136 CNS isolated from bovine mastitis. They found that 39 (37.9 %), 45 (43.7 %), 56 (54.4 %), 89 (86.4 %), 96 (93.2 %), 85 (82.5 %), 85 (82.5 %), 3 (90.3 %), 92 (89.3 %), 80 (77.7 %), 99 (96.1 %), 98 (95.1 %), 45 (43.7 %), 56 (54.4 %), 40 (38.8 %), S. aureus strains susceptible to penicillin G, ampicillin, amoxycillin, ampicillin/sulbactum, amoxycillin/clavulanic acid, methicillin, cloxacillin, cefuroxime, neomycin, lincomycin, enrofloxacin, danofloxacin, gentamicin, trimethoprim/sulphamethoxazole, oxytetracycline, respectively.

Rajendran et al. (2006) examined 48 milk samples and 19 (39.6%) yielded S. aureus. Result of antibiotic sensitivity testing revealed effectiveness in percentage as enrofloxacin (84.2%), amoxycillin (31.6 %), gentamicin (63.2%), chlortetracycline (15.8%), streptomycin (36.8%), oxytetracycline (21.2%) and chloramphenicol (47.4%). The study revealed that the antibiotics like enrofloxacin, gentamicin and chloramphenicol are the better drugs of choice for the treatment of mastitis.

Rodolpho et al. (2006) characterized 95 Staphylococcus spp. strains from 129 apparently healthy lactating cows from an organic farm situated in Sao Paulo State, Brazil. Resistance to penicillin was most frequently (96.8%) found, followed by resistance to streptomycin (67.3%), norfloxacin (31.5%) and vancomycin (5.2%). β-Lactamase production was found in 21.0% of the isolates. Multidrug resistant isolates were common and two patterns of phenotypes were found in 78.9% of the isolates. These results showed that cows not exposed to antimicrobial drugs for five years still presented a high level of antimicrobial resistance among the colonizing bacteria of these animals.

Bhanderi (2007) carried out antibiotic sensitivity pattern in S. aureus isolated from mastitic milk samples using 21 different types of antibiotic discs, out of them they found that oxacillin (100%), vancomycin (100%), methicillin (97.67%), cephalothin (93.02%), cephalexin (93.02%) and ciprofloxacin (88.57%) were most effective drugs, nalidixic acid (83.72%), tetracycline (79.06%), cotrimaxazole (79.06%) and
chloremphenicol (72.06%) were moderately effective, less effective drugs were pefloxacin (62.71%) and cloxacillin (55.81%), while streptomycin (39.53%), amoxicillin (38.23%), gentamicin (32.55%), enrofloxacin (27.90%), ampicillin/penicillin (25.58%), oxytetracyclin (20.94%), amikacin (16.28%) and erythromycin (11.62%) were most resistant drugs.

Patel (2007) reported that out of 80 isolated *S. aureus* from mastitic milk of different species of animals, 71 (88.75%) isolates were sensitive to oxytetracycline, 28 (35.00%) to ciprofloxacin, 71 (88.75%) to chloramphenicol, 65 (81.25%) to gentamicin, 38 (47.50%) to penicillin G, 54 (67.50%) to amoxycillin + clavulinic acid and 65 (81.25%) to oxacillin.

Suresh *et al.* (2007) performed antibiogram of 25 (55.5 %) isolates of *Staphylococcus* spp. obtained from 45 culturally positive mastitis milk samples of goat and found that 19 (76.0 %), 19 (76.0 %), 20 (80.0 %), 5 (20.0 %), 2 (8.0 %), 21 (84.0 %), 19 (76.0 %) and 25 (100.0 %) isolates were sensitive to cloxacillin, gentamicin, sulphadiazine, trimethoprim, chloramphenicol, oxytetracycline, ciprofloxacin, and enrofloxacin respectively.

Ochoa-Zarzosa *et al.* (2008) reported antibiogram of *S. aureus* isolates (31) from Mexico to show resistance against ampicillin, ceftazidime, dicloxacillin and penicillin. The resistant proportion to cefuroxime (35%), cefotaxime (29%) and cephalotin (29%) has been shown higher as compared to erythromycin, trimethoprim, pefloxacin, lincomycin and tetracycline (10% for each antibiotic).

Mohanasoundaram and Lalitha, (2008) determined methicillin resistance from *Staphylococcus aureus* isolates from different clinical samples. Out of 150 isolates, 33 were found to be MRSA by oxacillin disc diffusion. By MIC method, 13 per cent of the isolates had values 32 mg/ml, 6 per cent between 16-8 mg/ml and 2.7 per cent had a value of 4 mg/ml and reveled 100 per cent concordance was obtained between the oxacillin disc screening and MIC methods.

Adaleti *et al.* (2008) observed 210 isolates of *Staphylococcus aureus* were MRSA out of total 416 isolates based on oxacillin disk diffusion, cefoxitin disk diffusion and oxacillin agar screening test.

Awandkar and Khode (2009) carried out, *in vitro* antibiogram where ciprofloxacin to be the most effective drug (69.8%) followed by gentamicin (68.3%).
review of Literature...

enrofloxacin (58.7%), chloramphenicol (31.8%) and cephotaxim (23.8%). The sensitivity to cloxacillin was reported the lowest (1.6%) and with increasing trend to ampicillin (3.2%), streptomycin (4.8%), oxytetracycline (7.9%), amoxicillin (9.5%), doxycycline (9.5%) and ceftriaxone (14.3%).

Haque et al. (2009) studied to evaluate the antibiotic susceptibility pattern of *Staphylococcus epidermidis* isolated from different clinical specimens and healthy controls in the department of Microbiology. A total of 62 *Staphylococcus epidermidis* were isolated from 230 specimens. Among them 32(23%) *S. epidermidis* were isolated from 200 cases and 30(100%) were isolated from 30 healthy controls. Isolates of *S. epidermidis* from cases showed multidrug resistance as penicillin 94%, oxacillin 56%, gentamycin 44%, erythromycin 41%, doxycycline 37%, cephradine 34%, ciprofloxacin 28%, ceftriaxone 28%, fusidic acid 22% and cefuroxime 19%. On the other hand, isolates of *S. epidermidis* from controls were susceptible to all antibiotics except penicillin, which was only 10% resistant. A remarkable difference was observed in the resistance pattern of *S. epidermidis* isolated from cases and healthy controls. However in study no isolates of *S. epidermidis* was resistant to rifampicin and vancomycin, which can be used as last line of drugs to treat nosocomial *S. epidermidis* infection.

Kelman et al. (2011) identified antimicrobial resistance in *Staphylococcus aureus*, including Methicillin-resistant *S. aureus* (MRSA), recovered from raw retail meat products purchased in the Washington. A total 694 samples of ground beef (n = 198), ground pork (n = 300), and ground turkey (n = 196) were collected by random sampling from stores of three grocery chains. In total, 200 *S. aureus* isolates (29%) were recovered by direct plating. When tested for susceptibility to 22 antimicrobials, 69% of the *S. aureus* isolates were resistant to tetracycline, 26% to penicillin, 17% to ampicillin, 13% to methicillin, 8% to erythromycin, 4.5% to clindamycin, 1.5% to gentamicin, and 0.5% to chloramphenicol, oxacillin, cefoxitin, or quinupristin-dalfopristin. However, 27% of the isolates were susceptible to all tested antimicrobials.

Kumar et al. (2011b) determine antibiotic susceptibility test with Kirby-Bauer method revealed that, among the 107 isolates, higher resistance was observed to streptomycin (36.4% of the isolates), oxytetracycline (33.6%) gentamicin and ampicillin (29.9%), penicillin-G (28.9%), chloramphenicol, pristinomycin, ciprofloxacin (26.2% each), and rifampicin and lincomycin (25.2% each). However,
some isolates were highly susceptible to cepahlexin (84.1%), amoxicillin-clavulanate, ofloxacin and clindamycin (81.3%). Fourteen isolates were found to be methicillin resistant, while the remaining (93) were methicillin susceptible.

Haran et al. (2012) carried out antimicrobial susceptibility based on Kirby Bauer disk diffusion method in which two isolates (1.3%) from 150 milk samples were confirmed as methicillin resistant and of the 93 methicillin susceptible isolates 29% were resistant to cloxacillin, 17% to ampicillin, 16% to penicillin and tetracycline each, 7% to erythromycin, 5% to pirlimycin, 4% to novobiocin, 3% to streptomycin, and 1% to ceftiofur and cephalothin each.

Singh et al. (2012) reported that, out of 110 clinical specimens, 47(42.73%) isolates were confirmed as Staphylococci by phenotypic test. Of which, 43 (91.49%) isolates were S. aureus. Of these 43 isolates, 7 (16.27%) isolates were MRSA strains. Simultaneously, 110 nasal swabs of healthcare staff were collected, of which, 65 (59.09%) Staphylococci were identified. Out of these 65 isolates, 19 (29.23%) were S. aureus and 7 (36.84%) MRSA strains from nasal swabs were identified. The MRSA strains isolated from clinical specimen were highly sensitive to Co-trimoxazole (CT/25 mcg) while the MRSA strains isolated from carriers were highly sensitive to tetracycline (TE/30 mcg).

Frana et al., (2013) carried out antimicrobial susceptibility testing on 67 MRSA isolates from separate samples. Sources of MRSA isolates for AST included: pigs (n =31), environment (n= 28) and students (n= 8). Resistant levels to antimicrobials for all isolates included: chlortetracycline (n =58, 86.6%), clindamycin (n =31, 46.3%), enrofloxacin (n =11, 16.4%), florfenicol (n= 26, 38.8%), gentamicin (n =15, 22.4%), neomycin (n= 49, 73.1%), oxytetracycline (n =58, 86.6%), spectinomycin (n =67, 100%), sulfadimethoxine (n= 2, 3.0%), Tiamulin (n= 15, 22.4%), tilmicosin (n =23, 34.3%), trimethoprim/sulfamethoxazole (n= 0, 0.0%). Significant differences in level of resistance by source were seen only with enrofloxacin (p=0.024) and florfenicol (p=0.0006). The student isolates were more resistant than farm isolates for both antimicrobials.

Vishnupriya et al. (2014) isolated 96 Staphylococcus strains from 158 bovine mastitis cases with 22 coagulase-positive staphylococci and 74 coagulase-negative staphylococci, out of which 12 coagulase-positive staphylococci and 20 CNS isolates
showed methicillin resistance by Kirby- Bauer disc diffusion method. Simultaneously, 64 Staphylococcus strains from 126 nasal swabs were isolated. Out of these, 29 coagulase-positive staphylococci and 35 CNS; 13 coagulase-positive staphylococci and 15 CNS isolates showed methicillin resistance by disc diffusion method.

Chandrasekaran et al. (2014) determined antibiotic susceptibility test for 401 clinical mastitis samples with Kirby-Bauer method revealed that antibiotic resistant mastitis was detected in 235 (56.1%) out of 401 cows. The predominant resistant causative pathogen was E. coli (50.64%) followed by S. aureus (44.25%) and MRSA (5.11%) with different antibiotic coated disks in various concentration and MIC determination.

Abbas et al. (2015) detected 201 MRSA from human beings. They were then separated into two categories i.e. Community Acquired Methicillin-resistance Staphylococcus aureus (CA-MRSA) and Hospital Acquired Methicillin-resistance Staphylococcus aureus (HA-MRSA) according prescribed criteria. All isolates were 100 % sensitive to vancomycin and linezolid. About 61.19% of MRSA isolates were resistant to erythromycin, 52.73% to ciprofloxacin, 38.80 % to clindamycin, 37.81% to gentamicin, 29.35% to co-trimoxazole and 19.40% to tetracycline.

Gupta et al. (2015) analyzed 30 samples and susceptibility testing of MRSA isolates was done for various β-lactam, cephalosporins and methicillin antibiotics. MRSA was detected in 40% (12 out of 30 isolates). MRSA producing strains not only showed high-level resistance to β-lactam antimicrobial agents viz., ampicillin (82.35%) followed by ciprofloxacin (70.58%), co-trimoxazole (58.82), cefixime (52.94), doxycycline/ pefloxacin (41.17%), ofloxacin (35.29%) norfloxacin (29.41%), cefuroxime/ cephadroxil/ amoxiclave/ cefazolin/ cephalexin/ amikacin/ netilmicin (17.64%).

Garipcin and seker (2015) studied antimicrobial sensitivity of different MRSA isolates from Human and cattle origin. The sensitivity for oxacillin agar screening test, oxacillin and cefoxitin disc diffusion tests was found to be 75 %, 50 % and 100 %, respectively, while the specificity of the three tests was 100 %. According to the antibiotic susceptibility test results, high resistance rates were determined against clindamycin (46.1 %), kanamycin (46.1 %), mupirocin (46.1 %), erythromycin (38.5 %), fusidic acid (38.5 %) and tetracycline (38.5 %) in 13 MRSA isolates obtained from
humans. Resistance to clindamycin, erythromycin, fusidic acid, mupirocin, rifampicin and teicoplanin was 100% in all 3 MRSA strains isolated from cattle nasal swab samples.

Nimavat (2015) carried out antimicrobial sensitivity test as per the standard disc diffusion method. Different patterns of antibiotic-resistance were observed in the tested isolates. Based on antibiotic susceptibility test by disk diffusion method 10 methicillin resistant isolates were identified. Out of 10 isolates 2 were Methicillin-resistant Coagulase Positive Staphylococci (MRCPS) whereas rests 8 were Methicillin- resistant Coagulase Negative Staphylococci (MRCNS). All isolates showed higher susceptibility to chloramphenicol (87.09%), oxacillin (83.87%), oxytetracycline (79.03%), methicillin (83.87%), ofloxacin (69.35%) and rifampicin (67.74%).

Simultaneously, they have determined minimum inhibitory concentration by E-test, on which all isolates were shown methicillin resistance, majority of these isolates were found multidrug resistant in which each isolates were resistant to an average 17.3 antibiotics among selected 23 antibiotics and the multiple resistance index (MARI) were determined.

Etinosa et al, (2016) studied antimicrobial susceptibility profile of the Staphylococcus spp. showed that all the Staphylococcus spp. were 100% resistant to methicillin, penicillin, amoxicillin, clindamycin, chloramphenicol, and trimethoprim-sulfamethaxazole. S. aureus, S. chromogenes, S. epidermidis, S. haemolyticus, S. simulans, and S. pseudintermedius were 100% resistant to cloxacillin while S. saprophyticus and S. xylosus were 92.30% and 75% resistant to cloxacillin, respectively. S. haemolyticus, S. simulans, S. saprophyticus, and S. xylosus were 100% resistant to erythromycin while S. aureus, S. chromogenes, S. epidermidis, and S. pseudintermedius were 90%, 87.50%, 88.20% and 83.33% resistant to erythromycin, respectively. Moreover, S. chromogenes, S. haemolyticus, S. simulans, S. pseudintermedius and S. xylosus were 100% resistant to kanamycin while S. aureus, S. epidermidis, and S. saprophyticus were 90%, 94.12% and 92.31% resistant to kanamycin, respectively. Statistical analysis revealed that there was no significant difference observed between the antimicrobial susceptibility profiles of staphylococcal isolates.
Valle et al., (2016) isolated total 236 S. aureus isolates from the human clinical specimens from Makati City, Philippines. Out of total 236 isolates, 108 isolates (45.76 %) were found to be MRSA. Results showed that the MRSA strains were resistant to trimethoprim-sulfamethoxazole (20.37 %), azithromycin (10.19 %), gentamicin (5.56 %), and linezolid (4.63 %), while all were susceptible to vancomycin, nitrofurantoin, levofloxacin, minocycline, rifampin, and tetracycline. One isolate was found positive for inducible clindamycin resistance.

Het al (2016) studied antimicrobial sensitivity test from 7 bovine and 20 human methicillin resistant isolates were. Out of 7 bovine isolates and 20 human isolates 6 and 5 were Methicillin- resistant coagulase positive Staphylococcus aureus whereas rests 1 and 15 were Methicillin-resistant coagulase negative staphylococci (MRCONS) respectively. Among the 7 bovine and 20 human MRS isolates based on disk diffusion method and were found multidrug resistant among selected 14 antibiotics. In the same study, all bovine and human isolates shown higher susceptibility to chloramphenicol (87.24%) & (90.91%), gentamicin (87.24%) & (86.37%) and ampicillin/Sulbactam (93.62%) & (84.1%) respectively. Resistance to some of the antimicrobials (ceftriaxone/sulbactam, cefoperazone/sulbactam, amoxicillin/sulbactam, amoxyclov, and penicillin-G) was noticeably high in both, milk and human isolates.

2.4 PREVALENCE AND REPORTING OF METHICillin-RESISTANT STAPHYLOCOCCI AMONG DIFFERENT ANIMAL AND HUMANS.

The first MRSA isolates from bovine mastitis were described in 1972 (Devriese et al., 1972). MRSA has been reported in many studies (Vanderhaeghen et al., 2010b). The prevalence of MRSA has been reported 4.5% and 2.8% in Korean herds, respectively (Moon et al., 2007a). In herds of France and Belgium, it has been reported to be 3.2% and 9.3% of tested strains, respectively (Vanderhaeghen et al., 2010a). Turkyilmaz et al. (2010) reported 17.2% MRSA in Aydin region of Turkey. Only, 25MRSA were shown in 17 dairy farms in a recent report in Germany (Febler et al., 2010). Literature surveys reveal only a few reports on the prevalence of MRSA in bovine in India.

Kearns et al. (1999) developed mPCR to detect the coagulase gene (coa; pathognomic of Staphylococcus aureus) and the mecA gene (characteristically encoding for Methicillin-resistance in staphylococci) in a single, rapid test. Suitable
primers for the gene targets and an internal, amplification control were incorporated into a multiplex PCR assay, which was then optimized on a capillary air thermal cycler to improve the turnaround time of the test to approximately 1.5 hours. The assay was evaluated with 111 fresh clinical isolates of staphylococci. The multiplex PCR correctly distinguished between isolates of *S. aureus*, which were sensitive to methicillin (MSSA) and those resistant to it (MRSA). It also correctly differentiated between similar isolates of coagulase negative staphylococci (MSSE and MRSE respectively). They were concluded that this multiplex PCR was a rapid and reliable method for the detection of Methicillin-resistant staphylococci.

WHO (2000) reported that many types of microorganisms cause infection in humans and animals, so disease prevention and treatment strategies must be adapted to reflect infection risk factors and available treatment options. Over the past decades, most pathogenic species have developed resistance to one or more antimicrobials. Some of the species in which resistance was of greatest public health concern were listed. Among them *Staphylococcus aureus*, including community-associated MRSA (Methicillin-resistant *S. aureus*) was one of the pathogen.

Guerin-Faublee et al. (2003) analyzed 119 isolates of *S. aureus* collected between 1998 and 2000 in France from cows with clinical mastitis. For strains with an oxacillin MIC greater than 2 mg/L were employed for PCR for identification of *meca* but no MRSA was identified using PCR.

Farzana et al. (2004) analyzed 50 raw milk samples collected in 1992 in Pakistan. *S. aureus* was present in all the samples. Resistance to oxacillin and methicillin was assessed by disk diffusion (Bauer-Kirby) test. 10% of the isolates (eight of 77) were methicillin resistant.

Sakai et al. (2004) standardized a real-time PCR assay that uses two fluorescence resonance energy transfer probe sets and targeted the ‘*tuf*’ gene of staphylococci one probe set detected the *Staphylococcus* genus, whereas the other probe set was specific for *S. aureus*. Of 138 cultured isolates, which contained 41 isolates of staphylococci representing at least 9 spp., and 100 positive blood cultures that contained gram-positive cocci in clusters were tested. The assay was 100% sensitive and 100% specific for the detection of the *Staphylococcus* genus and of *S. aureus*.
Cuteri et al. (2004) characterized 32 (14.34%) from animals and 53 (63.8%) from men strains of *S. aureus* and showed resistance to methicillin. The microorganisms isolated from men revealed, among them, a high similarity while only two strains, from animals, were considered identical. The resistance to methicillin involved both human and veterinary pathology. The human MRSA strains were higher than the animal’s ones. The strains isolated from animals showed a large genomic variability while in man the number of indistinguishable microorganisms, induces to suppose the existence of a prevalent clone.

Baptiste et al. (2005) isolated MRSA from 67 horses, out of these, 11 were positive (16%) for carriage and 3 had MRSA associated clinical infections (pleuropneumonia, chronic septic arthritis, and chronic dermatitis). None of the isolates submitted from human at the equine hospital were positive for MRSA.

Baptiste et al. (2005) took swab from cats (n = 50) and dogs (n = 55) treated at the hospital. One cat was positive for Methicillin-resistant staphylococci, and 4 dogs were positive for MR-CNS, all of which were confirmed by PCR to be carrying the *meca* gene. The 2 samples were positive for MRSA from the human who remains in contact with this animals.

Paule et al. (2005) developed and validated real time polymerase chain reaction (PCR) for detection of the *femA* gene for *S. aureus* and the *meca* gene for methicillin resistance. Out of total 332 positive blood cultures tested, the assay had 100% sensitivity and specificity for identifying methicillin susceptible (n = 28) and methicillin resistant (n = 28) *S. aureus*. PCR detection yielded rapid (2-3 hours) results and accurate identification of *S. aureus* directly from signal-positive blood culture bottle samples.

Kwon et al. (2006) found an isolation rate of 0.18% of MRSA in 9,055 milk samples with more than 500,000 somatic cells/mL collected in 1999, 2000, and 2003 in the Republic of Korea. MICs of oxacillin were detected with a microdilution test of the NCCLS for the *S. aureus* isolates. All MRSA isolates harboured SCC*mec* type IV, revealed the same PFGE profile, and showed Sequence Type (ST) 5 with an allelic profile of 1-4-1-4-12-1-10.

Klevens et al. (2006) identified that the prevalence of MRSA among clinical *S. aureus* isolates varies between below 1% and above 40% worldwide.
Juhasz-Kaszanyitzky et al. (2007) characterized Methicillin-resistant Staphylococcus aureus (MRSA) from cows with subclinical mastitis from a person who worked with these animals. The bovine and human strains were indistinguishable by phenotyping and genotyping methods and were of a low frequency spa type. This finding indicates the first documented case of direct transmission of MRSA between cows and humans.

Moodley et al. (2008) assessed the MRSA on animal exposure and known MRSA risk factors from human participants (N=702) at conferences and revealed MRSA carriage was significantly (P<0.02) higher among the veterinary practitioners (3.9%) than among the participants not professionally exposed to animals (0.7%). Six of the nine MRSA strains isolated from veterinary practitioners belonged to clonal complexes (CC) previously associated with horses (CC8), small animals (CC22), and pigs (CC398). Although four of the nine positive veterinarians carried the CC associated with pigs, exposure to small animals, cattle or horses, but not to pigs, was found to be a significant risk factor. The results indicate that veterinarians were at risk of MRSA carriage.

Pu et al. (2009) investigated the prevalence of Staphylococcus aureus and Methicillin-resistant S. aureus (MRSA) in 120 retail meat samples. Of these, L.A. S. aureus strains were recovered from 45.6% of pork samples and 20% of beef samples, whereas MRSA strains were isolated from 6 meat samples (five pork samples and one beef sample).

Saleha and Zunita (2010) studied the occurrence of MRSA in animal and human. The studies reviewed a widespread occurrence of MRSA in animal species, which include pigs, horses, dogs, cats and chickens and in humans and showed transmissions in both directions. Thus, MRSA was of great concern in both veterinary and human medicine as it could serious illness in both sets of populations.

Vanderhaeghen et al. (2010b) reported that the occurrence of MRSA was also emerging in veterinary medicine. The livestock associated clone CC398 had been found not only in livestock but also in humans living in close contact with animals, like pig and dairy farmers, adding to the evidence of transfer of MRSA between animals and humans (and vice versa). In studying the epidemiological aspects of MRSA, up to now, mainly the food producing animals have been of concern.
Huber et al. (2010) investigated 2,662 samples from March to September 2009 in Switzerland, were tested for the presence of Methicillin-resistant *Staphylococcus aureus* (MRSA). The collection comprised nasal swabs from 148 pig farmers, 133 veterinarians, 179 slaughterhouse employees, 800 pigs, 300 calves, 400 cattle, 100 pooled neck skin swabs from chicken carcasses, and 460 food samples of animal origin. Moreover, 142 *S. aureus* strains, isolated from bovine mastitis milk, were included in the study. Twenty samples (< 1%; four veterinarians, 10 pigs, three calves, one young bull, and two mastitis milk samples) tested positive for MRSA.

Kumar et al. (2011b) found a 13.1% MRSA prevalence when analyzing 107 strains of *S. aureus* from 195 mastitic milk samples in India. Disk diffusion method on Mueller-Hinton agar according to CLSI guidelines was used to determine antibiotic susceptibility profile and molecular confirmation with PCR of 16S rDNA, *nuc*, and *mecA* genes.

Haran et al. (2012) collected total of 150 pooled BTM samples from 50 farms and revealed Herd prevalence of Methicillin-susceptible *S. aureus* (MSSA) was 84%, while MRSA herd prevalence was 4%. A total of 93 MSSA isolates and 2 MRSA isolates were recovered from 150 BTM samples. Out of this 2 MRSA isolates, one isolate was ST5-USA100-spa type 2 (traditionally reported as HA-MRSA) and the other isolates was ST8-USA300-t121 (traditionally reported as CA-MRSA).

Graveland et al. (2011) observed the mean MRSA prevalence was 38% in farmers and 16% in family members. Presence of MRSA in farmers was strongly related to duration of animal contact and was strongly reduced in periods with absence of animal contact (25%). Family members, especially children, were more often carriers when the farmer was a carrier (OR = 2, P, 0.05). Only 7% (n = 11) of the participants appeared to be persistent carriers.

Garcia-Graells et al. (2012) observed that the prevalence and risk factors associated with livestock-associated MRSA (LA-MRSA) carriage was examined in veterinarians. The MRSA and LA-MRSA carriage rates were 9.5% and 7.5% for MRSA and LA-MRSA, respectively, in Belgium and 1.4% in Denmark. All LA-MRSA isolates were resistant to tetracycline and 53.4% (7/13) showed a multi-resistant phenotype. Since carriage of MRSA ST398 may increase the risk of complications
during hospitalization, author underline that preventive measures may need to be developed for veterinary professionals, particularly for livestock veterinarians.

Gilbert et al. (2012) identified the livestock-associated Methicillin-resistant *Staphylococcus aureus* (LA-MRSA) carriage among workers in pig slaughterhouses and associated risk factors, including occupational exposure to LA-MRSA. Nasal swabs of participants were taken. Nasal swabs and surface wipes, air and glove samples were screened for presence of Methicillin-resistant *Staphylococcus aureus* (MRSA). A total of 11 of 341 (3.2%) participants were identified as nasal MRSA carriers. MRSA-positive workers were predominantly found at the start of the slaughter process. Major risk factors for carriage were working in the lairage and working in the scalding and dehairing area. Most nasal isolates (73%) belonged to the LA-MRSA clone ST398. MRSA ST398-positive environmental samples were found throughout the slaughter process. A clear decrease was seen along the slaughter line in the number of MRSA-positive samples and in the MRSA amount per sample.

Aquino et al. (2012) studied the prevalence of Methicillin resistant staphylococci in the animals and staff of a teaching and research farm in Brazil. Nasal swab samples were collected from healthy dairy cattle (n=36) and humans (n=13). Detection of *mecA* gene was performed by PCR. Antimicrobial resistance of *mecA*+ isolates was determined by disk-diffusion method (Kirby-Bauer) according with the CLSI breakpoints. No MRSA was isolated.

Frana et al. (2013) carried out isolation and characterization of Methicillin-resistant *Staphylococcus aureus* from pork farms and visiting veterinary students. Thirty (30) veterinary students were enrolled and 40 pork farms were visited. MRSA was detected in 30% of the pork farms and in 22% of the students following an exposure to a MRSA-positive pork farm. During study a total of 362 samples were collected from these sites including 194 from pigs and 168 from the environment. Overall MRSA was detected in 17.4% (63/362) of the samples tested including 17.5% (34/194) of the pig samples and 17.3% (29/168) of the environmental samples. In MRSA-positive farms, either animal or environmental samples were positive 60.1% (63/104) of the time. Of these, 69.4% (34/49) of pig samples and 52.7% (29/55) of environmental samples were MRSA-positive.
Garipcin and Seker, (2015) reported, 44 (29.3 %) and 8 (3.2 %) *S. aureus* isolates were obtained from the sampled 150 humans and 250 cattle, respectively. 16SrDNA specific bands were detected in all of 52 *S. aureus* isolates obtained from nasal samples, 16 (30.8 %) of these isolates harboured the *mecA* gene. The distribution of MRSA isolates among the nasal *S. aureus* isolates was 29.5 % (13/44) for humans and 37.5 % (3/8) for cattle. These MRSA isolates were obtained from 9 out of 35 farms. The nasal carriage rates of MRSA in humans and cattle were determined as 8.7 % (13/150) and 1.2 % (3/250), respectively. The MRSA strains isolated from humans and animals were not isolated from the same farms.

Etinosa *et al.* (2016) assessed 283 samples from cattle (137 milk samples and 146 nasal swabs, out of these, 50 Methicillin-resistant staphylococcal positive isolates from the nasal cavity and 50 Methicillin-resistant staphylococcal strains from the raw milk samples were recovered. The occurrence of Methicillin-resistant staphylococcal isolates from the samples were: *S. aureus* (30: 19 nasal cavity and 11 raw milk), followed by *S. epidermidis* (17: 13 nasal cavity and 4 raw milk), *S. haemolyticus* (15:0 nasal cavity and 15 raw milk), *S. saprophyticus* (13:8 nasal cavity and 5 raw milk), *S. chromogenes* (8: 0 nasal cavity and 8 raw milk), *S. simulans* (7:0 nasal cavity and 7 raw milk), *S. pseudintermedius* (6:6 nasal cavity and 0 raw milk) and *S. xylosus* (4:4 nasal cavity and 0 raw milk).

Fateh and Sharmin, (2016) collected and studied 36 chicken meat samples and analyzed to screen MRSA strains (25 samples out of 36 were MRSA positive; 69.4%). Eleven meat samples were negative for MRSA isolates and rest of the 25 isolates were identified as *S. aureus* strains using *nucA* specific primers and positive for the presence of *mecA* gene and confirmed as MRSA strains.

Bhagat *et al.* (2017) processed 165 milk samples from suspected cases of clinical mastitis in cows and buffaloes belonging to North Gujarat. Out of these, 55 *Staphylococcus aureus* isolates were recovered from bovine mastitis. Out of 55 *Staphylococcus aureus* isolates, 7 and 11 isolates were detected as methicillin resistant by Oxacillin screen agar and CHROM agar, respectively. CHROM agar was found superior to the oxacillin screen agar method. This provides an alternative for the detection of MRSA in clinical laboratories, especially when PCR is unavailable. All the 55 isolates yielded desired amplicon of *sa442* gene which confirmed as a
Review of Literature...

*Staphylococcus aureus*. Out of 11 MRSA isolates identified (CHROM agar), only 4 isolates were found to be positive for Methicillin resistance targeting a 533 bp fragment.

### 2.5 Molecular Detection of Virulence Genes (Toxic Genes) Including Panton-Valentine Leukocidin (PVL) Gene from MRS Organism

Gerard *et al.* (1999) screened a total of 172 *S. aureus* strains in human for PVL genes by PCR amplification from various ailments from human. Out of this, PVL genes were detected in 93% of strains associated with furunculosis, 85% of those associated with severe necrotic hemorrhagic pneumonia (all community-acquired), 55% of strain associated with cellulitis, 50% of cutaneous abscess strains, 23% of osteomyelitis strains and 13% of finger-pulp–infection strains.

Baptiste *et al.* (2005) observed that all MRSA isolates (1 from cat and 3 from dog) were positive for the *mecA* and *femA* genes, carried the SCCmec type IV cassette, and were *agr* operon group 1 strains but were negative for PVL genes. PFGE showed that the human and dog clinical MRSA isolates were identical to the human epidemic strain, EMRSA-15.

Yu *et al.* (2008) recovered 25 (12.8%) of 195 *Staphylococcus aureus* isolates were positive for Panton–Valentine leukocidin (PVL) genes in a teaching hospital in Wenzhou, China. Nineteen (11.9%) of 160 hospital-acquired isolates and six (17.1%) of 35 community-acquired isolates, harbored lukS’F-PV. Six sequence types (ST88, ST239, ST398, ST25, ST30 and ST59) were found among 18 PVL-positive methicillin-resistant isolates with SCCmec types I, III, IIIA or IV. Only ST88 was found among seven PVL-positive Methicillin-susceptible *S. aureus* isolates.

Hae-Kyung *et al.* (2008) detected four virulence genes, *mecA*, *PVL*, *bbp* and *icaA* genes for *S. aureus* and *S. epidermidis* via multiplex PCR from 750 infants having UTI. Out of these, *S. aureus* virulence genes were detected in 26 cases as *mecA* (16/26, 59.3%), *PVL* (17/26, 63.0%), *bbp* (7/26, 26.9%) and *icaA* (20/26, 76.9%), whereas *S. epidermidis* virulence genes were detected in 22 cases as *mecA* (17/22, 81.0%), *PVL* (15/22, 71.4%), *bbp* (3/22, 13.6%) and *icaA* (13/22, 50.1%). Therefore, *mecA*, *PVL* and *icaA* genes of MRSA and MRSE were detected with high positivity in urines from infants with fever. The results demonstrate that community-acquired MRSA or MRSE may be responsible for UTI incidence in febrile infants.
Pu et al. (2009) observed that, out of total 47 S. aureus isolates, 22 confirmed MRSA isolates, 3 isolates recovered from pork samples were positive for PVL, a two-component staphylococcal membrane toxin that targets leukocytes. Primarily associated with CA-MRSA but not HA-MRSA. The PVL was considered to be the principal virulence factor responsible for the spread of CA-MRSA in skin and soft-tissue infections.

Haran et al. (2012) analyzed 8 suspected MRSA isolates for their toxin production profiles and the presence of TSST and PVL toxin genes. Of this, 2 isolates were confirmed as MRSA by detection of the mecA gene and phenotypic expression of methicillin resistance, one MRSA isolateSp12 produced SE (Staphylococcal enterotoxin) B, SE C, and SE D, while isolate Sp19 produced SE C, SE D, and SE E. None of the 8 isolates showed the presence of the TSST gene, while the presence of the PVL toxin gene was detected in one MRSA isolate.

De Almeida et al. (2013) studied 270 milk samples from 135 sheep to ascertain the prevalence of the different accessory gene regulator (agr) groups and to evaluate the occurrence of encoding genes for cytotoxin, adhesins and toxins with superantigen activity in S. aureus isolates from milk of ewes with clinical and subclinical mastitis in sheep flocks raised for meat production. The presence of cflA gene was identified in 100% of the isolates. Whereas the frequency of hla and lukE-D genes was 77.3% and 82.8%, respectively and all these isolates from clinical mastitis presented these genes. The sec gene, either associated to tst gene or not, was identified only in isolates from subclinical mastitis. None of the following genes were identified: bbp, ebpS, cna, fnbB, icaA, icaD, bap, hlg and lukM-lukF-PV.

Bidya et al. (2014) screened 73 nosocomial isolates of S. aureus (46 MRSA and 27 MSSA isolates) from 2 hospitals and obtained an overall PVL-positivity rate of 35.6% among the hospital isolates. Out of 46 MRSA isolates 12 (26.1%) isolates were positive for PVL gene, whereas 14 (51.9%) isolates out of 27 MSSA found to be positive for PVL gene.

Alli et al. (2015) studied prevalence rate of virulent genes from 156 isolates of S. aureus obtained from human. Out of these, the proportion of S. aureus with the following genes was ascertained: hla (55.1%), icaA (42.3%), PVL (34.6%), fnbA (8.3%), bbp (4.5%) and eta (3.8%). All the isolates were etb and cna negative. The
prevalence of the PVL gene in Methicillin-susceptible *Staphylococcus aureus* (MSSA) was 53.3% compared with 9.1% of MRSA. An association between virulence genes (*eta* and *icaA*) and *mecA* positive *S. aureus*; and significant difference in the distribution of virulence genes in in-patients and out-patients were found.

Martins *et al.* (2015) collected 473 milk samples from 242 sheep on three farms. Out of these, 20 isolates are of *S. aureus* strains and none carried the *mecA* gene. The two *sec* gene-positive isolates and the isolates carrying the *tst* and *luk-PV* genes were positive by RT-PCR. *Staphylococcus aureus* isolated from the three flocks studied showed high susceptibility to the drugs tested and none was biofilm producer (*icaA* positive), indicating that biofilm formation was not a virulence factor causing infection by these strains. In this study multiple acquisition of virulent gene was reported and found only one isolate carried the complete *icaADBC* operon, whereas other genes of the operon, mainly *icaA* + *icaD*, were detected in most isolates. However, none of the isolates expressed the *ica* genes detected. The *bap* gene encodes a protein that plays an important independent role in biofilm formation (Cucarella *et al.*, 2004). This gene was detected in four strains. In same study, they detected exotoxin Panton-Valentine leukocidin (PVL) was detected in only one train and its expression was confirmed. The *PVL* is one of the most important virulence factors produced by *S. aureus*, contributing to the pathogenicity of this microorganism.

Fateh Rahimi and Sharmin Karimi (2016) studied presence of enterotoxin gene from 116 MRSA strains isolated from chicken meat samples and all these isolates were found positive for *sea*, *sek* and *seq* genes. All strains were negative for the presence of other enterotoxin genes including *PVL* gene.

### 2.6 IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF METHICILLIN-RESISTANT STAPHYLOCOCCI AND SCCMEC TYPING FROM ANIMAL AND HUMANS.

#### 2.6.1 Multiplex PCR for diagnosis of MRSA

Vannuffel *et al.* (1995) developed multiplex PCR strategy, in which 310 and 686 bp regions of the *mecA* and *femA* genes, respectively, were co-amplified to identify susceptible (lacking *mecA*) and resistant (mecA1) staphylococci and to differentiate *S. aureus* (*femA1*) from coagulase negative staphylococci (lacking *femA*). A third staphylococcal genomic sequence, corresponding to IS431 and spanning 444 bp, was
used as a PCR control. One hundred sixty-five staphylococcal strains were tested. All 72 methicillin resistant strains were found to be mecA1, and 92 of the 93 isolates were susceptible to lacked mecA gene. Only one coagulase-negative staphylococcus isolate carrying the mecA gene was highly susceptible to oxacillin. The femA was found in 100% of the S. aureus strains tested but was undetectable in all of the coagulase-negative staphylococci tested. In conclusion, co-amplification of mecA and femA determinants proved to be very reliable both for rapid detection of methicillin resistance and differential diagnosis between S. aureus and other staphylococci.

Schmitz et al. (1997) described a multiplex PCR on bacterial colonies picked directly from agar plates without preceding DNA preparation. Eubacteria and staphylococci were identified by 16S rRNA specific PCR products. In parallel, specific primers were used for the detection of staphylococcal coa and mecA genes. The results were compared with those of conventional susceptibility and typing methods. The specific 16srRNA PCR product for eubacterial isolates (n = 786) and staphylococci (686) was found in all strains tested. The coa gene was detected only in S. aureus (488) strains with a specificity of 100%, and was not detected in any of the coagulase-negative staphylococci (198). The mecA gene was detected in 98% of Methicillin-resistant staphylococci (393) and in 2% of all Methicillin-susceptible staphylococci (293). The multiplex PCR with co-amplification of different determinants provides rapid reliable information on staphylococcal identification and methicillin susceptibility supporting the diagnosis, treatment and control of staphylococcal infections.

Kearns et al. (1999) developed mPCR to detect the coagulase gene (coa; pathognomonic of Staphylococcus aureus) and the mecA gene (characteristically encoding for Methicillin-resistance in staphylococci) in a single, rapid test. Suitable primers for the gene targets and an internal, amplification control were incorporated into a multiplex PCR assay, which was then optimized on a capillary air thermal cycler to improve the turnaround time of the test to approximately 1.5 hours. The assay was evaluated with 111 fresh clinical isolates of staphylococci. The multiplex PCR correctly distinguished between isolates of S. aureus, which were sensitive to methicillin (MSSA) and those resistant to it (MRSA). It also correctly differentiated between similar isolates of coagulase negative staphylococci (MSSE and MRSE respectively). They were concluded that this multiplex PCR was a rapid and reliable method for the detection of Methicillin-resistant staphylococci.
Review of Literature...

Perez-Roth et al. (2001) describe a multiplex PCR assay for the detection of clinically relevant antibiotic resistance genes harbored by some *Staphylococcus aureus* isolates and for the simultaneous identification of such isolates at the species level. Conditions were optimized for the simultaneous detection of the 310, 456, and 651 bp regions of the *mecA* (encoding high-level methicillin resistance), *ileS-2* (encoding high-level mupirocin resistance), and *femB* (encoding a factor essential for methicillin resistance) genes, respectively, from a single colony in a single reaction tube. The *femB* PCR fragment allows the specific identification of *S. aureus*. Validation of the method was performed using 50 human isolates of Methicillin-resistant *S. aureus* (MRSA) and the appropriate control strains. This assay offers a rapid, simple, feasible, specific, sensitive, and accurate identification of mupirocin-resistant MRSA clinical isolates and could be systematically applied as a diagnostic test in clinical microbiology laboratories, facilitating the design and use of antibiotic therapy.

Maes et al. (2002) described a triplex PCR targeting the 16S rRNA, *mecA*, and *nuc* genes. It was developed for identification of staphylococci and detection of methicillin resistance. After validation of the assay with a collection of strains of Staphylococci and Enterococci (n=169), the assay was evaluated with cultures of blood with gram-positive cocci from 40 patients. Accurate results were obtained for 59 (98%) of 61 cultures within 6 h of growth detection.

Strommenger et al. (2003) described a multiplex PCR assay for the detection of nine clinically relevant antibiotic resistance genes of *Staphylococcus aureus*. Conditions were optimized to amplify fragments of *mecA* (encoding methicillin resistance), *aacA-aphD* (aminoglycoside resistance), *tetK*, *tetM* (tetracycline resistance), *erm*(A), *erm*(C) (macrolide-lincosamide-streptogramin B resistance), *vat*(A), *vat*(B), and *vat*(C) (streptogramin A resistance) simultaneously in one PCR amplification. An additional primer pair for the amplification of a fragment of the staphylococcal 16S rDNA was included as a positive control. The multiplex PCR assay was evaluated on 30 different *S. aureus* isolates, and the PCR results correlated with the phenotypic antibiotic resistance data obtained by the broth microdilution assay.

Mohanasoundaram and Lalitha (2008) have described multiplex PCR for determination of MRSA from 150 clinical isolates from hospital acquired infections based on *mecA* gene and *femB* gene detection. In the same year Rallapalli et al. (2008)
described Multiplex polymerase chain reaction (PCR) strategy for rapid identification of clinically relevant Methicillin-resistant *Staphylococcus aureus* (MRSA) that targets *mecA* and coagulase genes. Out of 55 *S. aureus* strains, three strains demonstrated *mecA* gene, which appeared to be oxacillin sensitive by disc diffusion. When (MS-CoNS) were evaluated, 10 isolates classified as oxacillin sensitive phenotypically, yielded positive results in PCR method.

Hassanain et al. (2009) developed pentaplex PCR assay for the rapid detection of MRSA. The assay simultaneously detected five genes, namely *16S rRNA* of the *Staphylococcus* genus, *femA* of *S. aureus*, *mecA* that encodes methicillin resistance, *lukS* that encodes production of Panton-Valentine Leukocidin (PVL), a necrotizing cytotoxin, and one internal control. The diagnostic evaluation of MRSA carried out using 230 clinical isolates, showed 97.6% of sensitivity, 99.3% of specificity, 98.8% of positive predictive value and 98.6% of negative predictive value compared to the conventional method.

Vanderhaeghen et al. (2010a) performed triplex PCR, targeting a *Staphylococcus*-specific *16S rRNA* sequence, the *mecA* gene, and the *S. aureus*-specific region of the thermonuclease gene (*nuc*) to identify MRSA, and found that nearly 10% of the *S. aureus* isolated from bovine subclinical and clinical mastitis (118 isolates from 118 different farms in Belgium collected from 2006 to 2007) in their study were MRSA.

Jian et al. (2011) developed a multiplex polymerase chain reaction (PCR) assay for simultaneous species identification and detection of penicillin, erythromycin and tetracycline resistance genes directly from bovine mastitis milk suggested that this mPCR assay can be used as an alternative diagnostic method in the routine diagnosis for rapid, sensitive, and specific detection of *S. aureus* and its associated antibiotic resistance genes in mastitic milk samples.

Duran et al. (2012) described multiplex PCR method, for detection of resistance gene in staphylococcal isolates. A total of 298 *staphylococci* clinical isolates were subjected to antimicrobial susceptibility testing. The genes implicated in resistance to oxacillin (*mecA*), gentamicin, erythromycin (*ermA, ermB, ermC*, and *msrA*), tetracyclin (*tetK, tetM*), and penicillin (*blaZ*) were amplified using multiplex PCR method, results showed that the phenotypic antibiotic susceptibility patterns were not similar to those obtained by genotyping done by multiplex PCR. Rapid and reliable methods for
antibiotic susceptibility are important to determine the appropriate therapy decisions. Multiplex PCR can be used for confirmation of the results obtained by conventional phenotypic methods.

Nimavat (2015) applied multiplex PCR assay to test a total of 100 clinical milk samples available at Veterinary Clinical Complex, Junagadh. Of these 100 milk samples, 69 staphylococci were identified based on amplification of 16S rRNA gene. out of this 69 samples, 20 samples were identified as coagulase negative staphylococci (CNS) based on amplification of only 16S rRNA gene, 29 samples were identified as coagulase positive Staphylococcus aureus based on amplification of three genes 16S rRNA, Nuc and Coa, 14 samples were identified as Methicillin-resistant coagulase negative staphylococci (MRCNS) based on amplification of genes 16S rRNA and meca and 6 samples were identified as positive for Methicillin-resistant staphylococcus aureus (MRSA) based on amplification of all four genes i.e. 16S rRNA, Nuc, coa and meca.

Hetal (2016) carried out multiplex PCR for the identification of staphylococcus using combination of various primers. Out of 50 milk samples tested, 47 staphylococci were identified based on amplification of 16S rRNA gene, 11 samples were identified as coagulase positive S. aureus based on amplification of Coa gene and 7 samples were identified as MRS based on amplification of meca gene. Out of these 7 MRS isolates, 3 isolates were Methicillin-Resistance Coagulase Positive Staphylococci (MRCoPS) and 4 were Methicillin-Resistance Coagulase Negative Staphylococci (MRCoNS). Eight isolates were identified as Methicillin-Sensitive Coagulase Positive Staphylococci (MSCoPS).

Similarly, she has studied 50 human nasal swabs for the identification of staphylococcus using combination of various primers. Out of 50 human nasal swab, 44 were identified as staphylococci based on amplification of 16S rRNA gene and 20 isolates among 44 staphylococci isolates were positive for meca (Methicillin resistance) gene. Out of total 20 methicillin resistant isolates, 7 isolates were Methicillin-Resistance Coagulase Positive Staphylococci (MRCoPS) and 13 were Methicillin-Resistance Coagulase Negative Staphylococci (MRCoNS). Five isolates were identified as Methicillin-Sensitive Coagulase Positive Staphylococci (MSCoPS).
2.6.2 Molecular characterization of major types of SCCmec in Methicillin-resistant staphylococci from animal and humans.

SCCmec is one of the largest bacterial mobile elements known to date, its size ranging from 21 to 67 kbp, and it is exclusive to staphylococci (Robinson and Enright, 2004). The SCCmec is considered to disseminate through horizontal transmission between staphylococcal species (Katayama et al., 2001). The molecular structure of SCCmec consists of the mec gene complex, a pair of ccr-genes, and three junkyard regions (J1-J3). The J regions are located between and around the mec and ccr complexes, and contain different genes or pseudo genes. SCCmec elements are classified into types based on the characteristics of the mec and ccr complexes, and the variants of each SCCmec type are defined by differences in the J regions.

Nadig et al. (2006) undertook the study to genotype a clinical MRSA isolates collected from hospitals in different parts of India. One hundred and eighty-six isolates were collected and characterized by phenotypic and genotypic methods using published protocols and they revealed majority of isolates were positive for mecA gene detected by PCR and possessed Staphylococcal Cassette Chromosome (SCCmec) type III or IIIA cassettes with the exception of two isolates from Nagpur, which had type II cassettes.

Walther et al. (2006) studied SCCmec typing on the isolates recovered from the dogs. Out of 16 isolates, 4 isolates were of SCCmec type IV and rest of the isolates were of ND (Not determine) types.

Kondo et al. (2007) developed six multiplex PCR for identifying the ccr gene complex (ccr), the mec gene complex (mec), and specific structures in the junkyard (J) regions. With the help of various combination of primer pairs they have identify type I and type IV SCCmec elements carrying class B mec, type II and type III SCCmec elements carrying class A mec and type V SCCmec elements carrying class C mec and specific primer pairs were used to subtype J1 regions of all five SCCmec type I or IV elements (type I.1, type IV.1, type IV.2, type IV.3, and type IV.4)

Boye et al. (2007) designed multiplex PCR with four primer-pairs to identify the five main known SCCmec types. Total 312 clinical isolates of MRSA from hospitalised patients or patients attending general practitioners were used to test the multiplex PCR. Out of these isolates, 306 (98%) were typeable by the new multiplex PCR and revealed that SCCmec type IV was the most common type (84% of the
isolates), followed by type V (6%), type I (4%) and type II (3%). SCCmec type III was found only in three imported isolates, whereas six isolates were classified as non-typeable according to the multiplex PCR.

Pu et al. (2009) Studied for SCCmec typing among the 22 MRSA positive isolates, of these 3 isolates were type IVa (a type IV subtype) and 19 isolates were type II. SCCmec type IV is predominately associated with CA-MRSA. It is characterized by its smaller size (20 to 24 kb) and carriage of a limited number of antimicrobial resistance genes. Type II is larger (52 kb) and contained additional resistance genes. Susceptibility testing revealed that isolates belonging to the two SCCmec types differed in the numbers of antimicrobials to which they were resistant.

Shabir et al. (2010) typed Methicillin-resistant Staphylococcus aureus (MRSA) isolates obtained from the hospital of Pakistan and India. Out of total 57 isolates, Thirty-four isolates carried SCCmec type III/IIIa and twenty-three carries SCCmec type IV. Among these SCCmec type, only SCCmec type III and subtype IIIa were identified in the isolates from India.

Romeeza et al. (2010) isolated Methicillin resistant Staphylococcus aureus from pus, blood, sputum and tracheal aspirates of human beings from various hospitals of Lahore. Out of total thirty five isolates, 21 (60%) isolates possessed SCCmec type IA while 14 (40%) isolates possessed SCCmec type IIC and these cassettes are shown to be multi drug resistance and have not been reported in other Asian countries so far.

Lulitanond et al. (2010) conducted studies to examine the antimicrobial susceptibilities and staphylococcal cassette chromosome mec-type for MRSA isolates collected from respiratory tract, pus, body fluid urine, eye, blood, soft tissue and lymph node from 237 patients treated in hospital at Thailand. Of these, Seventy-six isolates (97.4%) had ccr complex type III, consistent with SCCmec type III and 2 isolates (2.6%) had type II ccr complex corresponding with SCCmec type II. The mec operon was detected in 2 and 65 isolates with type II and type III SCCmec, respectively (85.9% total). The MLST for all 3 representatives with type III SCCmec were ST239, clonal complex 9 and the one representative strain with type II SCCmec was ST5, clonal complex 5.

Ehsanollah et al. (2010) developed multiplex PCR assay for the identification of major types and subtypes of staphylococcal cassette chromosome mec (SCCmec) in
Methicillin-resistant *Staphylococcus aureus* (MRSA) strains. The method used a novel 9 valent multiplex PCR plus two primer pairs for *S. aureus* identification and detection of methicillin resistance. All 389 clinical MRSA isolates from Malaysia and Europe were characterized by multiplex PCR assay and were found SCCmec type III and V were by far the most common types among both hospital and community acquired Malaysian MRSA isolates, with an apparent emergence of MRSA harbouring the IVh type.

Mert *et al.* (2011) determined the SCCmec types in Methicillin-resistant staphylococci and evaluate the diversity between methicillin-resistant CoNS (MRCoNS) and Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates. During the study, total 462 positive blood culture samples collected from human being, of these, a total 357 staphylococci were isolated, including 313 CoNS (261 MRCoNS, 52 Methicillin-susceptible coagulase negative staphylococci (MSCoNS)) and 44 were *S. aureus* (22 MRSA, and 22 Methicillin-susceptible *S. aureus* (MSSA)). Out of these, 22 MRSA and 261 MRCoNS isolates were tested for presence of SCCmec types using the TaqMan real-time method and were fond that, SCCmec type III was detected in all MRSA isolates. Whereas 261 MRCoNS isolates having SCCmec types I, II, III, IV, V, and some combinations of these types were detected in 61 (23.3%), 0 (0%), 31 (11.8%), 65 (24.9%), 37 (14.1%), and 46 (17.6%) isolates, respectively.

Inegol and Turkyilmaz (2012) conducted study to determine staphylococcal cassette chromosome mec (SCCmec) types of Methicillin-resistant staphylococci (MRS) isolated from cattle and farm workers. A total of 145 subclinical mastitic bovine milk and 91 nasal swab samples (56 from cows and 35 from farm workers) were studied for presence of MRS. Among 236 total samples, 181 staphylococci, 59 of which were found as methicillin resistant were isolated. A total 59 methicillin resistant isolates originated from bovine (37) and human (22). A total of 40 isolates including type II and III were hospital acquired whereas 19 isolates including type IV and V were community acquired. Almost all *S. haemolyticus* and *S. aureus* isolates carried hospital acquired type SCCmec. The similarity of SCCmec types between human and cattle isolates suggest evidence of transmission from animals to humans, or vice versa.

Nemeghaire *et al.* (2014) investigated the prevalence and types of MRSA present in the nose of healthy bovines of different age groups and rearing practices. Out
of total 81 isolates of MRSA, Forty-four (54.3%) isolates carried SCCmec type IV (2B) and nine (11.1%) isolates were of SCCmec type IV (2B&5), sixteen (19.8%) isolates carried SCCmec type V (5C2) and two (2.5%) SCCmec type III (3A), whereas ten (12.3%) isolates showed only the mecA gene but no ccr complex was detected with the PCR. These were thus considered non-typeable using these M-PCRs. Along with this, SCCmec type IV (2B and/or 2B&5) were found in isolates from veal (n = 37), beef (n = 12) and dairy farms (n = 4). SCCmec type V were also found in the three age groups with seven being found in isolates from veal, six from dairy and three from beef cattle. Type III cassette were found in from dairy (n = 1) and beef cattle (n = 1).

Saravanan et al. (2014) studied prevalence of SCCmec types among Methicillin-resistance coagulase negative staphylococci isolated from HIV patients in Chennai. Out of total 52 clinically significant MRCoNS isolates from HIV patients, SCCmec type I revealed (50%) to be predominant followed by type V (19%), type III (14%), type IV (7%) and type I & V (n=2, 4%). Three (6%) isolates were non typeable and type II was absent.

Bhutia et al. (2015) conducted study to determine the molecular characteristics of CA and HA-MRSA and Methicillin- sensitive S. aureus (MSSA) isolates in Sikkim. Out of total 150 isolates, 53 and 66 were positive for mecA (MRSA) and PVL genes, respectively. Thirty eight met the definition of CA-MRSA and 15 of HA-MRSA and the remaining 63 and 34 as CA and HA-MSSA, respectively. No significant difference was seen in the distribution of PVL toxin in MRSA and MSSA isolates, but it was significantly (P<0.001) high in overall MRSA isolates than in MSSA. The majority of the MRSA isolates showed a double amplification band of SCCmec type III plus V, and only a fewer isolates were amplified by single DNA fragments of type I, III, IVa and V. SCCmec types I, III, IVa were found only in HA-MRSA isolates, whereas type V in both the CA and HA-MRSA.

Govindan et al. (2015) studied Community Acquired MRSA (CA-MRSA) types (SCCmec type IV and V) as community based cross sectional study in the schools of Udupi taluka, Karnataka state, India. During the study, Staphylococcus aureus was isolated from 441 children out of 1503 subjects screened and the prevalence of Staphylococcus aureus colonization was estimated as 29.3%. Out of this 441 children’s the CA-MRSA colonization was seen in 17 (1.1%) children’s. Among these 17 CA-
MRSA isolates, seven isolates were found to be SCCmec type IV and none of them were type V.

Alli et al. (2015) carried out PCR for detection of the mecA gene including SCCmec typing of *S. aureus* isolates obtained from clinical specimen including wound swab, blood, eye/ear swab, urine etc. Out of total 156 *S. aureus* isolates obtained from clinical specimens, prevalence of mecA gene was 42.3% (66 out of 156 *S. aureus*). Out of 66 mecA positive isolates, 24 (36.4%) carried the SCCmec type II element, 4 (6.1%) with SCCmec type III, 10 (15.2%) with SCCmec IV and 28 (42.4%) harboured SCCmec type V and they concluded that SCCmec type II & V MRSA strain were dominated in south-western Nigeria.

Havaei et al. (2015) collected and studied 450 milk sample from clinical and subclinical mastitis samples. Out of total 450 samples, Fifty-four (12%) isolates were positive for *S. aureus*. Among 54 *S. aureus* isolates, 10 and 9 MRSA strains identified by cefoxitin disc diffusion and oxacillin agar screening methods, respectively. All 10 MRSA isolates identified by cefoxitin disc diffusion, were positive for mecA gene and all of them belonged to SCCmec type IV. The sea genes were detected in 19 isolates and only two isolates were positive for seb genes. One isolate possessed both sea and seb genes. All the 10 MRSA strains carried SCCmec type IV by multiplex PCR

Fateh Rahimi and Sharmin Karimi (2016) collected 116 colonies from chicken meat samples and subjected to SCCmec type. During the study all 116 isolates were detected as MRSA strains and all isolates harbored SCCmec type III and were classified as hospital acquired (HA)-MRSA strains.

Khaji and Shahreza (2017) collected total of 200 raw milk samples including bovine (n=80), ovine (n=60) and caprine (n=60) from supermarkets. Out of 200 milk samples, 60 (30%) raw milk samples were contaminated with MRSA. Bovine had the highest (20%), while caprine had the lowest (15%) prevalence of MRSA. The most prevalent alleles of SCCmec were SCCmec IVa (60%), SCCmec V (50%) and SCCmec IVb (25%). Bovine and caprine milk samples had the highest prevalence of SCCmec types.
2.7 CORRELATION STUDY OF SCCMEC TYPES WITH STAPHYLOCOCCAL ASSOCIATED VIRULENCE GENE.

Vandenesch et al. (2003) collected 117 different isolates of CA-MRSA from different continent. Among this methicillin resistance was conferred in all 117 CA-MRSA isolates by the truncated SCC mec type IV element and all the isolates contained the PVL locus. In addition, 112 isolates harbored the related lukE-lukD genes of another leukocidin frequently recovered from patients with all types of staphylococcal infections. Most (113 [97%] of 117) isolates were of agr type 3.

Kwon et al. (2005) isolated fourteen MRSA and a silent mecA-carrying Methicillin-susceptible S. aureus (smMSSA) were isolated from the milk of cows with an isolation ratio of 0.18%. SCC mec of 14 MRSA strains were designated as new subtype IVg, and one smMSSA strain was not classified. All 14 MRSA strains shared Panton-Valentine Leucocidin (PVL) and staphylococcal enterotoxin D (SED), SEI and SEJ, whereas smMSSA strain had only PVL. All MRSA and smMSSA isolates showed no multidrug resistance and had community acquired MRSA (CA-MRSA) characteristics.

Huang et al. (2006) isolated 283 individual MRSA-positive isolates out of 328 patients. Scientist revealed the proportion of MRSA infections among all S. aureus isolates was found to be 42%. During the study, 156 (55.1%) isolates of MRSA positive met the definition of HA-MRSA infections and the rest; 127 isolates (44.9%), were classified as CA-MRSA.

Moroney et al. (2007) screen sixty isolates by real-time PCR to confirm the identification of S. aureus (femA) and the presence of the mecA gene. Out of 60 MRSA isolates that were tested, the majority possessed the type II cassette (57% [34/60]) and most had met the criteria for HA-MRSA. All 34 of the SCC mec type II isolates were PVL negative (100%). During the study, out of 60 isolates, 25 isolates were determined to be CA-MRSA. Out of these, 23 isolates were SCC mec type IV and were PVL positive (92%) and 2 isolates were type IV were PVL negative (8%).

Jimenez et al. (2011) obtained sixty MSSA and MRSA isolates from paediatric patients and identified the genes encoding virulence factors, which included Panton-Valentine Leucocidine (PVL), staphylococcal enterotoxins A-E, exfoliative toxins A and B and toxic shock syndrome toxin 1. Typing of the staphylococcal chromosome
cassette mec (SCCmec) was performed in MRSA strains. The virulence genes were more diverse and frequent in MSSA than in MRSA isolates (83% vs. 73%). MRSA strains harboured SCCmec types IVc (60%), I (30%), IVa (7%) and V (3%). SCCmec type IVc isolates frequently carried the PVL encoding genes and harboured virulence determinants resembling susceptible strains while SCCmec type I isolates were often negative.

Machuca et al. (2013) collected 53 MRSA isolates from pediatric patients. The MRSA isolates were typed based on the SCCmec, MLST, spa and agr genes. The molecular characterization included the detection of Panton-Valentine Leukocidin, superantigenic and exfoliative toxins and adhesin genes. The correlation between themolecular types identified and the profile of virulence factors was determined for all isolates. The isolates were carriers of toxin genes, and hlg (100%), sek (92%) and PVL (88%) were the most frequent. Ten toxin gene profiles were observed, and the most frequent were seq-sek-hlg (22.6%), sek-hlg (22.6%), seb-seq-sek-hlg (18.9%) and seb-sek-hlg (15.1%). The adhesion genes were present in most of the MRSA isolates, including the following: clf-A (89%), clf-B (87%), fnb-A (83%) and ica (83%). The majority of the strains carried SCCmec-IVc and were identified as causing nosocomial infection. Some virulence genes were associated with specific molecular type’s sek and PVL were predominantly associated with SCCmec I and SCCmec IVc. No significant association between a molecular type and the virulence factors was found.

Asghar (2014) analyzed 206 S. aureus clinical isolates using standard microbiological methods. Multiplex PCR was performed on genomic DNA from MRSA isolates in order to identify the types of SCCmec and PCR was performed to detect the PVL gene among the isolates. Of the 206 S. aureus isolates, 114 (55.3%) were MRSA and 100 of the MRSA isolates carried the mecA gene. Results from SCCmec typing revealed that 3% were type I; 9% were type II; 47% were type III, and 29% were type IV. Nineteen per cent of the isolates harboured the PVL gene. Furthermore, there was a statistically significant correlation between the presence of the PVL gene and SCCmec type IV.

Su Jung Kim and Cheolin Park (2014) investigated staphylococcal cassette chromosome mec (SSCmec) and Panton-Valentine Leukocidin (PVL) gene were among presumptive community-associated Methicillin-resistant Staphylococcus aureus
MRSA (CA-MRSA). Among 100 samples collected from college students, 10 out of 15 samples of \textit{S. aureus} were resistant to oxacillin from antibiotic susceptibility test. Among 10 oxacillin resistant \textit{S. aureus} isolates, \textit{mecA} gene and \textit{PVL} gene primers were employed and PCR was carried out. Out of this, 3 MRSA samples are found as \textit{mecA (+)}. The frequency of MRSA from CA-MRSA of college students is only 3% (3/100) but no positive \textit{PVL} gene expression from MRSA isolates were observed. However, one \textit{PVL} positive gene is detected from MSSA, not MRSA samples.

Hu \textit{et al.} (2015) obtained 259 HA-MRSA isolates from the various ailments of human beings from hospital. Among this, 28.6% (74/259) were \textit{PVL} positive, as detected by PCR using a pair of primers spanning the \textit{lukS-PV} and \textit{lukF-PV} genes. The most frequent \textit{SCCmec} type associated with \textit{PVL} among all isolates was \textit{SCCmec IV} (60.9% [14/23]), followed by \textit{SCCmec V} (50.0% [4/8]), \textit{SCCmec I} (37.5% [3/8]), \textit{SCCmec III} (26.6% [41/154]) and \textit{SCCmec II} (17.7% [11/62]). Out of non typeable \textit{SCCmec} isolates, 25% (1/4) carried \textit{PVL}.

Valle \textit{et al.} (2016) isolated total of 236 \textit{S. aureus} from clinical specimens of human and 108 or 45.76 % were found to be MRSA. All of the 108 MRSA strains were confirmed to carry the \textit{mecA} and \textit{SCCmec} genes, while the \textit{PVL} genes were detected in 41 (38 %) of the isolates. Ninety-six isolates (89%) carried \textit{SCCmec} type IV, while the remaining isolates carried \textit{SCCmec} type I (11 isolates) or type III (one isolate).

Taherirad \textit{et al.} (2016) studied distribution of \textit{SCCmec} types among Methicillin resistant \textit{S. aureus} strains isolated from human, and to correlate the types into observed bacterial virulence factors. During the study scientist reveled most common \textit{SCCmec} type was type III, with a frequency rate of 76%, followed by types IV, I and V with frequency rates of 11.2%, 4.8% and 3.2%, respectively; three isolates (4.8%) were not typeable by this method. \textit{SCCmec} type I was only isolated from blood culture and types IV and V were mainly isolated from wounds and urine samples from human cases; \textit{SCCmec} type III was isolated from all of the clinically samples. All of the MRSA strains that were isolated from healthy carriers were type III. Multidrug resistance in the type III strains was higher compared to the other types. The frequencies of Panton-Valentine Leucocidin (\textit{PVL}) and biofilm production gene were significantly lower in the type III strains compared to the other \textit{SCCmec} types (P < 0.05).
Bhatta et al. (2016) collected 400 strains of *S. aureus* from human clinical specimens from hospital. Out of these, 139 isolates had been confirmed as MRSA by previous study. Out of 139 MRSA isolates, 79 (56.8 %) were *PVL* positive. The majority of the community acquired MRSA (90.4 %) were *PVL* positive, while *PVL* was detected only 4 (7.1 %) hospital associated MRSA strains. None of the MRSA isolates from hospital environment was found positive for the *PVL* genes. Antibiotic resistance among *PVL* negative MRSA isolates was found higher as compared to *PVL* positive MRSA.

Veni et al. (2016) studied 210 MRSA strains consisting of 103 (49%) CA-MRSA and 107 (51%) HA-MRSA. Among this, CA-MRSA isolates were significantly more susceptible to ciprofloxacin, clindamycin, co-trimoxazole, erythromycin and gentamicin. All the isolates were susceptible to linezolid, teicoplanin and vancomycin. Out of 103 CA-MRSA isolate, 24 (23%) were resistance to three or more antibiotics, whereas out of 107 HA-MRSA isolates, 79 (74%) were multidrug resistant. This difference was significant (*P*<0.050). Resistance to both erythromycin and clindamycin was observed in 4/103 (4%) CA-MRSA and 22/107 (21%), this difference was statistically significant (*P* <0.050). 23/103 (22%) CA-MRSA and 40/107 (37%) HA-MRSA were resistant to erythromycin but susceptible to clindamycin in the disk diffusion test.

Liu et al. (2016) investigated molecular characteristics and virulence genes of community-acquired Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates with skin and soft tissue infections (SSTIs) from human beings. A total of 203 *S. aureus* strains were isolated from 1400 outpatients with SSTIs and 21(10.3%) were CA-MRSA isolates. The positive rate of *PVL* genes among *S. aureus*, CA-MRSA and Methicillin-susceptible *S. aureus* (MSSA) isolates were 39.4%, 71.4% and 35.7%, respectively. CA-MRSA strains had greater sensitivity to non-β-lactam antimicrobial agents. All CA-MRSA isolates belonged to SCCmec IV and V, accounting for 47.6% and 52.4%, respectively. All CA-MRSA isolates were found to be positive for one or more virulence genes, 28.6% of isolates carried *PVL, seb, sek, seq, hla, hlb, hld* and *hlg*-2. CA-MRSA infections were relatively uncommon in outpatients with SSTIs, but they carried many virulence genes.
2.8 METHICILLIN-RESISTANT STAPHYLOCOCCI AND ITS ZOONOTIC IMPORTANCE

MRSA infected cattle act as a reservoir and later transmit the infections to other animals and humans (Spoor et al., 2013). MRSA colonization in cattle may be an occupational risk to the people in close contact with MRSA infected cattle such as veterinarians, farmers, milkers and people working at slaughterhouses (Paterson et al., 2012). Transmission of animal MRSA to veterinary personnel has been found and it is more common for large animal personnel than small animal personnel (Wulf et al., 2008).

Although, MRSA has been reported as transmissible zoonotic as well as humanotic importance, the direction and routes of transmission are superficially understood. Some authors have reported bidirectional transmission of MRSA (AVMA, 2014). Animal to human transmission occurs through direct contact, environmental contamination and through handling of infected animal’s product (Nunang and Young, 2007) whereas human to animal transmission is still unclear (Weese, 2010).

Long-term surveys and reviews on mastitis carried out in different herds have shown the significance of *S. aureus* transmission in the veterinary field and dairy industry. Infections due to staphylococci are difficult to eliminate from animal and herd. Eradication of Staphylococci in few dairy herds has been reported for some periods and prevalence of infection observed less than 1% of quarters (Hogan et al., 1989). However, still, Staphylococcal mastitis has proven difficult to be treated and effectively eliminated from many herds (Sutra and Poutrel, 1994). Average cure rates have been reported to be about 50% (17-95%) for subclinical cases, approximately 55% (26-92%) for clinical and around 60% (14-100%) for dry-cow therapy (Pyorala and Pyorala, 1997). Major problem associate with cure rate is less than 50% during lactation in cow. Under certain environmental conditions, many Staphylococcal strains can also be a zoonotic pathogen, even though it is generally regarded as rather host-specific. However, direct transmission of MRSA between cows and humans has also been reported (Juhasz-Kaszanyitzky et al., 2007).

Dairy cows, among other domestic artiodactyls, are considered to be temporary hosts of staphylococci, in which the microbe is frequently present as a contaminant that can multiply and persist for short periods. *S. aureus* has been isolated from practically...
all external surfaces of healthy cows, udder skin being the preferred site. Long-term colonization by staphylococci on teat skin and several other body sites, primarily mucosal external orifices, have been observed in heifers (Roberson et al., 1994) suggesting persistent colonization. Different types of MRSA may be distinguished based on epidemiological groups. This can be a simplistic approach since in some cases strains of MRSA have spread between the groups (Morgan, 2008).

LA-MRSA refers mainly to the clonal spread of a certain MRSA strain (ST398) that colonizes different food animal species (including horses) and may cause infections in humans. Companion animals and horses may be colonized with a variety of strains due to their close contact with humans. Thus these species may act as carriers of MRSA originating from humans (a so called “humanosis”) (Morgan, 2008). LA-MRSA belong to the clonal complex 398 (LA-MRSA; CC398) is considered to be zoonotically important because of its capacity to colonize a wide range of hosts (Paterson et al., 2012). Several studies have been done to determine the degree to which MRSA plays a role in zoonosis or humanosis. It has been observed that usually the strains originating from companion animals are originally human strains and that the infection with this MRSA type is considered humanosis.

On the other hand, the strains originating from livestock are often divergent from human strains and the infection with this type of LA-MRSA could be considered zoonosis, and in this case MRSA would be an emergent zoonotic agent (Morgan, 2008). Bovine and human MRSA strains indistinguishable by phenotyping and genotyping methods have been found providing evidence for MRSA transmission between human and cattle (Hata et al., 2010). MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans. Transmission of animal MRSA to veterinary personnel has been found and it is more common for large animal personnel than small animal personnel (Wulf et al., 2008; Hanselman et al., 2006). Animal to human transmission occurs through direct contact, environmental contamination and through handling of infected animal's product (Nunang and Young, 2007), whereas human to animal transmission is still unclear (Weese, 2010). Using various molecular tools, other authors have studied the similarity between human and bovine isolates.

Juhasz-Kaszanyitzky et al. (2007) isolated MRSA from cows with subclinical mastitis and from a person who worked with these animals. The bovine and human
strains were indistinguishable by phenotyping and genotyping methods and were of a low frequency spa type. This finding indicates the first documented case of direct transmission of MRSA between cows and humans.

Brody *et al.* (2008) reported that the human MRSA252 strain uniquely shares multiple DNA sequence blocks with three different etiological agents of contagious bovine mastitis, including *S. aureus*, but not with other human isolates.

Febler *et al.* (2010) investigated the genetic relationship of 25 MRSA ST-398 isolates from bovine mastitis and 2 isolates from farm personnel. The 2 human isolates were indistinguishable genotypically and phenotypically (broth microdilution antimicrobial resistance pattern) from mastitis isolates from the same farm.

Hata *et al.* (2010) first reported and analyzed 4 bovine milk MRSA isolates obtained in Japan between May 1998 and May 2005 and evaluated their relationship with 9 human MRSA isolates, where 3 of the bovine isolates showed identical genotypes to the human isolates.

Pantosti (2012) analyzed LA-MRSA ST398 can cause infections in humans in contact with animals and can infect hospitalized people, although at the moment this occurrence is relatively rare. Recently, ST130 MRSA isolated from bovine mastitis has been found to carry a novel *mecA* gene that eludes detection by conventional PCR tests. Similar ST130 strains have been isolated from human infections in UK, Denmark, and Germany at low frequency. It is possible that the increased attention to animal MRSA will reveal other strains with peculiar characteristics that can pose a risk to human health.

Widianingrum *et al.* (2016) studied antimicrobial resistance patterns and gene encoding for methicillin/oxacillin resistance (*mecA*) from 73 isolates of *S. aureus*. The isolates of *S. aureus* originated from bovine (39 isolates) or crossbred Etawa goats (24 isolates) and from patients of Sarjito hospital (10 isolates) in Indonesia. The identification of *S. aureus* was based on an amplification of a specific section of the 23S rRNA gene and thermonuclease (*nuc*) genes. The PCR amplification revealed 5 (12.82%) isolates for bovine, 1 (4.17%) isolates from goat and 9 (90%) isolates from human for methicillin/oxacillin resistant (*mecA*) genes. These isolates were identified as MRS.
CHAPTER-III
MATERIAL AND METHODS

The present investigation was carried out to optimize and standardize uniplex PCR for various types of Staphylococcus SCC\textit{mec} types from methicillin resistance staphylococci (MRS) prevalent in this region in animals and closely associated human beings that will facilitate the study of epidemiological correlation between human and animal isolates on the basis of hospital acquired methicillin resistance staphylococci (HA-MRS) or community acquire methicillin resistance staphylococci (CA-MRS). Staphylococcal isolates recovered from these animal samples and human nasal swabs were characterized for pathogenicity or virulence factors using molecular and biochemical tests. The complete spectrum of antibiotic resistance in staphylococcus can be deciphered insight into pathogenic potentials. Along with the study of virulence genes (toxin genes) prevalence in the population, there correlation with the SCC\textit{mec} types was established during this work. The materials and methods employed to achieve the aims and objectives of the present study are described as follows:

3.1 LOCATION OF THE STUDY

The present was carried out at the Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Junagadh Agricultural University, Junagadh.

3.2 AREA OF THE STUDY

The present study was carried out using samples from animals (Bovine) having clinical and subclinical mastitic milk samples, pus from various ailments brought at Veterinary Clinical Complex (VCC), Veterinary College, Junagadh and from organized dairy farm. Human nasal swabs were collected from the farm workers and closely associated personnel who were in close contact with these animals.

3.3 LAB WARES, MEDIA AND CHEMICALS FOR THE STUDY

All the chemicals and reagents used in the present study were of analytical and molecular grade and procured from Sigma-Aldrich (USA), Merck Co., Inc. (USA), Himedia Laboratories Pvt. Ltd. (India), Qiagen India Pvt. Ltd, Ranbaxy Labs Ltd.
Material and Methods...

(India), Thermo scientific (India) etc. All the oligonucleotide primers were synthesized at eurofins genomics (Bangalore).

During the course of study, all the glasswares used were of standard quality (Corning and Borosil) made and the plasticwares viz., micropipette tips, PCR tubes and centrifuge tubes (Tarson). All the glassware and reusable plasticwares were washed and sterilized as per the standard protocol.

All media required for isolation and biochemical characterization were used from Hi-Media Pvt. Ltd., Mumbai. Kit for isolation of bacterial genomic DNA was used from Genetics Biotech Asia Pvt. Ltd.

The reference strain of *Staphylococcus aureus* ATCC 43300 and ATCC 25923 (Himedia Pvt. Ltd., Mumbai) was used as negative and positive control, respectively for *mecA* gene. The reference strain of *E. coli* (MTCC-522) strain was used as SCCmec types negative.

The glassware used in the study were prepared by soaking them in detergent (Teepol) solution overnight. The following day, they were washed thoroughly in running tap water, followed by rinse in deionized/ distilled water. The oven dried glassware were packed and sterilized in hot air oven for one hr at 160º C as per Collee (1989). The new plasticware including microcentrifuge tubes and micropipette tips were sterilized by autoclaving at 121ºC for 15 min at 15 psi pressure.

3.4 COLLECTION OF SAMPLES FROM MILK, PUS/EXUDATE (ABSCESS) FROM ANIMAL AND HUMAN NASAL SWABS.

A total of 202 animal samples (167 milk and 35 pus/exudate samples from cattle and buffalo) were collected and examined from dairy cattle and buffaloes. Simultaneously, 100 nasal swabs were collected from the closely associated personnel and farm workers aseptically as per the guidelines given by Peacock *et al.*, 2001. The milk samples were collected from clinical and sub clinical mastitis as per the guidelines laid by European Food Safety Authority (EFSA, 2009). The samples from pus/exudate (abscess) were collected aseptically. Simultaneously, the isolates of Staphylococci previously recovered from subclinical and clinical mastitic milk from bovines and human nasal swabs and maintained at the Department of Veterinary Microbiology, Junagadh Agricultural University, were included in the study for SCCmec typing and to study the prevalence of virulence genes.
Material and Methods

Sampling was performed following standard procedures as per the European Food Safety Authority (EFSA, 2009). The nasal swabs were taken from the medial septum area of both the nostrils by gently rubbing mucosa approximately for 5 sec with sterile cotton swab moistened with sterile 0.9% saline solution as per the standard guideline (Peacock et al., 2001) and transported to the laboratory on the same day of sampling in cold chain. The microorganisms were stored at -86°C in a 50% glycerol till further use.

3.4.1 Sampling Technique for milk samples

- Loose dirt, bedding and hair from the udder and teats were removed with the help of brush. Then teats and udders were thoroughly washed and dry grossly before proceeding with sample collection.
- All quarters were dipped in an effective pre-milking teat disinfectant (70% alcohol) and were allowed at least 30 seconds contact time.
- Teats were dried thoroughly with an individual towel.
- After discarding few strip of milk from all the quarters the samples were collected in the tube; 1 to 2 ml of milk was collected from each quarter of the udder.
- After collecting the samples, teats were dipped in an effective germicidal teat disinfectant solution.
- Sample tubes were placed immediately on ice and transferred to laboratory.

3.4.2 Sampling technique for collection of pus/exudate (abscess) samples

- First the wound margins and superficial area were cleaned thoroughly with sterile saline and removed all superficial exudates and overlying debris with swabs.
- Using sterile technique, the samples were Aspirated/collection the deepest portion of the abscess/pus/exudate with a syringe and needle and bring into laboratory in a sterile tube.
- In those cases were pus/exudates were not discharged from wound where we used sterile cotton swab and Gently roll the swab over the surface of the wound.
approximately five times, focusing on an area where there is evidence of pus or inflamed tissue.

- After collection of the samples of swabs/pus/exudates, all were brought into laboratory by maintaining cold chain.

3.4.3 Procedure for nasal swab collection from human

- If the nose was found dry, collection swab was moistened with sterile 0.9% saline solution without touching the tip of the swab. Nasal samples were taken from the vestibulum nasi, which is the anterior nasal passage at the border between skin and mucosal tissue as described by Peacock, et al. (2001).
- Both nares were swabbed using the same swab to obtain adequate material and returned the swab to the container.
- The outside of the nostrils were rubbed after the procedure to alleviate the unpleasant sensation of swabbing.

3.5 ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI FROM ANIMAL AND HUMAN NASAL SWABS

The isolates were identified as per the methods described by Cowan and Steel, (1974). For isolation of Staphylococci, samples from animal and nasal swabs of human were primarily inoculated on Nutrient Agar (NA) and Mannitol Salt Agar (MSA) plates and were incubated at 37°C for 48 hours. The mannitol fermenting colonies indicative of Staphylococci were transferred to brain heart infusion (BHI) agar slants for further identification. Those staphylococci found positive for presence of methicillin resistance gene was inoculated on Blood Agar to study hemolytic pattern.

The culture smear of the isolates were prepared on microscopic glass slide and stained with Gram’s Method of staining. The stained smear was observed under microscope. Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes was considered to be staphylococci. Biochemically, for confirmation of staphylococcius, catalase positive reaction and oxidase negative reactions were considered as basis for identification.

A total of 202 samples from animal (167 milk samples and 35 pus/exudate samples from cattle and buffalo) subclinical/clinical mastitis cases and abscess/pus/wound samples were collected and examined and 100 samples from human
nasal swabs were cultured for primary isolation (Cowan and Steel, 1995) from closely associated personnel/farm workers aseptically. The isolates were subjected to culture on selective chromogenic media (Hichrom MeReSa Agar, Himedia) for further confirmation as per the colony characteristics. The isolates were confirmed as methicillin resistant staphylococci (MRS) using \textit{mecA}-positive gene amplification. The isolates confirmed as MRS were further subjected to SCC\textit{mec} typing using four primer-pairs designed to identify the five main known SCC\textit{mec} types.

### 3.5.1 Preparation of samples, medium plates and inoculation

The samples stored in ice were allowed to come at 16 to 18°C. Microorganisms reside in fat layer of milk, generally. Thus, samples were thoroughly mixed and aliquot of 100 μl from each milk sample and spread over nutrient agar plate. The samples from the abscess/pus and nasal swabs were streaked on to the surface of same medium followed by incubation at 37 °C for 24 hrs.

### 3.5.2 Isolation of presumptive staphylococci colonies

The white/yellow colonies grown on Nutrient agar were presumptive of staphylococci and colonies were examined for morphology. The distinct colonies of staphylococci were selected and processed for Gram staining, catalase test and oxidase test and culture on mannitol salt agar (MSA).

### 3.5.3 Gram staining:

One loop from colony was mixed with sterile water and spread over the surface of slide and fixed by gentle heating on flame. The slide was flooded with crystal violet stain for 2 min. The stain was poured off and the slide was washed gently with running water. Afterwards, Gram's iodine was flooded over the slide for 30 sec. The iodine solution was decanted and the slide was washed with running tap water. After the mordant step, slide was destained with alcohol (5 sec) and subsequently washed with tap water. Safranin was poured on the slide as counter stain for 45 sec, afterwards washed with tap water and dried in filter paper folds. The stained slide was examined under a microscope and organisms were classified into two groups as Gram positive and negative.
3.5.4 Catalase activity:

A drop of 3% (v/v) hydrogen peroxide was mixed with a loop of colony on a slide. Presence of effervescence, caused by the liberation of free oxygen as gas bubbles, indicated the presence of catalase (AOAC, 1995).

3.5.5 Oxidase test

A loopful of bacterial growth with the help of sterile platinum loop was rubbed on a moistened oxidase disc (Hi Media Ltd., Mumbai). Development of deep purple blue or mauve color within 10 seconds was considered as positive and no change in color was taken as negative reaction.

3.5.6 Determination of virulence factors of staphylococcal isolates

3.5.6.1 Mannitol fermentation

As per method described by Merchant and Packer (1967), the isolates of staphylococci, from the mastitis milk were inoculated on the MSA petri plates and was incubated at 37°C for 48 hours. The yellow coloration of colony along with media considered as mannitol fermenters.

3.5.6.2 Coagulase production

The coagulase production by Staphylococci was detected using tube coagulase test. All the isolates tested for coagulase production were incubated overnight in BHI broth at 37°C. The tube coagulase test was performed by adding 0.1 ml of the overnight BHI broth culture to 0.5 ml of 1: 5 diluted human plasma in a small test tube. After gentle mixing, the tubes were incubated at 37°C and examined after 1, 3, 6 and 24 hours. Simultaneously negative control was maintained to interpret the results. The interpretation was made as per the Gillespie (1943). The test was considered positive showing any degree of clot formation, often the plasma converted into stiff gel that remained in place when tube was tilted or inverted but sometimes clots were also seen floating in the field. No degree of clotting would be taken as negative.

A simple slide coagulase test was also performed. The drop of human plasma mixed with an equal volume of freshly cultured broth (or emulsified colony in a drop of water) on a microscopic slide. A positive result was indicated by macroscopically clumping of the bacterial cells within five seconds.
3.5.6.3 Hemolysin production

Hemolytic activity of the Staphylococci was detected adding 5 per cent defibrinated sheep blood in to melted Blood agar base (Hi Media). The methicillin resistance staphylococci isolates were streaked on the medium and incubated aerobically overnight at 37°C for 24 hours. Result was interpreted after keeping all the plates at 4°C for 1-2 hours. Isolates showing hemolytic zones were taken as positive. Interpretation was made as follows as per Quinn et al. (1994).

- Alpha hemolysis- positive strains, which showed a wide zone of complete hemolysis with blurred edges.
- Beta hemolysis- positive strains, which showed a wide zone of incomplete hemolysis with sharp edges.
- Alpha-beta hemolysis- Hot and cold hemolysis.
- Gamma hemolysis- No hemolysis

3.6 IN VITRO ANTIBIOGRAM PATTERN OF STAPHYLOCOCCUS FROM ANIMAL AND HUMANS

All the Staphylococcal isolates obtained were subjected to in vitro antibiotic sensitivity test as per The Kirby-Bauer (Bauer et al. 1966) method is usually used for antimicrobial susceptibility testing.

3.6.1 Disk diffusion test

3.6.1.1 Preparation of the medium

Mueller Hinton Agar medium was prepared using dehydrated powder according to the directions specified on the label (HiMedia Laboratories).

3.6.1.2 Preparation of Inoculum

For Preparation of Inoculum direct colony suspension method was used. Direct colony suspension was prepared from 18-24 hour old grown nutrient agar plate in BHI broth. Adjust the turbidity to that of standard 0.5 McFarland (HiMedia Laboratories).

1. Plates were prepared with suitable make of Mueller Hinton Agar (Himedia Laboratory) according to the directions specified on the label.
2. Sterile non-toxic cotton swab on a wooden applicator was dipped into the 5-6 hrs bacterial culture broth and was rotate the soaked swab firmly against the upper inside wall of the tube to express excess fluid. This inoculum was swabbed on the entire agar surface of the plate.

3. Antimicrobial discs (Table 3.3) were dispensed with the help of applicator and sterile forceps on the surface of inoculated plate.

4. Plates were transferred in the incubator 35-37°C for 18 hrs.

Zones of inhibition were measured and compared with zone size interpretative table furnished by the CLSI and manufacturer and graded as sensitive and resistant.

3.6.2 Determination of minimum inhibitory concentration (MIC)

MIC of all methicillin resistant staphylococcal isolates were determined by E-test. Here MIC values were determined using commercial MIC determination paper strips Ezy MIC® strips obtained from Himedia Laboratories, Mumbai (Table 3.1). These were discs with pre-coated antibacterial in a concentration gradient manner capable of showing MICs in the range of 0.016 μg to 256μg/ml or 0.01 μg to 240μg/ml, on testing against the test organism. Following antibiotics were used for determination of MIC for MRS and results were interpreted as per Clinical Laboratory Standards Institute (CLSI, 2011).

1. Cefoxitin (Range in μg : 0.016 - 256 mcg/ml)
2. Oxacillin (Range in μg : 0.016 - 256 mcg/ml)
3. Vancomycin (Range in μg : 0.016 - 256 mcg/ml).

**TABLE 3.1:** Interpretive criteria for susceptibility categorization based on MIC value (mcg/ml) for different antibiotics

<table>
<thead>
<tr>
<th>Name of antibiotic</th>
<th>Name/Type of microorganism</th>
<th>Interpretative Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; S</td>
</tr>
<tr>
<td>Cefoxitin (mcg/ml)</td>
<td><em>Staphylococcus aureus</em></td>
<td>4</td>
</tr>
<tr>
<td>Oxacillin (mcg/ml)</td>
<td><em>Staphylococcus aureus</em></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Coagulase-negative Staphylococci</td>
<td>0.25</td>
</tr>
<tr>
<td>Vancomycin (mcg/ml)</td>
<td><em>Staphylococcus</em> spp.</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Coagulase-negative Staphylococci</td>
<td>4</td>
</tr>
</tbody>
</table>
3.6.2.1 Protocol for the determination of MIC values

1. Preparation of the inoculum and inoculation to Mueller Hinton Agar (MHA) plates were carried out in a similar manner as mentioned in section 3.6.1.

2. Ezy MIC® strips stored at 2 – 8°C were first brought to room temperature or 25°C before they were used.

3. The strips were applied aseptically with sterile forceps on to the agar surface with MIC scale facing upwards and the plates were incubated at 37°C for 18 – 24 hrs.

4. Then, the plates were examined for the zone of inhibition in the form of an ellipse.

5. MIC value was read as the value at which the zone convenes the comb-like projections of the strips and not the handle. When no zone of inhibition was observed, the MIC was recorded as greater than the highest concentration of the strip. When the zone of inhibition was below the concentration, then the MIC was recorded as less than the highest concentration of the strip.

The MIC values recorded were compared with the interpretative chart (supplied with the MIC strips) which was in accordance with the performance standards for antimicrobial disc susceptibility tests, Clinical Laboratory Standards Institute (CLSI, 2011).

3.7 IDENTIFICATION OF MRS USING SPECIFIC MEDIA

The isolates presumptive of staphylococci obtained from various samples from animals (milk and pus/exudate) and human nasal swabs were streaked on the chromogenic media, MeReSa® Agar medium (HiMedia, HiMedia laboratory Pvt. Limited) and incubated for 18-24 hours at 37°C. After the incubation, the results were observed for greenish blue on the agar medium.

3.8 MOLECULAR BASED EVALUATION OF STAPHYLOCOCCAL ISOLATES FROM ANIMAL AND HUMANS

The PCR with positive reference strain (ATCC 43300) for detection of mecA gene selected for detection of MRS from isolates obtained from milk, pus and nasal swab from various animal and humans was carried out. The E. coli (MTCC-522) strain was used as negative control.
3.8.1 PCR for identification of coagulase positive MRS (CoPMRS) and coagulase negative MRS (CoNMRS)

Primers previously described by various authors were used for m-PCR. All the primer sets (reverse and forward) were analyzed for their compatibility using Primerstat software and evaluated for secondary structures including hairpins, self-dimers, cross-dimers in primer pairs and primer dimers. Primers are enlisted with their base sequences, product size and reference in Table 3.4 with cycling conditions Table 3.8.

The primers procured in lyophilized form were dissolved (100pm/μl) in autoclaved HPLC grade water and further diluted to 10pm/μl. The vials were tapped gently, spin for proper mixing and kept in a laminar flow hood for 3-4 hrs. Subsequently these were stored in -20°C, until further use.

3.8.2 Molecular characterization of major SCCmec types among MRS from animal and humans

The worldwide increase in the number of infections caused by Methicillin-Resistant Staphylococci (MRS) has emphasized the need for fast and reliable identification and typing methods. In addition to genotyping, characterization of the staphylococcal cassette chromosome mec (SCCmec) type has led to better discrimination of hospital acquired MRSA (HA-MRSA) and community acquires MRSA (CA-MRSA) (Aires et al., 2003 and Boyle-Vavra et al., 2005). The multiplex PCR for discrimination of SCCmec types I–IV developed by Oliveira and de Lencastre, 2002 has been used widely, but was designed primarily to characterize HA-MRSA carrying SCCmec type’s I–III. Currently, an increase in CA-MRSA is occurring worldwide, caused mainly by CA-MRSA belonging to the same multi locus sequence type lineage as HA-MRSA and carrying the smaller SCCmec types IV or V (Vandenesch et al., 2003 and Charlebois et al., 2004). In order to discriminate the classic HA-MRSA with SCCmec type I–III from CA-MRSA with SCCmec types IV and V, the present study designed to carry out PCR for use in routine SCCmec type determinations.

Four pairs of primer sets for four genes were designed to ensure amplification of two DNA targets genes from SCCmec type IV and two targets genes from SCCmec type V. The targets were chosen so that one target would be amplified from each of SCCmec type I–III (Boye et al., 2007) as described in table Table 3.6. Primers are
enlisted with their base sequences, product size and reference in Table 3.5 with their cycling condition (Table 3.9).

3.8.3 Isolation of bacterial genomic DNA from bacterial culture

The genomic DNA of staphylococci from culture was extracted according to Sambrook and Russell (2001) with minor modifications.

3.8.3.1 Preparation of broth culture of staphylococci

The culture was prepared by inoculating the isolate in BHI broth and incubating at 37°C for 24 hrs. in an incubator.

3.8.3.2 Following solutions were used for extraction

1. Tris-EDTA (TE) (pH 8.0)
2. 10mM Tris-HCL
3. 1mM EDTA
4. SDS (10 % w/v)
5. Proteinase K solution (20 mg/ml, w/v)
6. 5M Sodium chloride
7. CTAB (Cetyl trimethyl ammonium bromide, 10 % solution in 0.7M NaCl)
8. Saturated phenol (pH 8.0)
9. Chloroform
10. Isoamyl alcohol
11. 7.5M Ammonium acetate
12. Chilled absolute ethanol

3.8.3.3 Isolation of bacterial genomic DNA by Proteinase K-SDS method

1. First 10 ml of broth culture was centrifuged at 10,000 rpm for 10 min. at 37°C. The supernatant was discarded and the pellet was used for extraction of nucleic acid.
2. Pellet containing bacterial cells was suspended in 2 ml Tris EDTA (pH-8.0), 250 µL SDS (10 % w/v) and 10 µl of proteinase K solution (20mg/ml, w/v) and incubated overnight at 37°C.

3. Subsequently, 500 µl of 5M NaCl followed by 100 µl CTAB (Cetyl trimethyl ammonium bromide, 10 % solution in 0.7M NaCl) was added and incubated in water bath for 10 min at 65°C.

4. The solution was spun at 8,000 rpm for 10 min after mixing with equal volume of chloroform: isoamyl alcohol (24:1) and upper phase was transferred to clean microfuge tube.

5. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by inverting, spun for 10 min at 10,000 rpm and upper aqueous phase was transferred again to a clean microfuge tube.

6. In the collected supernatant, the DNA was precipitated with double volume of chilled absolute ethanol with 1/10 volume of ammonium acetate (7.5M) and double the volume. Then kept the tube at -4°C for 30 min.

7. Tube was centrifuged for 10 min at 11,000 rpm and ethanol was discarded.

8. The pellet was washed in 70 per cent ethanol and again spun for 15 min at 11,000 rpm.

9. Ethanol was discarded and pellet was dried.

10. DNA was resuspended in 200µl sterile distilled water and kept in water bath at 65°C for one hour and stored at -20°C till use.

3.9 MOLECULAR DETECTION OF MRSA ISOLATES FROM ANIMAL AND HUMANS.

Four predominant genes (16S rRNA, Nuc, Coa & MecA gene targets) identified based on the outcome of phenotype-based speciation were considered to be included for detection by m-PCR. From all genes, 16S rRNA was used to identify genus i.e. Staphylococci, Nuc gene encodes for thermonuclease was used to identify species i.e. Staphylococcus aureus, Coa gene encodes for coagulase was used as pathogenicity indicator and mecA gene target encodes for modified penicillin binding protein 2B was used for genotypically identification of methicillin resistance in Staphylococcus spp. So
this can provide advantage to diagnose both Coagulase Positive Methicillin Resistant Staphylococci (CoPMRS) as well as Coagulase Negative Methicillin Resistant Staphylococci (CoNMRS) at the same time in one single reaction as per the primers described (Table 3.4) with cycling condition (Table 3.8).

3.10 MOLECULAR DETECTION OF VIRULENCE GENES (TOXIC GENES) FROM MRS ISOLATES.

The distribution of various virulence genes among clinical methicillin resistant staphylococci isolates were investigated. The detection of the Panton–Valentine Leukocidin gene complex (PVL) which produces toxins that cause necrosis and leukocyte breakdown. The occurrence of PVL is usually indicative of CA-MRSA, while the complex rarely occurs in HA-MRSA (Vandenesch et al., 2003 and Naimi et al., 2003). Alpha toxin/Alpha hemolysin (hla) is known to play a role in the pathogenesis of staphylococcal disease, as S. aureus mutants lacking hla display reduced virulence in invasive disease models (Patel et al., 1987). Detection of intercellular adhesion (ica gene) locus gene required for biofilm formation and associated with the adhesion of S. aureus and S. epidermidis. For the detection of all these three virulence genes, the sets of three primer pairs of respective genes were used (Table 3.7) with thermal cycling condition as described by Alli et al., (2015) (Table 3.10).

PCR products (5 µL) were analysed by electrophoresis on agarose 1.5% w/v gel followed by staining with ethidium bromide. The virulence gene was determined on the basis of the band pattern obtained (Table 3.7) with cycling condition described in Table 3.10. Isolates with no visible bands, were considered to be negative for that particular virulence genes.

3.11 MOLECULAR DETECTION OF SCCMEC TYPES FROM METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMANS.

The isolates which were confirmed for the presence of methicillin gene were used for the SCCmec typing. To identify SCCmec types I to V, the multiplex PCR protocol were considered (Boye et al., 2007) and by using this m-PCR protocol, uniplex PCR for all this genes were used and individual PCR reaction was carried out for the identification of various SCCmec types (Table 3.5) with cycling condition described in Table 3.9.
PCR of MRS colonies was carried out in final reaction volume of 25 µl in thermal cycler (verity, Applied Biosystems by life technology, Singapore). Quantity and concentration of various components used for PCR were as per Table 3.2. Steps and conditions of thermal cycling for different primer pairs in PCR was as described by Boye et al., (2007).

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Components</th>
<th>PCR Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>PCR Master Mix (2X)</td>
<td>12.5 µl</td>
</tr>
<tr>
<td>2.</td>
<td>Forward Primer (10 pmol/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>3.</td>
<td>Reverse Primer (10 pmol/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>4.</td>
<td>Template DNA</td>
<td>3 µl</td>
</tr>
<tr>
<td>5.</td>
<td>NFW (Nuclease Free Water)</td>
<td>7.5 µl</td>
</tr>
</tbody>
</table>

PCR products (5 µL) were analyzed by electrophoresis on agarose 1.5% w/v gel followed by staining with ethidium bromide. The SCC\textsubscript{mec} type was determined on the basis of the band pattern obtained (Table 3.6). Isolates with no visible bands, or with a band pattern that was not in agreement with one of the five predicted band patterns, were classified as non typeable (NT).

**3.12 DNA CONFIRMATION BY AGAROSE GEL ELECTROPHORESIS**

**3.12.1 Equipments**

1. Weighing balance (Shimazdu Philippines Manufacturing Inc., Japan)
2. Horizontal electrophoresis apparatus with power pack (Tarson, India)
3. Microwave oven (M/s. BPL Pvt. Ltd.)
4. Gel documentation unit (Biorad, USA)

**3.12.2 Reagents**

1. Agarose (M/s. Sigma Aldrich, USA)
2. Tris Borate EDTA buffer (10X, pH 8.2)
   
   Tris base 108.0 g

   Boric acid 55.0 g
Material and Methods...

EDTA disodium salt 8.3 g
Double distilled water up to 1000.0 mL

The stock solution was sterilized by autoclaving and made to 0.5X before use.

3. Gel loading dye (6X)
   - Bromophenol blue 0.25% (w/v)
   - Xylene cyanol 0.25% (w/v)
   - Sucrose 40% (w/v) in distilled water

The dye was procured from M/s. Bangalore Genei, Bangalore and stored at 4°C.

4. Ethidium bromide (10 mg / mL)
   - Ethidium bromide (Bangalore Genie) 100 mg
   - Double distilled water 10 mL

The suspension was stirred to ensure that the dye had dissolved. The vial was then wrapped in aluminium foil.

3.12.3 Procedure

Required quantity of agarose was weighed and dissolved in proportionate volume of 1X Tris Borate EDTA buffer (TBE) and melted in a microwave oven until a clear uniform suspension was obtained. (About 0.2 g of analytical grade agarose was dissolved in 20 mL of 1X TBE buffer by heating in microwave oven to obtain uniform solution). Prior to casting the gel, the molten agarose was allowed to cool to about 50°C, after which ethidium bromide was added to a final concentration of 0.5 g/mL and mixed thoroughly. Gel was casted on an appropriate gel casting tray fitted with acrylic comb and left for setting. The acrylic comb was carefully removed after the gel was set. The tray with gel was then submerged in an electrophoresis tank containing 1X TBE buffer.

The DNA to be analyzed was mixed with appropriate volume of 6X DNA gel loading dye and charged into wells alongside 100 bp DNA molecular weight marker. Electrophoresis was carried out at 5 V/cm until the tracking dye (Bromophenol Blue) had just passed out of the gel. Following the electrophoresis, DNA bands were visualized and the images were captured by using Gel Doc (Bio-Print ST4, Vilber lourmet).
TABLE 3.3: Commercially available antibiotics discs with their respective concentration used for staphylococcal isolates.

<table>
<thead>
<tr>
<th>Antibiotic group</th>
<th>Name of antibiotic used</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beta lactam</strong></td>
<td>Methicillin (5 mcg)</td>
</tr>
<tr>
<td></td>
<td>Penicillin-G (10 unit)</td>
</tr>
<tr>
<td></td>
<td>Ampicillin/Cloxacillin (10 mcg)</td>
</tr>
<tr>
<td><strong>Amino penicillin</strong></td>
<td>Amoxicillin/Salbactam (20 mcg)</td>
</tr>
<tr>
<td><strong>Cephalosporins</strong></td>
<td>Ceftriaxone/Salbactum (30/15 mcg)</td>
</tr>
<tr>
<td></td>
<td>Cefixime (10 mcg)</td>
</tr>
<tr>
<td></td>
<td>Ceftizoxime (30 mcg)</td>
</tr>
<tr>
<td></td>
<td>Cefoxitin/Cloxacillin (30/200 mcg)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime (30 mcg)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime/Clavulanic acid (30/10 mcg)</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td>Gentamicin (10 mcg)</td>
</tr>
<tr>
<td></td>
<td>Amikacin (30 mcg)</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td>Ofloxacin (5 mcg)</td>
</tr>
<tr>
<td></td>
<td>Levofloxacin (5 mcg)</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacin (10 mcg)</td>
</tr>
<tr>
<td></td>
<td>Norfloxacin (10 mcg)</td>
</tr>
<tr>
<td><strong>Tetracyclins</strong></td>
<td>Oxytetracyclins (30 mcg)</td>
</tr>
<tr>
<td><strong>Rifampin</strong></td>
<td>Rifampicin (5 mcg)</td>
</tr>
<tr>
<td><strong>Amphenicol</strong></td>
<td>Chloramphenicol (30 mcg)</td>
</tr>
<tr>
<td><strong>Sulfa group</strong></td>
<td>Co-Trimoxazole (Trimethoprim/ Sulphamethoxazole) (25 mcg)</td>
</tr>
</tbody>
</table>
TABLE 3.4: Nucleotide sequences of primers used for m-PCR along with their product size and references

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of primer</th>
<th>Primer sequence (5’-- 3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1       | TStaG422F1     | 5’- GGC GTG GTT GAA CGT GGT CAA ATC A-3’  
           | TStaG765R1     | 5’- TIA CCA TTT CAG TAC CTT CTG GTA A-3’ | 370 bp      | Martineau et al., (2001) |
| 2       | Nuc F          | 5’- GCG ATT GAT GGT GAT ACG GTT -3’  
           | Nuc R          | 5’- ACG CAA GCC TTG ACG AAC TAA AGC -3’ | 280 bp      | Brakstad et al., 1992 |
| 3       | Coa F1         | 5’- GTA GAT TGG GCA ATT ACA TTT TGG AGG -3’  
           | Coa R1         | 5’- CGC ATC AGC TTT GTT ATC CCA TGT A -3’ | 117 bp      | Moon et al. (2007b) |
| 4       | mecA F1        | 5’- GAGT TGT AGT TGT CGG GTT TGG-3’  
           | mecA R1        | 5’- GGC CAA TTC CAC ATT GTT TC-3’ | 454 bp      | Malik et al. (2006) |

TABLE 3.5: Nucleotide sequences of primers used for the identification of SCCmec types of MRS

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of primer</th>
<th>Primer sequence (5’-- 3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
</table>
| 1       | β F1           | 5’- ATTCCTTGATAATAGCCYTCT -3’  
           | α 3 R1          | 5’- TAAAGGCATCAATGCAACACT-3’ | 937 bp      | Ito et al. (2001) |
| 2       | ccrCF1         | 5’- CGTCTATTACAAGATGTITAAGGATAAT-3’  
           | ccrCR1          | 5’- CCTTTTATAGACTGGATTATTTAAATAT-3’ | 518 bp      | Ito et al. (2004) |
| 3       | 1272F1         | 5’- GCCACTTCATAACATATGGAA-3’  
           | 1272R1          | 5’- CATCCCGTGAACCACCAAAA-3’ | 415 bp      | Boye et al. (2007) |
| 4       | 5RmecAF1       | 5’- TATACCAAAAAACCGAACAATAC-3’  
           | 5R431R1         | 5’- CGGCTACAGTGATACCATCC-3’ | 359 bp      | Boye et al. (2007) |
### TABLE 3.6: Primers used in SCCmec typing PCR and the resulting gel band patterns of SCCmec types I-V.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of primer</th>
<th>Product size</th>
<th>Target Gene</th>
<th>SCCmec type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I II III IV V</td>
</tr>
<tr>
<td>1</td>
<td>β F1 α 3 R1</td>
<td>937 bp</td>
<td>ccrA2-B</td>
<td>✓ ✓ ✓ ✓ ✓</td>
</tr>
<tr>
<td>2</td>
<td>ccrCF1 ccrCR1</td>
<td>518 bp</td>
<td>ccrC</td>
<td>✓ ✓ ✓ ✓ ✓</td>
</tr>
<tr>
<td>3</td>
<td>1272F1 1272R1</td>
<td>415 bp</td>
<td>IS1272</td>
<td>✓ ✓ ✓ ✓ ✓</td>
</tr>
<tr>
<td>4</td>
<td>5RmecAF1 5R431R1</td>
<td>359 bp</td>
<td>mecA–IS431</td>
<td>✓ ✓ ✓ ✓ ✓</td>
</tr>
</tbody>
</table>

✓ - Desired amplification of gene.

### TABLE 3.7: Nucleotide sequences of primers used for the PCR amplification of the virulence genes of staphylococci spp.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Primer name</th>
<th>Sequence (5’—3’)</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>luk-PV-1</td>
<td>5’-ATCATTAGTAAAAATGTCTGGACATGATCCA-3’</td>
<td>433 bp</td>
<td>Lina et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>luk-PV-2</td>
<td>5’-GCATCAATTGTATTGGATAGCAAAAGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>icaA F icaA R</td>
<td>5’-GAT TAT GTA ATG TGC TTG GA - 3’</td>
<td>769 bp</td>
<td>Peacock et al., (2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-ACT ACT GCT GCG TTA ATA AT - 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>hla F hla R</td>
<td>5’-CAA TCA AAC CGC CAA TTT TT - 3’</td>
<td>174 bp</td>
<td>Shukla et al., (2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’-CCT GGC CTT CAG CAT TTA AG - 3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Material and Methods...**

**TABLE 3.8:** Thermal cycling condition for different primer pairs in PCR for identification of MRSA organism.

<table>
<thead>
<tr>
<th>Primers (forward and reverse)</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>16S rRNA Nuc Coa MecA</td>
<td>94°C 5 min.</td>
</tr>
</tbody>
</table>

Repeated for 40 cycles

**TABLE 3.9:** Thermal cycling condition for different primer pairs in PCR used for SCC\(_{mec}\) typing.

<table>
<thead>
<tr>
<th>Primers (Forward and Reverse)</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial denaturation</td>
</tr>
<tr>
<td>β F1 α 3 R1</td>
<td>94 °C for 4 min</td>
</tr>
<tr>
<td>ccrCF1 ccrCR1</td>
<td></td>
</tr>
<tr>
<td>1272F1 1272R1</td>
<td></td>
</tr>
<tr>
<td>5RmecAF1 5R431R1</td>
<td></td>
</tr>
</tbody>
</table>

30 Cycle
**TABLE 3.10**: Thermal cycling condition for different primer pairs in PCR for detection of virulence gene.

<table>
<thead>
<tr>
<th>Primers (Forward and Reverse)</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial denaturation</td>
</tr>
<tr>
<td><strong>luk-PV-1</strong></td>
<td>94°C for 2min</td>
</tr>
<tr>
<td><strong>luk-PV-2</strong></td>
<td>94°C for 2min</td>
</tr>
<tr>
<td><strong>icaA F</strong></td>
<td>94°C for 2min</td>
</tr>
<tr>
<td><strong>icaA R</strong></td>
<td>94°C for 2min</td>
</tr>
<tr>
<td><strong>hla F</strong></td>
<td>94°C for 2min</td>
</tr>
<tr>
<td><strong>hla R</strong></td>
<td>94°C for 2min</td>
</tr>
</tbody>
</table>

35 cycle
Animal milk samples (n=167), Pus/exudate (n=35) and Human nasal swabs (n=100)

Samples transported to the laboratory, Department of Veterinary Microbiology, JAU, Junagadh and inoculated on nutrient agar

24-48 hrs incubation at 37°C

Growth observation

Ready for routine microbiological work and use

### ISOLATION AND IDENTIFICATION OF BACTERIA

- Culturing on nutrient agar
- Morphological identification by Grams staining
- Culturing on mannitol salt agar
- Culturing on blood agar
- Culturing on MeReSa agar for identification of methicillin resistant

### CHARACTERIZATION

- Viewing colony morphology, pigment production and hemolytic activity
- Staining and focusing under microscope

### BIOCHEMICAL AND ANTIBIOMGRAM PROFILE

- Catalase, oxidase and coagulase test
- Disk diffusion method to study antibiogram
- MIC study of MRS isolates by E-test

### MOLECULAR CHARACTERIZATION

- m-PCR for the detection of MRSA organism.
- Molecular detection of virulence genes including Panton-Valentine leukocidin (PVL)
- Staphylococcal Cassette Chromosome (SCCmec) typing of MRSA

**FIGURE 4.1:** Research Design for isolation, identification and molecular characterization of MRSA isolates for SCCmec typing
CHAPTER-IV
RESULTS AND DISCUSSION

*Staphylococcus aureus* is a versatile pathogen of humans and animals that causes a wide variety of the disease (Abebe et al., 2013). The bacterium is a colonizer on the skin and mucosae from which it can invade multiple organs. In livestock *Staphylococcus aureus* is an important cause of bovine mastitis, skin and soft tissue infections and to a lesser extent infections of the locomotory system. *S. aureus* is also being isolated and reported from surgical site. (Normanno et al., 2007).

The routine use of penicillin for staphylococcal infections resulted in a selection pressure for penicillin-resistant *S. aureus*, which spread rapidly and became pandemic in both hospitals and the community as such (Jovetic et al., 2010). This development has been described as “the first wave of *Staphylococcus aureus* resistance” by Chambers and Deleo (Chambers and Deleo, 2009). “The second wave” was initiated by the introduction of methicillin. Again driven by selection pressure, the use of this semi-synthetic beta-lactam antibiotic led to the spread of MRSA. There is now increasing concern about the public health impact of MRSA associated with food producing animals, because MRSA containing resistant genes can spread from animal isolates to human isolates by direct contact or through the food chain (Kluytmans, 2010). MRSA strains have been isolated in many countries from cows’ or small ruminants’ milk and also various dairy products (Unal et al., 2012).

MRSA was initially reported as a nosocomial pathogen in human hospitals (hospital-associated MRSA) and was isolated from patients with compromised immune systems undergoing medical procedures. MRSA accounts for 30 to 40% of all hospital-acquired infections and for 40% to 70% of *S. aureus* infections in intensive care units (Gordon and Lowy, 2008). In the 1990s, a major change in the epidemiology of MRSA had been observed, with the appearance of cases affecting people with no epidemiological connection to hospitals; strains that cause such infections are referred to as community-acquired or community associated MRSA (EFSA, 2009). In contrast to livestock, most companion animals live in close contact to their owners, a fact that is of special concern with regard to the possibility of zoonotic transmission of pathogens (Walther et al., 2012). Several case reports from the recent past demonstrated the
zoonotic potential for MRSA between companion animals and their owners (Ferreira et al., 2011), raising the concern about these animals might serve as a MRSA infection source for humans.

Epidemiological studies revealed the transmission of *S. aureus* from cow to cow, the primary source of which is the milk from infected glands and also from dairy cows to humans and humans to cows (Melchior et al., 2007). In many cases, colonization or infection by MRSA in animal species appears to be due to zoonotic transfer from human owners or caretakers (Smith and Pearson, 2010). Once exposed to Methicillin-resistant staphylococci, animals become reservoir of pathogen for human beings. So the increased antimicrobial resistant of the organisms in animals treated with antibiotics and their zoonotic transmission continues to be a matter of great concern globally (Unakal and Kaliwal, 2010).

In the beginning, MRSA were restricted to hospitals and human healthcare settings and therefore denominated as healthcare associated (HA-MRSA). HA-MRSA became one of the most important nosocomial pathogens in human medicine (Enright et al., 2002). It was not before the late 1990s that MRSA were also regularly described in the community. At that time, these community-associated (CA-MRSA) were distinguished from HA-MRSA by their genetic profile (Herold et al., 1998). At present, various different definitions are available to distinguish between HA- and CA-MRSA. In 2000, the U.S. Centers for Disease Control and Prevention (CDC) published a definition based on defined risk factors for the development of HA-MRSA. Thus, CA-MRSA included all MRSA infections from patients without exposure to risk factors for HA-MRSA infections (Morrison et al., 2006). Other attempts to differentiate between HA- and CA-MRSA were based on a genotypic differentiation like the SCCmec type (CA-MRSA harbor smaller SCCmec types IV or V), a lower number of antibiotic resistances beside beta-lactams (described for CA-MRSA) or the occurrence of the virulence factor Panton-Valentine leukocidin (PVL) (frequent carriage in CA-MRSA) (Chambers and Deleo, 2009). However, these lines are increasingly blurred and differentiation between classical HA- and CA-MRSA is becoming more and more difficult.

Identification of a bacterial pathogen by bacteriological methods from samples are time consuming. Identification of bacteria in most of the clinical laboratories is
currently based on analysis of phenotypic characteristics utilizing biochemical tests, serotyping and enzymatic profiles. However, there are several disadvantages associated with current microbiological methods such as a negative culture may result from residual antibiotics following antibiotic therapy or from low numbers of pathogens in the sample. Current methods of mastitis/pus/nasal swab pathogen identification are time consuming and generally require more than 72 hours to obtain results. Inadequate pathogen detection or confirmation techniques have often delayed timely intervention in disease control.

Considering the limitations of conventional approaches, the DNA-based assays are being used which focus on the unique nucleic acid composition of the bacterial genome rather than on phenotypic expression of proteins that nucleic acids encode. Also, DNA-based identification assays are subject to less variability when compared to diagnostic methods based on phenotypic characterization. The DNA-based identification systems are targeted for specific virulence factors of pathogens, allow for rapid screening of a large number of genes of interest simultaneously and provide definitive confirmation of pathogens. The development of PCR based methods provides a promising option for the rapid identification of bacteria. Real time PCR based methods have added advantage in terms of time required and identification of bacterial species can be made in hours, rather than days for conventional culture methods (Espy, et al., 2006).

In the present study, along with the conventional methods like isolation and biochemical characterization, the more rapid, accurate and sensitive molecular method, PCR was adopted for the early and accurate detection of MRSA in mastitic milk/pus/human nasal swab samples. Genetic analysis of MRS strains from animals and humans has given incite about the overall evolution, transfer and adaptability of MRS strains across these species. These analysis may also be inferred to determine \textit{mecA} gene of species \textit{i.e.} MRS in the area of study and also may aid as a useful tool for the epidemiology of the MRS between the farms and its zoonotic potential. Further, the outcome may be useful in establishing the effective control strategies as MRS has got very high public health importance. Simultaneously, the MRS isolates were categorized as HA-MRS and CA-MRS based on SCC\textit{mec} typing to study the correlation of animal and human MRS isolates.
Total 202 samples from animal (milk, pus/exudate) and 100 human nasal swabs were analyzed with conventional microbiological methods as well as multiplex PCR for MRSA identification and PCR for the SCCmeC typing. The results were analyzed and studied correlation with the animal and human isolates in terms of healthcare associated and community associated MRS.

4.1 ISOLATION AND IDENTIFICATION OF STAPHYLOCOCCI FROM ANIMAL AND HUMANS AND THEIR BIOCHEMICAL CHARACTERIZATION

The various conventional methods are used to identified and characterized S. aureus on the basis of phenotypic properties e.g., colony morphology, staining characteristics, growth pattern at different temperatures, fermentation of carbohydrates and production of different enzymes like oxidase, coagulase and catalase, (Kloos and Schleifer, 1986; AOAC, 1995).

The 202 animal samples (milk, pus/exudate) and 100 human nasal swabs were processed for isolation of bacteria as per standard procedures (Quinn et al. 1994) (Table 4.1). The isolated bacteria were identified up to genus level based on colony characteristics of individual primary isolate, growth on mannitol salt agar (Plate 4.3) and Gram’s staining (Plate 4.2). Further, these isolates were subcultured on plain nutrient agar plates and primary biochemical tests viz., catalase, coagulase and oxidase were carried out using pure young cultures and were preserved at 4°C on BHI agar slants. Out of total 202 animal and 100 human samples collected 86 (42.57%) and 62 (62%) isolates, respectively were identified as Staphylococcus spp. based on biochemical and growth patterns Table 4.2.

TABLE 4.1: Details of the samples from various sources

<table>
<thead>
<tr>
<th>Sample origin</th>
<th>Types of sample collected</th>
<th>No. of sample collected</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cow</td>
<td>Milk</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Pus/Exudate (abscess/wound)</td>
<td>20</td>
</tr>
<tr>
<td>Buffalo</td>
<td>Milk</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Pus/Exudate (abscess/wound)</td>
<td>15</td>
</tr>
<tr>
<td><strong>Human</strong></td>
<td>Human beings</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Nasal swabs</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>302</strong></td>
</tr>
</tbody>
</table>
During current study, all 86 isolates of animal (milk, pus/exudate) and 62 isolates of human (human nasal swabs) origin *Staphylococcus* spp. formed entire and low convex colonies on nutrient agar as well as BHI agar. Of these, 38 of animal isolates and 28 human isolates produced yellow pigmented colonies while rest of the isolates grown with white colony. The biochemical profiles of all the bacterial isolates were examined as previously described (FDA bacteriological analytical manual, 2001; Collee *et al*., 2008 and Cowan and Steel, 1995). Biochemical characteristics of animal and human samples were described in Table 4.2. The staphylococcal isolates were inoculated on mannitol salt agar. Out of 86 animal isolates, 60 isolates and out of 62 human isolates, 21 isolates have fermented mannitol and produced yellow colonies, whereas other isolates were non mannitol fermenter and produced white colonies. All 86 isolates of animal and 62 isolates of human were catalase positive and oxidase negative. The study revealed 12 isolates from animals and 12 isolates from humans were positive for coagulase gene and 74 isolates and 50 isolates were coagulase negative by PCR from 86 *Staphylococcus* spp. from animals and 62 *staphylococcus* spp. from humans, respectively.

For the purpose of identification of Methicillin-resistant staphylococcus (MRS), all the 86 animal isolates and 62 human isolates were streaked on Hi Chrom MeReSa® agar (HiMedia, HiMedia laboratory Pvt. Limited) which is selective media for detection of MRS. Out of these, 15 isolates from animal and 29 isolates from human produced dark greenish blue colonies (Plate 4.4) and identified as Methicillin-resistant staphylococcus. Of these 15 animal and 29 human isolates, 5 animal and 14 human isolates colonies appeared within an incubation period of 48 hours and in rest 10 animal isolates and 15 human isolates colonies appeared in the time period of 72 hours. Culture on selective chromogenic media is an important criterion for selective isolation and diagnosis of MRS as reported in previous works (Al-Mussawi, 2014; Nimavat, 2015; Hetal, 2016) but when we consider time, it may be time consuming because in present investigation incubation period for isolation of MRS from animal samples and human nasal swabs was more than 48 hours which is same as in normal primary isolation and biochemical characterization, but it can be used for confirmation from pure culture.
TABLE 4.2: Biochemical characteristics of *Staphylococcus* spp. isolates (Animal and human nasal swabs)

<table>
<thead>
<tr>
<th>BIOCHEMICAL TEST</th>
<th>N= 86 (Animal samples)</th>
<th>N=62 (Human nasal swabs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Mannitol Fermentation</td>
<td>60 (69.77%)</td>
<td>26 (30.23%)</td>
</tr>
<tr>
<td>Catalase test</td>
<td>86 (100%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Oxidase test</td>
<td>0 (0%)</td>
<td>86 (100%)</td>
</tr>
<tr>
<td>Growth on MeReSa media</td>
<td>15 (17.44%)</td>
<td>71 (82.86%)</td>
</tr>
<tr>
<td>Coagulase gene (PCR)</td>
<td>12 (13.95%)</td>
<td>74 (86.05%)</td>
</tr>
</tbody>
</table>

During the study, total 9 MRS isolates from 86 *Staphylococcus* spp. and 20 MRS isolates from 62 *Staphylococcus* spp. were identified from animal and human samples, respectively. The coagulase and hemolytic pattern were studied from MRS positive isolates. Out of 9 MRS isolates from animal, only 1 (11.11%) isolate was coagulase positive (MRCoPS – Methicillin-Resistant Coagulase Positive Staphylococcus) by tube coagulase test, slide coagulation test (Plate 4.5. 1, 2) and PCR, whereas 8 (88.89%) isolates were coagulase negative (MRCoNS – Methicillin-Resistant Coagulase Negative Staphylococcus). Similarly, out of total 20 MRS isolates from human, 2 (10%) isolates were coagulase positive (MRCoPS – Methicillin-Resistant Coagulase Positive Staphylococcus) on tube coagulase test, slide coagulation test and PCR and 18 (90%) isolates were coagulase negative (MRCoNS – Methicillin-Resistant Coagulase Negative Staphylococcus) (Table 4.3).
TABLE 4.3: Coagulase Negative (MRCoNS) and coagulase positive (MRCoPS) MRS isolates from animal and humans

| Sample origin | No. of samples collected | No. of sample found positive for *Staphylococcus* spp. No. (%) | Methicillin-Resistant Staphylococcus (out of positive staphylococcus) No. (%) | No. (%) Methicillin-Resistant Staphylococcus
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals (Milk+Pus)</td>
<td>202</td>
<td>86 (42.57)</td>
<td>9 (10.47)</td>
<td>8 (88.89) 1 (11.11)</td>
</tr>
<tr>
<td>Human (Nasal swabs)</td>
<td>100</td>
<td>62 (62)</td>
<td>20 (32.26)</td>
<td>18 (90) 2 (10)</td>
</tr>
</tbody>
</table>

MRCoNS – Methicillin-Resistant Coagulase Negative Staphylococcus
MRCoPS – Methicillin-Resistant Coagulase Positive Staphylococcus

These isolates were also evaluated for hemolytic patterns of the isolates identified as MRS on blood agar. Out of 9 MRS isolates from animal, 5 isolates produced complete hemolysis (alpha), 2 isolates produced partial hemolysis (beta) and 2 isolates had not produced hemolysis (No hemolysis) around colony, whereas out of 20 MRS isolates from human, 7 isolates produced complete hemolysis, 9 isolates produced partial hemolysis and 4 isolates had not produced hemolysis (No hemolysis) around colony (Table 4.4).

TABLE 4.4: Hemolysis patterns of Methicillin-resistant staphylococci isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Animal samples (Total: 9)</th>
<th>Human samples (Total: 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alpha Hemolysis (Complete)</td>
<td>Beta Hemolysis (Partial)</td>
</tr>
<tr>
<td>Positive</td>
<td>5 (55.56%)</td>
<td>2 (22.22%)</td>
</tr>
</tbody>
</table>

In present investigation it was observed that prevalence of *Staphylococcus* spp. is highest in animal milk, pus/exudate samples (42.57%) and human nasal swabs (62%) among all other bacteria (Table 4.3). Similarly, Hetal, (2016) reported higher
prevalence of Staphylococcus spp. in milk samples (66%) and human nasal swabs (70%) as compared to all other bacterial spp. Nimavat, (2015) also reported higher prevalence of Staphylococcus spp. (62%) among all other bacterial spp. from milk samples. The previous reports also suggest that, it is one of the most frequently isolated contagious pathogens as compared to other microbes in bovine mastitis and as a nosocomial infections worldwide (Verma, 1988; Kaya, et al., 1998; Das and Kanna 1994; Datta and Rangenkar 2001; Megra et al., 2006; Karsten et al., 2006; El-Jakee et al., 2008; Ali et al., 2008; Alzohairy 2011; Marcela et al., 2011; Abera et al., 2013; Sharma et al., 2015; Sun et al., 2017). The prevalence of S. aureus in bovine mastitis were reported between 29.34% to 78.54% by various authors (Ebrahimi et al., 2007; Sharma and Maiti, 2010; Ali et al., 2011) in cows and 27.36% to 70.32% (Sharma, 2004; Sharma et al., 2007; Beheshti et al., 2010) in buffaloes. Therefore detailed analysis of previous studies revealed that highest prevalence of clinical and sub-clinical mastitis in cattle and buffaloes was due to S. aureus with mean of 46.72%.

In current study, the 62% of isolates were staphylococcus among human nasal swabs. When compared to the prevalence rates detected by other workers worldwide, the prevalence rate of S. aureus in milk and in the anterior nares of animal worker population depending upon the size, geographical area and management practices of the herd. Little lower rates of staphylococcus were reported by various authors. A 13.2% prevalence rate of S. aureus from nasal swabs of 68 dairy farm workers at four different farms around Addis Ababa (Mekuria et al., 2013), 41% and 40% among industrial and antibiotic free livestock operational workers respectively in North Carolina (Rinsky et al., 2013) and 21.6% from hog slaughter and processing plant workers were reported positive for S. aureus (Neyra et al., 2014). In India prevalence rate of MRS in human samples were reported to be 32.8% (Mathur et al., 1994), 51.6% (Vidhani et al., 2001), 35% (Krishna et al., 2007) and high prevalence rate of 70% were recorded by Hetal, (2016) as compared to present investigation, in human samples carring Staphylococcus spp.

During the current study, on biochemical characterization, the proportion of coagulase and clumping factor was found in isolates recovered from the animal (13.95%, 12/86) and human nasal swabs (19.35%, 12/62) (Table 4.2). Similarly, other researcher across the globe have reported coagulase activity among S. aureus isolates from animal and human beings (Chatterjee et al., 1990; Kamata et al., 1990; Das and
Results and Discussion

Khanna, 1994; Kumar et al., 2011a; Tyagi et al., 2013; Rusenova et al., 2013; Nimavat, 2015. Hetal, 2016). Staphylococcal coagulase converts fibrinogen to fibrin and causes clotting of cells (Phonimdaeng et al., 1990).

Hemolysins have been shown to be involved in maintenance or continuation of infection and act as cytotoxic agents that damage and/or destroy cells of the defense system (Bramley et al., 1989; Dinges et al., 2000). The alpha-hemolysin toxic to bovine mammary cells, causes erythrolysis, disturbs the ion balance in host cells, is dermonecrotic and neurotoxic and is also needed in biofilm formation (Bramley et al., 1989; Dinges et al., 2000; Caiazza and O’Toole, 2003). Conversely, beta hemolysin also probably is produced in most of the bovine strains and functions as sphingomyelinase of the erythrocytes (Aarestrup et al., 1999; Akineden et al., 2001).

During the present study, the hemolytic patterns of MRS isolates from animal and human were studied. A total 55.56%, 35% isolates, 22.22%, 45% isolates and 22.22%, 20% isolates were produced alpha hemolysis, beta hemolysis and no hemolysis on blood agar medium from the animal and human MRS isolates, respectively (Table 4.4).

Similar to present finding, Munch-Peterson and Gardiner (1965) identified 227 isolates of staphylococci on ox blood agar. They found 43 isolates (18.9 %) were alpha hemolytic (lower to present study), 54 (23.8 %) isolates were beta hemolytic, 17 (7.5 %) isolates were alpha-beta type and 56 (24.7 %) isolates found to be non-hemolytic strains of Staphylococcus aureus. Da Silva et al. (2000) isolated staphylococci from 29 goat’s mastitis milk from Brazilian dairy herds. Out of total isolates studied, 80% demonstrated hemolytic activity. In same study, Staphylococcus aureus isolates showed hemolytic activity for all hemolysin types studied 76.7% of them produced alpha hemolysin, 74.4 per cent beta hemolysin and 83.7% produced gamma hemolysin. Bhanderi (2007) reported 27 (62.79 %) isolates of S. aureus exhibited alpha hemolysis, whereas 7 (16.27 %) isolates showed beta hemolysis and 9 (20.93 %) isolates were non-hemolytic on 5% sheep blood agar. During the same year, Patel (2007) reported that out of total 80 (20 isolates each from sheep, goats, cattle and buffaloes) isolates, number of isolates showing alpha, beta, gamma and alpha-beta hemolysin production were 19 (23.75 %), 48 (60.00 %), 13 (16.25 %) and 23 (28.75 %) on sheep blood agar, respectively.
In present finding, majority of MRS isolates showed hemolytic activities and are in agreement with earlier studies as majority of the *S. aureus* isolates were positive for hemolysin activity phenotypically (Aarestrup *et al*., 1999; Fitzgerald *et al.* 2000; Akineden *et al*., 2001; Ghosh, 2003). The evaluation and presence of hemolysins showed their importance in mastitic infection. The observations of the present study are in agreement with earlier studies (Younis *et al*., 2000; Turkyılmaz and Kaya, 2006). It may be inferred that the presence of coagulase clumping factor and hemolysins are crucial for establishment of infection in mammary glands.

In contrast to present finding, higher rate of hemolytic activities were showed by some of the researcher. Matsunaga *et al.* (1993) examined 58 *Staphylococcus aureus* isolated from bovine mastitis milk and revealed 74.1%, 65.5% and 12.1% isolates produced alpha, beta and gamma hemolysins, respectively. Whereas, lower rate of hemolysis were reported by Ali-Vehmas *et al.* (2001), they identified 12 (24%) isolates out of 50 isolates of *Staphylococcus aureus* recovered from healthy and mastitic bovine milk for beta and delta hemolysins. Ghosh (2003) tested 58 coagulase positive *Staphylococcus aureus* isolates for hemolysin production and they reported that 6.9%, 15.5%, 10.3%, 18.9%, 12.1% and 13.8% produced the alpha, beta, gamma, alpha-beta, beta-gamma and gamma-alpha hemolysins respectively, while five strains (8.6 %) were found to be non-hemolytic in nature.

The isolates showed diverse characteristics on testing and the biochemical assays and majority of isolates could be identified by these test but very few of the isolates showed unique biochemical pattern which were not reported to be the identifying markers for designating the species level identification of *Staphylococcus* spp. However, a few of the biochemical techniques showed indistinctness occasionally in results due to flexibility in bacterial nature and growth depending on laboratory environment (Stephan *et al*., 2004). The atypical biochemical patterns (gram positive cocci in bunch but oxidase positive, catalase negative, mannitol nonfermenter *S. aureus*, etc.) in bovine and human isolates have been reported in earlier studies (Aarestrup *et al*., 1999; Boerlin *et al*., 2003; Rabello *et al*., 2005; Nimavat, 2015; Hetal, 2016). The understanding of atypical biochemical patterns is important as misidentification could lead to a decrease in the true number of isolates in the study. Therefore, isolates with atypical patterns were excluded only after molecular identification.
The *Staphylococcus* spp. is particularly important because it causes mainly subclinical and clinical forms of infectious mastitis and wound infection and specifically subclinical form of mastitis are often difficult to detect by the herd men. As observed in the present study, majority isolates were observed to be from milk having normal physical properties. As several other workers reported that Staphylococci were the predominant isolates in subclinical and clinical mastitis cases (kang-Hee et al., 2001; Abdel-Rady and Sayed, 2009; Rahman et al., 2010; Sharma and Maiti, 2010; Nimavat, 2015; Hetal, 2016) and *S. aureus* is a commensal organism, persistently or transiently present in up to 80% of healthy humans and it is most common cause of skin and soft tissue infections and remains an important cause of nosocomial infections worldwide (Popovich and Hota, 2008; David and Daum, 2010) with severe consequences for patients and significant healthcare costs.

In the present study, 86.05% (74/86) of *Staphylococcus* spp. from animal and 80.64% (50/62) of *Staphylococcus* spp. from human beings were CoNS (Table 4.2) which is highly indicative of an emerging mastitis pathogen which should be taken in to consideration as emerging pathogenic species of Staphylococcus. The identification of CoNS isolates was based on colony morphology, staining characteristics, catalase reaction, coagulase test and finally confirmed by the PCR. Prior to the 1970’s, clinicians and microbiologists generally regarded coagulase negative staphylococci (CoNS) as contaminants in clinical specimens and *S. aureus* as the only to be pathogenic. Today 41 different *Staphylococcus* species have been described to be potent pathogens. CoNS have often been considered as minor udder pathogens, causing relatively negligible udder health problems. The finding is in accordance with the earlier reports, wherein the udder quarter prevalence of CoNS intramammary infections varied between 4% to 50% (Trinidad et al., 1990) and it was 16.6% in Finland (Pitkala et al., 2004).

In the present study majority of MRS isolates were MRCoNS from animal (8, 88.89%) and human beings (18, 90%) (Table 4.3). Similarly, Vishnupriya et al. (2014) reported 27% of the isolates were Methicillin-resistant coagulase negative staphylococci (MRCoNS) from bovine mastitis and 42.86% were MRCoNS from nasal swabs, whereas lower rate (7.27%) of MRCoNS were isolated by Baptiste et al. (2005). However, CoNS infections may cause substantial herd problems due to high prevalence of sub-clinical and/or clinical mastitis (Wilson et al., 1997). CoNS are now, a frequent cause of bovine mastitis in modern dairy herds as well as in the human nasal carrier in
many countries. In a nationwide survey in Finland, CoNS were isolated from 17% of the quarters and from 50% of the quarter’s positive for bacterial growth (Pitkala et al., 2004). Similarly, Lim et al. (2007) reported CoNS were the most common bacteria (51%) causing intramammary infection (IMI) at drying off period. The study in USA reported prevalence of CoNS infections among all sampled cows at the rate of 11% and 23% of the bacteria isolated from the milk samples (Wilson et al., 1997).

4.2 ANTIBIOTIC SUSCEPTIBILITY PROFILE OF STAPHYLOCOCCUS SPP. FROM ANIMAL AND HUMANS

In recent years, there has been increased concern about antibiotic resistance strains of S. aureus. Development of resistance has been attributed to the extensive therapeutic use of antimicrobials and using antibiotic as growth promoters in food animal production. The resistance to methicillin in staphylococci is mediated by the meca gene. An organism with this type of resistance is referred to as Methicillin-resistant S. aureus. The meca gene resides on a staphylococcal chromosomal cassette (SCCmec). MRSA was initially reported as a nosocomial pathogen in human hospitals (hospital-associated MRSA) and was isolated from patients with compromised immune system undergoing medical procedures. In the 1990s, a major change in the epidemiology of MRSA has been observed, with the appearance of cases affecting people with no epidemiological connection to hospitals; strains that cause such infections are referred to as community-acquired or community associated MRSA.

Treatment of staphylococcal infections in humans and mastitis in bovines is becoming difficult due to the increasing trend of antibiotics resistance. So, antibiotic sensitivity profile provides vital information that can be helpful in evolving a strategy for prevention and treatment of mastitis in cattle and buffaloes and nosocomial infections in humans. In a particular region antibiotic-susceptibility/resistance in bacterial isolates varies due to different strategies and policies in containment programs, availability and usage patterns of antibiotics to treat mastitis. Therefore, isolates of S. aureus were analyzed to find the status or information on the resistance patterns among the strains both from animals and human beings in this particular region.

Several conventional methods, such as the oxacillin agar screening test and the oxacillin and cefoxitin disc diffusion tests, have been developed to detect MRSA
isolates. The reliability of these phenotypic methods has been reported to be between 80 % and 100 % (Tiwari et al., 2009). Alternatively, molecular techniques, mostly based on PCR amplification of the mecA gene, have been used for the rapid detection of MRSA (Kalhor et al., 2012).

In present study, antimicrobial sensitivity test was carried out as per the standard disc diffusion method (Bauer et al., 1966). Different patterns of antibiotic-resistance were observed in the tested isolates (Table 4.5, 4.6). Nine animal and 20 human Methicillin-resistant isolates were identified. Out of 9 animal and 20 human isolates, 1 and 2 were Methicillin-resistant coagulase positive Staphylococcus (MRCoPS) and rests 8 and 18 were Methicillin-resistant coagulase negative staphylococci (MRCoNS), respectively (Table 4.3).

The minimum inhibitory concentration (MIC) of animal and human MRS isolates was determined by E-test (Table 4.7, 4.8) and majority of these isolates were multidrug resistance based on MIC value of oxacillin strip. Majority of the isolates were found multidrug resistance against 20 selected antibiotics and multiple resistance index (MRI) were determined (Table 4.10, 4.11) by dividing the number of antibiotics to which the isolate is resistance by the total numbers of antibiotics tested (Olayinka et al., 2009).

In present study, all animal MRS isolates showed higher susceptibility to amikacin and rifampicin (100%) followed by oxytetracyclin (77.78%) and chloramphenicol (66.66%) (Table 4.5 and Figure 4.1), whereas overall sensitivity of all the staphylococcal isolates including MRS from animal was higher in chloramphenicol (88.37%) followed by ceftriaxone/salbactum (86.05%), oxytetracyclin (84.88%), levofloxacin (77.90%), amoxicillin/salbactum (79.07%), gentamicin (69.79%) and little lower rate of sensitivity of these isolates were observed again methicillin (55.81%) (Table 4.6 and Figure 4.2).

Similarly, the MRS isolates from human showed higher susceptibility to methicillin (98%) followed by rifampicin and gentamicin (90%) chloramphenicol (80%) and ofloxacin and levofloxacin (70%) (Table 4.5 and Figure 4.1), whereas overall sensitivity of all the staphylococcal isolates including MRS from human was higher in gentamicin (96.77%) followed by methicillin (93.55%), chloramphenicol (91.94%) and levofloxacin (80.65%) and little lower rates were observed again...
amoxicillin/salbactum and oxytetracyclin (50%) and ceftriaxone/salbactum (40.32%) (Table 4.6 and Figure 4.2).

During the present study, the higher rates of methicillin, gentamicin, ofloxacin and levofloxacin sensitivity were observed in human isolates as compared to animal MRS isolates, where as in contrary to this, higher rates of amikacin, rifampicin, oxytetracyclin sensitivity were observed in animal isolates as compare to human MRS isolates. This change in the percentage of sensitivity in animal and human isolates attributed to the most frequent use of antibiotic in respective species may developed higher resistance compare to least use of this antibiotics in other species. This might be attributed to continuous selection pressure of antibiotics on the isolates because of extensive and repeated use of same class of antibiotics.

Resistance to some of the antimicrobials (Penicillin-G, ampicillin/cloxacillin, enrofloxacin, norfloxacin, co-trimoxazole Cefoxitin/cloxacilln, ceftizoxime) was noticeably high in both, animal and human MRS isolates (Table 4.5). As in previous reports of our lab all 100 milk isolates showed higher susceptibility to chloramphenicol (87.09%), oxacillin (83.87%), oxytetracycline (79.03%), methicillin (83.87%), ofloxacin (69.35%) and rifampicin (67.74%) reported by Nimavat, (2015). Hetal, (2016) reported higher susceptibility to chloramphenicol (87.24%) & (90.91%), gentamicin (87.24%) & (86.37%) and ampicillin/Sulbactam (93.62%) & (84.1%) from animal and human isolates, respectively.

All over results of the present study were in concurrence with the results reported by various researchers across the globe (Kabir et al., 2005; Parmar et al., 2006; Rajendran et al., 2006; Bhanderi, 2007; Suresh et al., 2007; Frana et al., 2013). Similarly, Lower levels of sensitivity as compared to present study to staphylococcus spp. of different origin was also reported by several authors (Awandkar and Khode 2009; Kelman et al., 2011; Kumar et al., 2011b).

The results of ABST showed in present study were in concurrence with many of the published reports with few exceptions. Resistance in isolates remained higher to common antibiotics from milk and human samples (Sabour et al., 2004; Moon et al., 2007a; Rajaduraipandi et al., 2006). However, the commonness of resistance by strains to some of the antibiotics those are not frequently used in veterinary practices indicated possible reason of dissemination of these isolates in animals from milkman/ farmer/herd
worker. In present investigation same phenomena was observed and majority of isolates were resistance to one or more above mentioned antibiotic groups which is strongly suggesting that the resistance might be bear of and transfer from milkman/farmer/herd worker.

The antibiogram reported in the present studies showed variation in the resistance when compared with strains of different geographical regions. Such variations in antibiotic-resistance patterns in isolates of different regions could be due to diverse antibiotic policies and conditions which was also supported by various authors (Gentilini et al., 2000; Erskine et al., 2002; Moon et al., 2007a; Wang et al., 2008; Ochoa-Zarzosa et al., 2008; Turutoglu et al., 2009). The most common resistance mechanism is beta lactamase production, which results in resistance to penicillin G and aminopenicillins (Chambers and Hackbarth, 1989, Hetal, 2016). However, in most of the countries penicillin-resistance is the commonest form, similarly 100% of MRS isolates from animal and human resisting penicillin in the present study. Earlier investigations from Argentina, Belgium, Brazil, Finland, Germany, Great Britain, Ireland, Portugal, Switzerland, United States and Uruguay have showed approximately one-third to two-thirds of bovine S. aureus isolates were resistance to penicillin (Das and Khanna, 1995; De Oliveira et al., 2000; Giannechini et al., 2002; Makovec and Ruegg, 2003; Tenhagen et al., 2006; Rabello et al., 2007, Nimavat, 2015). S. aureus was commonly resistance to penicillin (94.4%) and amoxicillin (36.1%), while susceptible to other antimicrobial agents such as chloramphenicol and gentamicin (Abera et al., 2013).

The present study revealed 100% of MRS isolates from animal and human were resistance to penicillin-G, whereas 100% isolates from animal and 75% isolates from human were resistance to ampicilline/cloxacillin. The antimicrobial sensitivity tests of S. aureus as seen in the present study agree with earlier reports that heightened resistance of S. aureus to penicillin and ampicillin (Kalmus et al., 2011). There is no scientific evidence as to the resistance pattern of mastitis pathogens to penicillin and the cephalosporin group, but there were widespread reports of antimicrobial drug resistant among Staphylococcus pathogens for over 2-3 decades as earlier reported in Germany (Botrel et al., 2010), Estonia (Kalmus et al., 2011), Sweden (Persson et al., 2011), Iran (Sahebekhtiari et al., 2011), India (Chaudhary and Payasi, 2013) and Brazil
The trend of resistance patterns to antibiotic use over time showed a long-term effect of over 3-7 years (Rall et al., 2013).

During the present study, the higher rates of methicillin, gentamicin, ofloxacin and levofloxacin sensitive isolates were observed in human beings as compared to animal MRS isolates, whereas high resistance towards penicillin, cefixime, ampicillin/cloxacillin, Cefoxitin/cloxacillin and amoxicillin/subbactam noticed among both the species. Similarly in human, S. aureus isolates showed 97.8% resistance to gentamicin (Qureshi et al., 2004) which is higher to present study. Rajaduraipandi et al. (2006) reported 99.6% isolates were resistance to penicillin, 93.2% to ampicillin, 62% to gentamicin, 60.8% to cephalaxin and 60% to erythromycin among 250 MRS isolates screened. At present, less than 90% of S. aureus strains are resistant to most penicillin derivatives (Freeman-Cook and Freeman-Cook, 2006) and ordinary antimicrobial agents like drugs from the family of aminoglycosides, macrolides, chloramphenicol, tetracyclines and fluoroquinolones (Lee, 2003).

In contrary to present study, in India 76% MRSA strains were resistant to gentamicin (Saxena et al., 2003) and 60% were resistant to gentamicin among MRS isolated in Saudia Arabia (Baddour et al., 2006). Whereas, 58.3% MRS were resistance to tetracycline (Sharma et al., 2013) that is lower to our study. Similarly in Maharashtra MRS were highly resistance to ampicillin, tobramycin, erythromycin, kanamycin and 91 per cent strains were resistant to gentamicin. But all the isolates were sensitive to vancomycin (Kandle et al., 2003). In present study out of 20 human MRS isolates, 13 isolates showed resistance to more than 50% of the antibiotic designated as multidrug resistant. Unlike reported that 30% resistant to all the antibiotics tested (Goyal et al., 2002; Deshpande et al., 2002). Prates et al., 2010 reported that it is alarming to note that 60% (6/10) isolates were resistant to ciprofloxacin though it had been proposed as an alternative for treatment of MRS infection.

In present investigation, human isolates showed higher resistance towards fluoroquinolone as compared to animal isolates and also the resistance to gentamicin was low. Similar results were obtained in a survey conducted in UK and Ireland in 2001-2002. They had reported that fluoroquinolone resistance emerged very rapidly in human associated MRS after widespread utilization of this antibiotic and studies showed that fluoroquinolone resistance increased from 7% in 1988 to 83% in 1990.
Results and Discussion

(Hershow et al., 1998). The present study revealed higher percentage of sensitivity to gentamicin in human isolates. This was a rather unexpected result because of the earlier MRS were known to be resistance to gentamicin and so the use of gentamicin gradually decreased until it was no longer used for therapy, while the use of fluoroquinolones and macrolides increased. This change in antibiotic usage trend had led to the development of gentamicin sensitive. These findings were substantiated by the studies where the predominanace of gentamicin sensitive isolates of MRS in hospitals after 1997 was reported (Robert et al., 2006). In France in 1992 the frequency of gentamycin resistance MRS was about 7.4% from 1992 to 1998. This prevalence rate increased progressively to reach between 46.8 and 94.4% in French hospitals. These strains were then replaced by gentamicin sensitive MRS (Lemaitre et al., 1998).

In current study, the 80% of the isolates from human revealed sensitive to chloramphenicol. Similar results had been reported by Baddour et al. (2006), they revealed 80% of the isolates were chloramphenicol sensitive. Similarly, report showed full susceptibility of the isolates to chloramphenicol (Panhotra et al., 2005). In contrary to current finding, susceptibility to chloramphenicol in Japanese isolates ranged from 3.8% to 5.1% (Yamaguchi and Ohno, 2005) which is far lower than the results obtained in our study.

Earlier the tetracycline was not used directly to treat mastitis because of its irritant effects. But now a days, slowly this drug has been introduced for the treatment of mastitis and because of this the resistance to this drugs was observed 22.22% in present study with animal isolates, therefore the resistance frequencies in different countries can significantly vary, from very low to very high and reflect the usage pattern of antimicrobials in these countries. In context to the observations of the present investigation, higher rate of resistance were reported in several earlier Indian studies (Parmar et al., 2006; Joshi and Gokhale, 2006; Awandkar et al., 2009) to tetracycline. At present, less than 90% of S. aureus strains are resistance to various groups of antibiotics including tetracycline group (Lee, 2003).

Detection of MRS from milk, exudate/abscess/pus cattle and buffaloes was other significant finding of this study. During the study, 10.47% (9/86) isolates were found MRS positive among total staphylococcal isolates recovered. Smith and Pearson, (2010) reported that the MRS isolates were found rarely to be associated with bovine
Results and Discussion…

mastitis. However, MRS has been reported in many studies (Monecke et al., 2007; DeVries et al., 2009; Febler et al., 2010; Vanderhaeghen et al., 2010a) among milk samples. The prevalence of MRS might be location specific and antibiotic usage pattern dependent as various observations reported in different regions.

In contrary to present investigation, higher rates of MRS were reported by Turkyılmaz et al. (2008) (17.2%) and Febler et al. (2010) (25%), whereas little lower rate of MRS isolates were reported in different studies conducted by Lee, (2003) and Moon et al., (2007b), they reported 2.8% and 4.5% occurrence of MRS in Korean herds, respectively. Whereas, in herds of France and Belgium, it was showed 3.2% and 9.3% MRS (Alves et al., 2009; Vanderhaeghen et al., 2010b), respectively. In Switzerland and Germany, only 0.2% of MRS has been reported (Monecke et al., 2007).

In present investigation, 32.26% (20/62) isolates from human were found MRS positive among staphylococcal isolates recovered, which was higher in compare to animal MRS isolates recovered. In contrary to present investigation, lower recovery rate of MRS were reported in human beings by Mathanraj et al., 2009, they reported 8.5% overall MRS carriage rate from South India. Similarly, a prevalence of only 0.7% MRS among human patients were reported by Munckhof et al. (2009), 24.15% MRS recovered in USA (Currie et al., 2008) and 13.6% and 14.85% of MRS reported from Taiwan and Nigeria reported respectively, from anterior nares of healthy population (Onanuga et al., 2011).

During the study, the correlation of phenotypic test with meca gene amplification were studied. Total 29 isolates from animal and human were identified as Methicillin-resistant staphylococcus based on meca gene amplification. Out of this 29 isolates, 26 (89.67%) isolates showed phenotypic oxacillin susceptibility test correlation with meca gene amplification. Among these, 100% (0.5 to ≥256 µg/ml) isolates from human and 66.67% (0.75 to ≥256 µg/ml) isolates from animal showed correlation of phenotypic oxacillin susceptibility with meca gene amplification among MRS isolates (CLSI, 2011). (Table 4.9). Since detection of meca gene is considered the gold standard for identification of MRSA strains, but when facilities for such molecular techniques are not available for detection of MRS organism, the oxacillin disc diffusion testing is far superior and can be used as alternative technique for diagnosis of such organism.
Similar to present study, the same result were obtained by Havaei et al., (2015), they reported 90% and 100% sensitivity and specificity, respectively of Oxacillin agar screening and was completely in agreement with the PCR for meca gene detection. Similarly Turkyilmaz et al., (2008) reported similar kind of results to this present study.

The MIC (µg/ml) of all the MRS isolates from animal and human were studied during the present investigation. The MIC results showed 100% (0.5 to 4 µg/ml) and 55% (1.5 to 4 µg/ml) isolates were sensitive to vancomycin from animal and human respectively, whereas 45% (8 to 16 µg/ml) of isolates from animal showed intermediately sensitive to vancomycin as per the standards of CLSI, (2016). This results shows alarming situation about the increase in susceptibility of vancomycin from sensitive to become intermediately sensitive in human isolates and may become resistance in near future. The same trends might progress with the animal MRS isolates in near future.

For many years vancomycin has been considered the drug of choice for the treatment of S. aureus infections in human due to strains that had become resistance to methicillin. However, in July 2002, things changed when the Centers for Disease Control (CDC) in the USA published the first documented report of S. aureus that was resistance to vancomycin as well as being resistant to methicillin.

Similarly during the present study, the MIC of cefoxitin showed 33.34% (0.75 to 1 µg/ml) and 40% (2 to 4 µg/ml) isolates were sensitive and 66.67% (8 to ≥256 µg/ml) and 60% (8 to ≥256 µg/ml) isolates were resistant to this antibiotics in animal and human MRS isolates, respectively as per the standards of CLSI, (2013).

In accordance with the present study, Alli et al. (2015) showed 66 (42.3%) isolates were meca MRSA positive showing 100% correlation with the phenotypic cefoxitin susceptibility test results. All the isolates were susceptible to vancomycin (0.2 to 1 µg/ml). MIC to cefoxitin showed that the MIC50 and MIC90 of the MRSA strains were ≥256 µg/ml indicating high level resistance to methicillin.

During the present study, the MRS was isolated from animal and human beings sharing some of the common SCCmec types among the farm labors/workers-associated personel working in close contact with the animals, indicating colonization of MRS infection from animal to human or vice versa.
Identification of MRS in various species and in food has led to concerns about the roles of animals, both pets and livestock, in the epidemiology of MRS infection and colonization in humans. There is evidence of the role of food animals in human MRS infections in some countries and of pets as a possible source of human infection. Some groups of individuals who work closely with animals, such as veterinarians, have high MRS colonization rates (Weese, 2010). In many cases, colonization or infection by *S. aureus* in animal species appears to be due to zoonotic transfer from human owners or caretakers.

However, some species have the capacity to act as reservoirs of the organism and potentially transmit the bacterium to humans who work in close contact with colonized animals (Smith and Pearson, 2010). Spread of such strains in milk of infected dairy cows represents a risk to public health (Juhasz-Kaszanyitzky *et al.*, 2007; Smith and Pearson, 2010). The epidemiology of MRSA isolates from human and animal sources showed that for certain strains, a cross-infection might have happened (Seguin *et al.*, 1999; Strommenger *et al.*, 2006; Weese *et al.*, 2006). Studies conducted by Feirrera *et al.* (2011) and Verkade and Kluytman (2014) suggested that animals can be a potential source of MRS infection to humans.

In present investigation, several MRS isolates from human and animal were found to be multidrug resistant (*Table 4.10, 4.11*) because of continues pressure of antibiotics lead to acquisition of resistance genes. Therefore, the treatment of mastitis and nosocomial infections should be based on testing of the antimicrobial susceptibility of the causative agents. As a possible consequence of changes in the treatment strategies, multidrug resistance in *S. aureus* have become common. Exposure of *S. aureus* to one antibiotic might lead to acquisition of multiple-resistance determinants by the bacterial strain. Since the determinants of antibiotic-resistance often co-exist in mobile genetic elements, which can transfer as entire elements from one strain to another in a single event. In the long run, multi-resistance is considered to be a burden for microbes, including *S. aureus* and continuous production of resistance factors confers no additional fitness in an environment where selection pressure from antimicrobials is low.
TABLE 4.5: Antibiotic resistance patterns of Methicillin-resistant *Staphylococcus* spp. from animal and human

<table>
<thead>
<tr>
<th>Antibiotic group</th>
<th>Name of antibiotic used</th>
<th>Antibiotic resistance of animal isolates (%) (n=9)</th>
<th>Antibiotic resistance of Human isolates (%) (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Beta lactam</strong></td>
<td>Methicillin</td>
<td>88.89 %</td>
<td>2 %</td>
</tr>
<tr>
<td></td>
<td>Penicillin-G</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>Ampicillin/Cloxacillin</td>
<td>100 %</td>
<td>75 %</td>
</tr>
<tr>
<td><strong>Amino penicillin</strong></td>
<td>Amoxicillin/Salbactam</td>
<td>66.67 %</td>
<td>60 %</td>
</tr>
<tr>
<td><strong>Cephalosporins</strong></td>
<td>Ceftriaxone/Salbactum</td>
<td>55.56 %</td>
<td>65 %</td>
</tr>
<tr>
<td></td>
<td>Cefixime</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>Ceftizoxime</td>
<td>77.78 %</td>
<td>60 %</td>
</tr>
<tr>
<td></td>
<td>Cefoxitin/Cloxacillin</td>
<td>77.78 %</td>
<td>90 %</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>55.56 %</td>
<td>50 %</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime/Clavulanic acid</td>
<td>55.56 %</td>
<td>55 %</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td>Gentamicin</td>
<td>88.89 %</td>
<td>10 %</td>
</tr>
<tr>
<td></td>
<td>Amikacin</td>
<td>0 %</td>
<td>50 %</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td>Ofloxacin</td>
<td>100 %</td>
<td>30 %</td>
</tr>
<tr>
<td></td>
<td>Levofloxacin</td>
<td>77.78 %</td>
<td>30 %</td>
</tr>
<tr>
<td></td>
<td>Enrofloxacin</td>
<td>100 %</td>
<td>65 %</td>
</tr>
<tr>
<td></td>
<td>Norfloxacin</td>
<td>100 %</td>
<td>75 %</td>
</tr>
<tr>
<td><strong>Tetracyclins</strong></td>
<td>Oxytetracyclins</td>
<td>22.22 %</td>
<td>70 %</td>
</tr>
<tr>
<td><strong>Rifampin</strong></td>
<td>Rifampicin</td>
<td>0 %</td>
<td>10 %</td>
</tr>
<tr>
<td><strong>Amphenicol</strong></td>
<td>Chloramphenicol</td>
<td>33.34 %</td>
<td>20 %</td>
</tr>
<tr>
<td><strong>Sulfa group</strong></td>
<td>Co-Trimoxazole (Trimethoprim/Sulphamethoxazole)</td>
<td>77.78 %</td>
<td>80 %</td>
</tr>
</tbody>
</table>
FIGURE 4.2: Antimicrobial resistance patterns of Methicillin-resistant staphylococcus from animal and humans.

![Per cent of Antibiotic resistance](image)

TABLE 4.6: Antibiotic resistance patterns of *Staphylococcus* spp. (Including MRS) from animal (n=86) and human (n=62)

<table>
<thead>
<tr>
<th>Antibiotic group</th>
<th>Name of antibiotic used</th>
<th>Antibiotic Resistance of animal isolates (%) (n=86)</th>
<th>Antibiotic Resistance of Human isolates (%) (n=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beta lactam</td>
<td>Methicillin</td>
<td>44.19 %</td>
<td>6.45 %</td>
</tr>
<tr>
<td>Amino penicillin</td>
<td>Amoxicillin/Salbactam</td>
<td>20.93 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Ceftriaxone/Salbactum</td>
<td>13.95 %</td>
<td>59.68 %</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Gentamicin</td>
<td>30.23 %</td>
<td>3.23 %</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Levofloxacin</td>
<td>22.10 %</td>
<td>19.35 %</td>
</tr>
<tr>
<td>Tetracyclins</td>
<td>Oxytetracyclins</td>
<td>15.12 %</td>
<td>50 %</td>
</tr>
<tr>
<td>Amphenicol</td>
<td>Chloramphenicol</td>
<td>11.63 %</td>
<td>8.06 %</td>
</tr>
</tbody>
</table>
**FIGURE 4.3:** Antimicrobial resistance patterns of *staphylococcus* spp. with different antibiotics in animal and human isolates.

**TABLE 4.7:** Minimum inhibitory concentrations of selected antibiotics for Methicillin-resistant *Staphylococcus* spp. from animal isolates

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Antibiotics(μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cefoxitin</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>54A</td>
<td>8</td>
</tr>
<tr>
<td>75</td>
<td>0</td>
</tr>
<tr>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>85</td>
<td>0.75</td>
</tr>
<tr>
<td>86</td>
<td>10</td>
</tr>
<tr>
<td>P3</td>
<td>8</td>
</tr>
<tr>
<td>P21</td>
<td>1</td>
</tr>
<tr>
<td>P27</td>
<td>10</td>
</tr>
</tbody>
</table>
Results and Discussion...

**TABLE 4.8:** Minimum inhibitory concentrations of selected antibiotics for Methicillin-resistant *Staphylococcus* spp. from human isolates.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Cefoxitin (μg/ml)</th>
<th>Vancomycin (μg/ml)</th>
<th>Oxacillin (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9</td>
<td>3</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>H14</td>
<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>H17</td>
<td>32</td>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>H19</td>
<td>0</td>
<td>8</td>
<td>24</td>
</tr>
<tr>
<td>H24</td>
<td>16</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>H32</td>
<td>3</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>H42A</td>
<td>8</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>H45</td>
<td>12</td>
<td>4</td>
<td>1.5</td>
</tr>
<tr>
<td>H49</td>
<td>3</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>H52</td>
<td>4</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>H55A</td>
<td>48</td>
<td>3</td>
<td>64</td>
</tr>
<tr>
<td>H57</td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>H59</td>
<td>2</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>H63</td>
<td>8</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>H68</td>
<td>32</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>H70</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>H71A</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>H78A</td>
<td>8</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>H79</td>
<td>8</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>H80</td>
<td>3</td>
<td>4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**TABLE 4.9:** Correlation of antibiotic resistant V/S presence of *mecA* gene.

<table>
<thead>
<tr>
<th>PCR <em>mecA</em></th>
<th>No. of isolates</th>
<th>Oxacillin</th>
<th>Cefoxitin</th>
<th>Vancomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R%</td>
<td>S%</td>
<td>R%</td>
</tr>
<tr>
<td>Positive</td>
<td>29</td>
<td>26 (89.67)</td>
<td>3 (10.33)</td>
<td>18 (62.06)</td>
</tr>
<tr>
<td>Negative</td>
<td>119</td>
<td>Not tested for this antibiotics by MIC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R - Resistance; S- Sensitive
**TABLE: 4.10:** Multiple resistance indices of Methicillin-resistant staphylococci from human nasal swabs.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Multiple Resistance Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>H9</td>
<td>0.7</td>
</tr>
<tr>
<td>H14</td>
<td>0.6</td>
</tr>
<tr>
<td>H17</td>
<td>0.6</td>
</tr>
<tr>
<td>H19</td>
<td>0.65</td>
</tr>
<tr>
<td>H24</td>
<td><strong>0.8</strong></td>
</tr>
<tr>
<td>H32</td>
<td>0.45</td>
</tr>
<tr>
<td>H42A</td>
<td>0.5</td>
</tr>
<tr>
<td>H45</td>
<td>0.6</td>
</tr>
<tr>
<td>H49</td>
<td>0.6</td>
</tr>
<tr>
<td>H52</td>
<td>0.5</td>
</tr>
<tr>
<td>H55A</td>
<td>0.6</td>
</tr>
<tr>
<td>H57</td>
<td>0.65</td>
</tr>
<tr>
<td>H59</td>
<td>0.65</td>
</tr>
<tr>
<td>H63</td>
<td>0.5</td>
</tr>
<tr>
<td>H68</td>
<td>0.35</td>
</tr>
<tr>
<td>H70</td>
<td>0.55</td>
</tr>
<tr>
<td>H71A</td>
<td>0.4</td>
</tr>
<tr>
<td>H78A</td>
<td>0.5</td>
</tr>
<tr>
<td>H79</td>
<td>0.45</td>
</tr>
<tr>
<td>H80</td>
<td>0.4</td>
</tr>
</tbody>
</table>
**TABLE 4.11:** Multiple resistance indices of Methicillin-resistant staphylococci from animal isolates.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Multiple Resistance Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.7</td>
</tr>
<tr>
<td>54A</td>
<td>0.7</td>
</tr>
<tr>
<td>75</td>
<td>0.8</td>
</tr>
<tr>
<td>84</td>
<td><strong>0.85</strong></td>
</tr>
<tr>
<td>85</td>
<td>0.6</td>
</tr>
<tr>
<td>86</td>
<td>0.6</td>
</tr>
<tr>
<td>P3</td>
<td>0.6</td>
</tr>
<tr>
<td>P21</td>
<td>0.6</td>
</tr>
<tr>
<td>P27</td>
<td><strong>0.8</strong></td>
</tr>
</tbody>
</table>
PLATE 4.1: Colonies of *Staphylococcus* spp. on Nutrient agar (NA)

A. Golden yellow colour colonies on NA  
B: White colonies on NA

PLATE 4.2: Gram positive cocci in bunch shown distinguishing gram staining pattern of *Staphylococcus aureus*
PLATE 4.3: Mannitol fermentation of *Staphylococcus* spp. on mannitol salt agar (24 hours at 37 °C)

PLATE 4.4: Methicillin-resistant *Staphylococcus* spp. colonies on selective chromogenic media (MeReSa media) after incubation for 48-72 hrs at 37 °C
Results and Discussion

1. Tube coagulation test  
2. Slide coagulation test

**PLATE 4.5:** Tube coagulate test (1) and Slide coagulation test (2) using rabbit plasma

**PLATE 4.6:** Antibiotic sensitivity test (disc diffusion method) with different antibiotics
PLATE 4.7: Determination of minimum inhibitory concentration using E-test.

PLATE 4.8: Hemolysis patterns of *staphylococcus* spp. on sheep blood agar (48 hours at 37 °C).
4.3 M-PCR FOR THE DETECTION OF MRSA FROM ANIMAL AND HUMAN ISOLATES.

Rapid nucleic acid amplification and detection technologies are quickly replacing the traditional assays which are based on pathogen phenotype rather than genotype. The polymerase chain reaction (PCR) has increasingly been described as the latest gold standard for detecting some microbes, but such claims can only be taken seriously when each newly described assay is suitably compared to its characterized precursors. PCR is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods. Polymerase chain reaction protocols have been developed for identification of various pathogenic microorganism.

Keeping above points in observance, in present study the m-PCR was used successfully as described earlier in this laboratory (Nimavat, 2015; Hetal, 2016) to identify the *Staphylococcus* genus (*16S rRNA*), *S. aureus* species specific (*Nuc*), methicillin resistant (*mecA*) and coagulase (*Coa*) genes simultaneously from the animal and human nasal swab.

The multiplex PCR gave amplification of all four genes successfully by adding additional 0.5 U/Reaction *Taq* polymerase and 52°C annealing temperature in standard reaction mixture. Components and conditions for this m-PCR is described in Table 4.14. In the present study, performed combined molecular test for the rapid identification and discrimination of the *Staphylococcus* genus from others, with simultaneous discrimination of Methicillin-resistant from susceptible staphylococcal strains, *S. aureus* from CoNS and concomitant detection of coagulase genes. The PCR assay performed using DNA extracted from a pure culture of bacterium

The DNA of all the isolates were extracted with conventional DNA isolation method using phenol-chloroform-isoamyl alcohol mixture (Sambrook and Russell, 2001). The analytical specificity of the m-PCR assay for all genes were determined using ATCC 43300 *Staphylococcus* spp. reference strain with one well characterized staphylococci as positive control and one non-staphylococcal Gram-positive and one Gram-negative strains obtained previously from bovine mastitis as negative control. The reference strain gave appropriate amplicons for the *Staphylococcus* genus (*16Sr RNA*), *S. aureus* species specific (*Nuc*), Methicillin resistance (*mecA*) and coagulase
Results and Discussion…

\( \text{(Coa) genes. S. aureus (ATCC 43300) control strain was positive for all the four gene selected in the study and none of the extracted DNA from the other species strains cross-reacted with all four primer pairs, showing that the selected primer set were 100% specific for identifying Methicillin-resistant S. aureus isolates so overall the analytical specificity of multiplex PCR was 100% for the detection of MRSA reference strain.} \)

A total of 302 samples were collected, comprising 202 samples from animal milk/exudate/abscess/pus and 100 samples from human nasal swab. All the samples were collected from the animal of organized dairy farm and clinical cases from the Veterinary Clinical Complex, College of Veterinary Science and Animal Husbandry, Junagadh Agricultural University, Junagadh. The samples from human were collected from farm laborer/farm workers/personnel working on the farm and remains in close proximity with these animals. Out of 202 animal samples, 86 staphylococci were identified based on amplification of \( 16S \) rRNA gene, of these 74 isolates were identified as Coagulase Negative Staphylococci (CoNS) based on amplification of only \( 16S \) rRNA gene and 12 isolates were identified as Coagulase Positive Staphylococci (CoPS) based on amplification of both \( 16S \) rRNA and \( \text{Coa} \) gene. Based on amplification of three genes \( 16S \) rRNA, \( \text{Coa} \) and \( \text{mecA} \), 8 isolates were identified as Methicillin-resistant Coagulase Negative Staphylococci (MRCoNS) and 1 isolates were Methicillin-Resistant Coagulase Positive Staphylococci (MRCoPS), whereas only one isolate was identified (based on all four gene amplification pattern) as Methicillin-Resistant Coagulase Negative Staphylococcus \( \text{aureus} \) (MRCoNSA) from the animal samples (Table 4.12). Based on amplification of genes \( 16S \) rRNA and \( \text{mecA} \) gene, 9 isolates were identified as positive for Methicillin-Resistant Staphylococci (MRS) (Plate 4.9).

During the study, the 100 samples were collected from the human nasal swabs which had remained in contact with these animals. Of these, 62 isolates were identified as staphylococcus based on amplification of \( 16S \) rRNA gene and 50 isolates were identified as Coagulase Negative Staphylococci (CoNS) based on amplification of only \( 16S \) rRNA gene and 12 isolates were identified as Coagulase Positive Staphylococci (CoPS) based on amplification of both \( 16S \) rRNA and \( \text{Coa} \) gene. Based on amplification of three genes \( 16S \) rRNA, \( \text{Coa} \) and \( \text{mecA} \), 18 isolates were identified as Methicillin-Resistant Coagulase Negative Staphylococci (MRCoNS) and 2 isolates were identified as Methicillin-Resistant Coagulase Positive Staphylococci (MRCoPS), whereas only 2 isolates were identified (based on all four gene amplification pattern) as MRSA. Of this
2 isolates, one isolate was Methicillin-Resistant Coagulase Negative *Staphylococcus aureus* (MRCoNSA) and one isolate was Methicillin-Resistant Coagulase Positive *Staphylococcus aureus* (MRCoPSA) from the human samples (*Table 4.13*). Based on amplification of genes *16S rRNA* and *mecA* gene, 20 isolates were identified MRS positive (*Plate 4.10*).

The presence of MRSA in animal and human in the many countries is of veterinary and public health concern. Nasal carriage has an important role in the epidemiology and pathogenesis of MRSA infection in human and animal (Kluytmans *et al.*, 1997). Several reports shown the prevalence of MRSA in cattle with mastitis (Spoehr *et al.*, 2011; Turkyilmaz *et al.*, 2008; Bhagat *et al.*, 2017). In a study made in farm animals, the nasal MRSA prevalence was found to be 0.3 % and 1 % in 400 cattle and 300 calves, respectively (Huber *et al.*, 2010) which was lower as compared to present findings. In comparison to current finding, higher rates of MRSA was reported by, Erdem and Turkyilmaz (2013) reported 7.14% (4/56) bovine and 17.64% (6/34) human nasal isolates were positive for MRSA. In another study from Turkey, Methicillin resistant was detected in 26.8 % (15/56) and 62.8 % (22/35) of bovine nasal and human nasal isolates, respectively (Inegol and Turkyilmaz, 2012). Garipcin and Seker, (2015) revealed the nasal carriage rates of MRSA in 150 humans and 250 cattle as 8.7 % and 1.2 % respectively.

In agreement with this study, Nimavat, (2015) applied multiplex PCR assay to test a total of 100 clinical milk samples. Of these, 69 staphylococci were identified based on amplification of *16S rRNA* gene. Out of this 69 samples positive for staphylococci, 20 samples were identified as Coagulase Negative Staphylococci (CNS) based on amplification of only *16S rRNA* gene, 29 samples were identified as coagulase positive *Staphylococcus aureus* based on amplification of *16S rRNA*, *Nuc* and *Coa* gene, 14 samples were identified as Methicillin-resistant coagulase negative staphylococci (MRCoNS) based on amplification of *16S rRNA* and *mecA* gene and 6 samples were identified as positive for Methicillin-resistant *Staphylococcus aureus* (MRSA) based on amplification of all four genes *i.e. 16S rRNA, Nuc, Coa and MecA*. Similarly, Hetal, (2016) carried out multiplex PCR for the identification of *Staphylococcus* spp. using combination of various primers. Out of 50 milk and 50 human nasal swabs tested, 47 and 44 isolates were identified as *Staphylococcus* spp. based on amplification of *16S rRNA* gene, 7 and 20 isolates were identified as MRS.
based on amplification of 16S rRNA and mecA gene, 11 and 3 isolates were identified as coagulase positive *S. aureus* based on amplification of 16S rRNA, Nuc and Coa gene from bovine and human, respectively. Of these 7 and 20 MRS isolates from bovine and human nasal swabs, 3 and 7 isolates were Methicillin-Resistant Coagulase Positive Staphylococci (MRCoPS) and 4 and 13 isolates were Methicillin-Resistant Coagulase Negative Staphylococci (MRCoNS), respectively. Whereas, 8 and 5 isolates were identified as Methicillin-Sensitive Coagulase Positive Staphylococci (MSCoPS) from bovine and human, respectively.

Various researchers have worked on molecular identification of coagulase and Methicillin resistant genes among *Staphylococcus* spp. using various gene specific primers and amplified using uniplex or multiplex PCR in different regions and countries (Kearns *et al.* 1999; Perez-Roth *et al.* 2001; Maes *et al.* 2002; Mohanasoundaram and Lalitha 2008; Vanderhaeghen *et al.* 2010a; Duran *et al.* 2012).

Similar to present study, other scientist have carried out same studies to identify the toxic and mecA gene. Vannuffel *et al.* (1995) reported only one coagulase-negative staphylococcus isolate carried the mecA gene and highly susceptible to oxacillin was identified. Schmitz *et al.* (1997) identified 686 staphylococci by multiplex PCR. Of these, the coa gene was detected only in *S. aureus* (488) strains with a specificity of 100% and was not detected in any of the coagulase-negative staphylococci (198). The mecA gene was detected in 98% of Methicillin-resistant staphylococci (393) and 2% of all Methicillin-susceptible staphylococci (293).

The possibilities of occurrence of higher or lower antibiotics resistant patterns in present study in compare to previous study, could be because of usage of different patterns/choice of antibiotics in different geographical region in animal and human beings.
TABLE 4.12: Culture and PCR based identification of staphylococci from different sources of animals

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>ID No.</th>
<th>Species</th>
<th>Type of sample</th>
<th>Growth on MSA</th>
<th>Growth on MeReSa</th>
<th>mPCR-Animal samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16S rRNA</td>
</tr>
<tr>
<td>1</td>
<td>ATCC 43300</td>
<td>S. aureus</td>
<td>Reference strain</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
</tr>
<tr>
<td>2</td>
<td>MTCC 522</td>
<td>E. coli</td>
<td>Reference strain</td>
<td>-</td>
<td>NG</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>9</td>
<td>21</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>10</td>
<td>22</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>11</td>
<td>23</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>12</td>
<td>24</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>14</td>
<td>27</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>15</td>
<td>29</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>16</td>
<td>33</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>17</td>
<td>35</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>18</td>
<td>40</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>19</td>
<td>48</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>20</td>
<td>49</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>21</td>
<td>50</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>22</td>
<td>53</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>23</td>
<td>54</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>24</td>
<td>61</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>25</td>
<td>65</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>26</td>
<td>75</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>27</td>
<td>77</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>28</td>
<td>83</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>29</td>
<td>84</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>30</td>
<td>85</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>31</td>
<td>86</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>32</td>
<td>87</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>33</td>
<td>19-A</td>
<td>Cow</td>
<td>Milk</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
</tr>
<tr>
<td>34</td>
<td>20-A</td>
<td>Cow</td>
<td>Milk</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
</tbody>
</table>
### Results and Discussion

<table>
<thead>
<tr>
<th>No.</th>
<th>ID</th>
<th>Animal</th>
<th>Milk Color</th>
<th>Condition</th>
<th>Growth</th>
<th>Abscess/Pus</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>116</td>
<td>33</td>
<td>Cow</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>117</td>
<td>34</td>
<td>Cow</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>118</td>
<td>35</td>
<td>Cow</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>36</td>
<td>Cow</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>37</td>
<td>Cow</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>38</td>
<td>Cow</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>39</td>
<td>Cow</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>123</td>
<td>40</td>
<td>Cow</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>124</td>
<td>41</td>
<td>Cow</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>42</td>
<td>Cow</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>43</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>127</td>
<td>44</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>128</td>
<td>45</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>129</td>
<td>46</td>
<td>Buffalo</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>130</td>
<td>47</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>131</td>
<td>48</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>49</td>
<td>Buffalo</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>133</td>
<td>50</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>134</td>
<td>51</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>135</td>
<td>52</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>136</td>
<td>53</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>137</td>
<td>54</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>138</td>
<td>55</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>139</td>
<td>56</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>140</td>
<td>57</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>141</td>
<td>58</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>142</td>
<td>59</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>143</td>
<td>60</td>
<td>Buffalo</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>61</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>145</td>
<td>62</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>146</td>
<td>63</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>147</td>
<td>64</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>148</td>
<td>65</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149</td>
<td>66</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>67</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>151</td>
<td>68</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>152</td>
<td>69</td>
<td>Buffalo</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>70</td>
<td>Buffalo</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>154</td>
<td>71</td>
<td>Buffalo</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>155</td>
<td>72</td>
<td>P1</td>
<td>Cattle</td>
<td>Abscess/Pus</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
</tr>
<tr>
<td>156</td>
<td>73</td>
<td>P2</td>
<td>Cattle</td>
<td>Abscess/Pus</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
</tr>
</tbody>
</table>
Results and Discussion

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Sample ID</th>
<th>Species</th>
<th>Type of sample</th>
<th>Growth on MSA</th>
<th>Growth on MeReSa</th>
<th>mPCR-Human samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>P3</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>P4</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P7</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>P11</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>P12</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P13</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P17</td>
<td>Cattle</td>
<td>Abscess /pus</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P21</td>
<td>buffalo</td>
<td>Abscess /pus</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>P22</td>
<td>buffalo</td>
<td>Abscess /pus</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P24</td>
<td>buffalo</td>
<td>Abscess /pus</td>
<td>yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>P27</td>
<td>buffalo</td>
<td>Abscess /pus</td>
<td>yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>P30</td>
<td>buffalo</td>
<td>Abscess /pus</td>
<td>yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
</tr>
</tbody>
</table>

- Amplified desired products

**TABLE 4.13:** Cultural and PCR based identification of staphylococci from human nasal swabs.
<p>| | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>H19</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>H20A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>12</td>
<td>H21A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>H22</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>H24</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>15</td>
<td>H25</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>H28</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>H29</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>H29A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>H30A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>H32</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>H33</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>H35</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>H36</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>H38</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>H41</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>H42</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>27</td>
<td>H42A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>H43</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>H43A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>H45</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>H46</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>H47</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>H49</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>H52</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>H55A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>H57</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>H58</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Yellow</td>
<td>NG</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>H58A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>H59</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>H60</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>H62</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>H62A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>H63</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td>H64</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>yellow</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>H64A</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>H65</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>H67</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>H68</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>49</td>
<td>H69</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>NG</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>H70</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>Pink</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>51</td>
<td>H71</td>
<td>Human</td>
<td>Nasal Swab</td>
<td>yellow</td>
<td>Growth</td>
<td>√</td>
<td>√</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 4.14: Composition of reaction mixture for multiplex PCR with simple Master Mix

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Components</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2 X PCR Master Mix*</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>(0.05 U/μL Taq DNA polymerase)</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>16S rRNA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer (10pmole/μl)</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer (10pmole/μl)</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Nuc</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer (10pmole/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer (10pmole/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Coa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer (10pmole/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer (10pmole/μl)</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>MecA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward Primer (10pmole/μl)</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer (10pmole/μl)</td>
<td>1.25</td>
</tr>
<tr>
<td>3.</td>
<td>Taq Polymerase(5U/ μl)*</td>
<td>0.1</td>
</tr>
<tr>
<td>4.</td>
<td>DNA Template</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td><strong>Grand Total</strong></td>
<td><strong>25.0</strong></td>
</tr>
</tbody>
</table>

* Thermo scientific

- Amplified desired products
**PLATE 4.9:** PCR amplicons of Methicillin-resistant coagulase negative/positive *Staphylococcus aureus* by mPCR *(16S rRNA gene = 370bp, MecA = 454bp, Coa = 280bp and Nuc = 117bp)* from animal isolates.

20, 75, 86, P3, 54A = Clinical samples from animals

L1 = 50bp ladder

L2 = 100 plus bp ladder

P = ATCC 43300 reference strain (*S. aureus*)

Neg. = Negative sample

C = Negative control (*E. coli*)
PLATE 4.10: PCR amplicon of Methicillin-resistant coagulase negative/positive *Staphylococcus aureus* by mPCR (16S rRNA gene = 370bp, *MecA* = 454bp, *Coa* = 280bp and *Nuc* = 117bp) from human isolates.


L1 = 50bp ladder

L2 = 100 plus bp ladder

P = Positive control (ATCC 43300 reference *S. aureus* strain)

N. = Negative sample

C = Negative control (*E. coli*)

4.4 MOLECULAR DETECTION OF VIRULENCE GENES (TOXIC GENES) FROM MRS ISOLATES.

*Staphylococcus aureus* is a versatile pathogen capable of causing a wide range of human diseases due to its extensive armamentarium of virulence factors located on the plasmid or bacterial genome. There are about 40 virulence genes/factors that are known to be involved in almost all processes from colonization of the host to nutrition and dissemination. The most remarkable feature of *S. aureus* is that the virulence genes
are expressed at various periods during colonization and infection. MRSA virulence factors are generally not unique to MRSA and MRSA is not necessarily more virulent than MSSA. Nevertheless, some MRSA strains contain factors or genetic backgrounds that may enhance their virulence or may enable them to cause particular clinical syndromes. So to decipher the toxic gene in Staphylococcus spp. isolates circulating in the region specific PCR assay were carried out to asses all possible toxic gene among MRS isolates from animal and human beings.

During the current study, total 235 Staphylococcus spp. were isolated and studied. Of these, 148 staphylococcal spp. (86 from animal and 62 from human nasal swab) were from the present study and 87 Staphylococcus spp. (47 from animal and 40 from human nasal swab) from department were included for the study of virulence gene and SCCmec typing (Table 4.16).

During the current study, a total 133 staphylococcal isolates from animal and 102 from human nasal swabs were identified. Of these, 16 (12.03%) and 40 (39.21%) isolates were having mecA gene which were classified as Methicillin-resistant staphylococci from animal and human isolates, respectively. Out of this total animal and human staphylococcal isolates, 3 (2.26%) different isolates were found positive for all these virulence genes i.e. PVL, hla and icaA from animal isolates, whereas 7 (6.86%), 6 (5.88%) and 4 (3.92%) isolates were found positive for PVL, hla and icaA gene from human isolates, respectively (Table 4.16).

During the study, Overall percentage of virulence genes among animal and human were studied. There was varying distribution of the virulence genes among the Staphylococcus spp. isolates were investigated, the mecA gene accounting for the highest proportional distribution (19.59%), followed by PVL gene (4.26%), hla gene (3.83%) while icaA gene was detected only in 1.70% of isolates (Table 4.16).

The present results were in concurrence with those reported by Tel et al. (2012) who analyzed 110 S. aureus strains isolated from cases of sheep clinical mastitis and reported positive for the icaA and icaD genes.

In contrary to present study, higher prevalence rate was reported in previous studies. Martineau et al. (2000) reported 30.6% of S. aureus and 79.3% of S. epidermidis strains harbored the mecA gene. Hae-Kyung et al. (2008) showed a higher percentage of mecA, with a 59.3% rate in S. aureus and an 81.0% rate in S. epidermidis.
Whereas mecA gene was not detected in any of the *S. aureus* strains reported by Martins, *et al.*, (2015). Vyletelova *et al.* (2011) also did not find the mecA gene in *S. aureus* strains isolated from sheep milk, whereas the gene was detected in 20 (6.1%) *S. aureus* strains isolated from cow milk.

In present finding, the occurrence of PVL gene among MRS isolates was 4.26%. In comparison to current finding, higher prevalence rate of PVL gene was reported in human having various ailments by Gerard *et al.* (1999), they reported 93% of strains associated with furunculosis, 85% of those associated with severe necrotic hemorrhagic pneumonia (all community-acquired), 55% of strain associated with cellulitis, 50% of cutaneous abscess strains, 23% of osteomyelitis strains and 13% of finger-pulp–infection were positive for PVL gene. Yu *et al.* (2008) recovered 12.8% (25) of PVL positive isolates among 95 *Staphylococcus aureus* isolates in a teaching hospital. Similar percentage of isolation rate was reported by Hae-Kyung *et al.* (2008), they revealed 59.3% mecA (16/26), 63.0% PVL (17/26) and 76.9% icaA (20/26) positive from *S. aureus*. Whereas *S. epidermidis* virulence genes were detected in 22 cases as 81.0% mecA (17/22), 71.4% PVL (15/22) and icaA 50.1% (13/22) gene positive, which is much higher than the current study. De Almeida *et al.* (2013) reported high frequency of hla (77.3%) and lukE-D (82.8%) genes from clinical mastitis in sheep.

During the current finding, icaA gene was detected in 1.70% of MRS isolates. In contrary to the present study Martins *et al.* (2015) reported none of the isolates were ica (biofilm producer) and mecA gene positive, indicating that biofilm formation was not a virulence factor causing infection by these strains in same study. Similarly, Fateh Rahimi and Sharmin Karimi (2016) reported that, all the strains were negative for the presence of enterotoxin genes including PVL gene. Aguilar *et al.* (2001), reported that *S. aureus* strains isolated from mastitis cases are surrounded by a biofilm layer that facilitates adhesion and colonization of the mammary gland epithelium. However, the 20 isolates from sheep did not expressed the genes studied (*icaADBC* and *bbp*). De Almeida *et al.* (2013) reported none of the following genes (*bbp, ebpS, cna, fnbB, icaA, icaD, bap, hlg and lukM-lukF-PV*) were identified during the study.

In current study, the overall percentage of the isolates for all these 3 genes from MRS were studied and were found to be 18.75% (3/16) isolates from each gene were found positive for one of this virulence gene *i.e.* PVL, hla and icaA from animals and
17.50% (7/40), 15% (6/40) and 10% (4/40) isolates were found positive for $PVL$, $hla$ and $icaA$ gene from human, respectively (Table 4.16, Plate 4.11).

Similar results were obtained by Haran et al. (2012), they reported one (12.5%) MRSA isolate were positive for $PVL$ toxin gene.

The higher rates of toxic genes were prevailed in the study conducted by Bidya et al. (2014) as compared to current study. They obtained an overall $PVL$-positivity rate of 35.6% among the hospital isolates. Out of 46 MRSA isolates, 12 (26.1%) isolates were positive for $PVL$ gene, whereas 14 (51.9%) isolates from 27 MSSA found to be positive for $PVL$ gene. Alli et al. (2015) studied prevalence of toxic genes, of these $hla$ (55.1%), $icaA$ (42.3%), $PVL$ (34.6%), $fnbA$ (8.3%), $bbp$ (4.5%) and $eta$ (3.8%) were identified, whereas none of the isolates were $etb$ and $can$ positive from 156 $S. aureus$ isolates. They also stated that, prevalence of the $PVL$ gene in Methicillin-susceptible $Staphylococcus aureus$ (MSSA) was 53.3% compared with 9.1% in MRSA and concluded that an association between virulence genes ($eta$ and $icaA$) and $mecA$ positive $S. aureus$ and significant difference in the distribution of virulence genes in in-patients and out-patients were found.

During the present finding, the overall percentage of Methicillin-resistant staphylococcus were found lower in animal (12.03%) isolates as compared to human (39.21%) isolates. Similarly, the percentage of virulence gene from staphylococcal isolates from animal were low as compared to human isolates, but the percentage of virulence gene among Methicillin-resistant staphylococcus were found nearly similar in animal and human during the study.

The current study revealed, multiple virulence gene acquisition among MRS isolates. Out of total MRS isolates from animal and human, 5 isolates from animal and 15 isolates from human found positive for one of this three virulence gene. Of this, one isolate to $PVL$ and $hla$, one isolate to $PVL$ and $icaA$ and one isolate was positive for all this three virulence gene ($PVL$, $hla$ and $icaA$) from animal, whereas from human, one isolate to $PVL$ and $icaA$, one isolate to $PVL$, $hla$ and $icaA$, two isolate to $PVL$ and $hla$ and two isolate to $hla$ and $icaA$ found positive from human isolates (Table 4.15).

Similar reports were published by Martine et al., (2015) they detected the complete $icaADBC$ operon in one of the isolates. The $icaA + icaD + icaB$ were concomitantly present in five isolates, $icaA + icaD$ in six isolates, three isolates only
carried the *icaD* gene and one isolate carried the *icaB* gene. Simultaneously they have studied other virulence genes and they found Seven (35%) of the *S. aureus* isolates carried one or more exotoxin genes. One isolate carrying the sec+tst genes expressed the two genes concomitantly, one isolate carrying the sea+seb+luk-PV genes expressed only the *luk-PV* gene and the third isolate positive for the sea+sec genes expressed only the sec gene. In same study, they have detected exotoxin Panton-Valentine leukocidin (*PVL*) in only one strain and its expression was confirmed. *PVL* is one of the most important virulence factors produced by *S. aureus*, contributing to the pathogenicity of this microorganism. This toxic genes were also is associated with different diseases in humans, such as pneumonia and necrotizing dermatitis (Giudice et al., 2009). The presence of *luk-PV* gene in *S. aureus* strains from mastitis was reported by various scientist (Zecconi et al., 2006; Aires-de-Souza et al., 2007; Unal et al., 2012).

During the study, the overall 15% (6/40) of isolates were found *hla* toxic gene among MRS positive isolates. Alpha toxin (*hla*) is known to play a role in the pathogenesis of staphylococcal disease, as *S. aureus* mutants lacking *hla* display reduced virulence in invasive disease models (Patel et al., 1987). In comparison to present study, Alli et al., 2015 revealed 86 (55.1%) isolates were *hla* gene positive which was higher to present finding. Furthermore, it was detected in MRSA (33.3%) and MSSA (24.4%) strains indicating that the gene is relatively conserved. Overall, the high prevalence of this gene suggestive of playing a significant role in the pathogenesis of *S. aureus* infection. The *hla* gene was found mainly in wound and blood samples suggesting that the *hla* gene is important for the survival of *S. aureus* at these sites. Staphylococcal pathogenicity is not due to a single virulence factor as infections occur in a stepwise manner, each step involving one or several virulence factors (Foster, 2005).

**TABLE 4.15: Multiple virulence genes (Toxin genes) in animal and human MRS isolates.**

<table>
<thead>
<tr>
<th>Virulence genes</th>
<th>No. of isolates resistance (Animal)</th>
<th>No. of isolates resistance (Human)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVL, hla, icaA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PVL, icaA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PVL, hla</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>hla and icaA</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>
**TABLE 4.16**: Prevalence of SCC<em>mec</em> types among Methicillin-resistant staphylococci and associated virulence genes among animals and human isolates.

<table>
<thead>
<tr>
<th>SCC&lt;em&gt;mec&lt;/em&gt; types</th>
<th>Methicillin-resistant isolates from Animals</th>
<th>Methicillin-resistant isolates from Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC&lt;em&gt;mec&lt;/em&gt; type</td>
<td>Positive &lt;em&gt;SCCmec&lt;/em&gt; type No. (%)</td>
<td>Virulence gene positive</td>
</tr>
<tr>
<td></td>
<td>PVL No. (%)</td>
<td>&lt;em&gt;hla&lt;/em&gt; No. (%)</td>
</tr>
<tr>
<td>I</td>
<td>2 (12.5)</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>1 (6.25)</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>5 (31.25)</td>
<td>1 (33.33)</td>
</tr>
<tr>
<td>V</td>
<td>6 (37.5)</td>
<td>2 (66.67)</td>
</tr>
<tr>
<td>UT</td>
<td>2 (12.5)</td>
<td>-</td>
</tr>
<tr>
<td>Total (Out of 133 staphylococcus)</td>
<td>16 (12.03)</td>
<td>03 (2.26)</td>
</tr>
<tr>
<td>I</td>
<td>7 (17.50)</td>
<td>2 (28.57)</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>3 (7.50)</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>9 (22.50)</td>
<td>3 (42.84)</td>
</tr>
<tr>
<td>V</td>
<td>9 (22.50)</td>
<td>2 (28.57)</td>
</tr>
<tr>
<td>UT</td>
<td>12 (30.00)</td>
<td>-</td>
</tr>
<tr>
<td>Total (Out of 102 Staphylococcus)</td>
<td>40 (39.21)</td>
<td>07 (6.86)</td>
</tr>
</tbody>
</table>

**Plate 4.11 A**
PLATE 4.11: PCR amplicons of ($icaA$, $hla$ and $PVL$) of virulence genes among Methicillin-resistant staphylococci from animal and humans.

Plate 4.11 A. $hla$ gene = 174 bp

Plate 4.11 B. $icaA$ gene = 769 bp

Plate 4.11 C. $PVL$ gene = 433 bp

54A, P21, H9, H24, 75, H68, 84, H55,

L = 100 plus bp ladder

P = Positive control (ATCC 43300 reference $S. aureus$ strain)

N. = Negative sample

C = Negative control ($E. coli$)
4.5 MOLECULAR CHARACTERIZATION OF MAJOR SCCMEC TYPES AMONG METHICILLIN-RESISTANT STAPHYLOCOCCI FROM ANIMAL AND HUMANS.

The evolution of MRS from animals and human are long been studied in detail. Myriad reports suggest circulation of these isolates in their epidemiological and geographic region and are also being carried out on region basis across the globe. The MRSA has been known to be one of the most common nosocomial pathogens causing a wide range of hospital-linked infections throughout the world since the 1980s. Since the mid-1990s MRSA has spread outside the hospital environment and appeared in community populations without identifiable healthcare-associated risk factors. Besides its importance as a hospital and community pathogen, MRSA has also been considered as an emerging problem in veterinary medicine in recent years. The primary colonization site of MRSA is known as the nasal mucosa in humans and animals. Therefore, the nasal MRSA carriage has an important role in the epidemiology and pathogenesis of infections. Various researchers have reported that it colonizes the nasal mucosa of different animal species, their owners, caretakers or veterinarians and emphasized the importance of nasal MRSA carriage in terms of public and animal health. These epidemiological studies indicate that MRSA colonizing the nasal mucosa may be transmitted between animals and humans. Accordingly, this agent has been accepted as a zoonosis and/or humanosis pathogen nowadays.

The Panton-Valentine leukocidin (PVL) known as lukFPV/lukS-PV gene (a virulence gene) has been associated with community-acquired MRSA (CA-MRSA) in many parts of the world, but number of investigations have provided evidence that the prevalence of the PVL gene is high among Methicillin-susceptible S. aureus (MSSA) than MRSA. Therefore presence of PVL gene among MRS was no valid tools to consider as CA-MRSA. However, there is paucity of data on the epidemiology of CA-MRSA in animal and there correlation with human isolates are available in India and in this region, so to decipher the total epidemiological outset of MRS isolates in the present study, SCCmec typing was carried out for all the MRS bacterial isolates recovered from animal and human beings. Moreover, the distribution of various virulence genes among S. aureus isolates had been investigated.
In present study, occurrence of staphylococcal cassette chromosome mec types (SCCmec type) were investigated from Methicillin-resistant staphylococcal isolates obtained from animal and human samples. Out of total 133 and 102 Staphylococcus spp. isolates, 16 and 40 isolates from animal and human were identified as Methicillin-resistant staphylococci, respectively. Of these 16 animal MRS isolates, 14 isolates having one of the SCCmec types (SCCmec type I, 2; SCCmec type II, 0; SCCmec type III, 1; SCCmec type IV, 5; SCCmec type V, 6), whereas 2 isolates were untypable, whereas from 40 human MRS isolates, 28 isolates having one of the SCCmec types (SCCmec type I, 7; SCCmec type II, 0; SCCmec type III, 3; SCCmec type IV, 9; SCCmec type V, 9), whereas 12 isolates were Untypable (Table 4.17).

**TABLE 4.17:** Occurrence and correlation between SCCmec type isolates based on HA-MRS and CA-MRS among animal and human MRS isolates.

<table>
<thead>
<tr>
<th>SCCmec types of MRS</th>
<th>MRS isolates from Animal</th>
<th>MRS isolates from Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive for SCCmec type No. (%)</td>
<td>MRS type</td>
</tr>
<tr>
<td>SCCmec type I</td>
<td>2 (12.5)</td>
<td>HA-MRS</td>
</tr>
<tr>
<td>SCCmec type II</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>SCCmec type III</td>
<td>1 (6.25)</td>
<td>HA-MRS</td>
</tr>
<tr>
<td>SCCmec type IV</td>
<td>5 (31.25)</td>
<td>CA-MRS</td>
</tr>
<tr>
<td>SCCmec type V</td>
<td>6 (37.5)</td>
<td>CA-MRS</td>
</tr>
<tr>
<td>UT</td>
<td>2 (12.5)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>40</td>
</tr>
</tbody>
</table>

During the current study, 18.75% (3/16) isolates were classified as hospital associated methicillin-resistant staphylococci (HA-MRS) (SCCmec type I, 2; SCCmec type III, 1), whereas 68.75% (11/16) isolates were classified as community associated methicillin resistant staphylococci (CA-MRS) (SCCmec type IV, 5; SCCmec type V, 6) and 12.5% (2/16) isolates remained untypable from animal MRS isolates. Similarly, study revealed 25% (10/40) isolates were classified as hospital associated methicillin...
Results and Discussion

Resistant staphylococci (HA-MRS) (SCC\textit{mec} type I, 7; SCC\textit{mec} type III, 3), whereas 45% (18/40) isolates were classified as community associated methicillin resistant staphylococci (CA-MRS) (SCC\textit{mec} type IV, 9; SCC\textit{mec} type V, 9) isolates and 30% (12/40) isolates remained untypable from human MRS isolates. The overall percentage of CA-MRS (63.04%) was higher as compare to HA-MRS (28.26%) among both the species (Table 4.17, Plate 4.12 and Plate 4.13).

As compare to present finding, little higher percentage were reported by Inegol and Turkyilmaz (2012), they revealed 67.80% (40/59) isolates including type II and III were hospital acquired (HA-MRS) whereas 32.20% (19/59) isolates including type IV and V were community acquired (CA-MRS), which was lower as compared to present finding, but they had reported similarity of SCC\textit{mec} types between bovine and human isolates, suggestive of evidence of transmission from animals to humans, or vice versa. Bhutia et al. (2015) reported 25.33% (38/150) isolates met the definition of CA-MRSA and 10% (15/150) of HA-MRSA, which was lower as compare to present study. Similarly lower rate of recovery were also reported by Govindan et al. (2015), they revealed only 1.1% (17/1503) CA-MRSA colonization in children. Of these 17 CA-MRSA isolates, 7 isolates were found to be SCC\textit{mec} type IV and none of them were type V. Huang et al. (2006) reported higher percentage of isolates met the definition of HA-MRSA (55.1%, 156) and 127 isolates (44.9%) were classified as CA-MRSA, which was opposite to current investigation.

During the current finding, out of total 16 clinically significant MRS isolates from animal, SCC\textit{mec} type V revealed (37.50%) to be predominant followed by type IV (31.25%), type I (12.5%), type III (6.25%), 2 (6%) isolates were non typeable and type II was absent, whereas out of total 40 MRS isolates from human, SCC\textit{mec} type IV and V revealed (22.50% each) to be predominant SCC\textit{mec} types followed by type I (17.5%), type III (7.5%), whereas 30% isolates were non typeable and type II was absent (Table 4.17).

In present study, out of total 16 and 40 MRS isolates, 1 (6.25%) isolates from animal and 2 (5%) isolates from human were identified as MRSA. The one MRSA isolates, was SCC\textit{mec} type V from animal, whereas from 2 human MRSA isolates, one isolates of SCC\textit{mec} type I and one was of SCC\textit{mec} type IV from 2 human were identified (Table 4.17).
In agreement with this study, Strommenger et al. (2006) reported 25% (4/16) isolates were SCCmec type IV and rest of the isolates were of ND (Not determine) types. Boye et al., (2007) used multiplex PCR and revealed SCCmec type IV was the most common type (84%), followed by type V (6%), type I (4%) and type II (3%). Nemeghaiare et al. (2014) investigated the prevalence and types of MRSA present in the nose of healthy bovines. Out of total 81 isolates of MRSA, Forty-four (54.3%) isolates carried SCCmec type IV (2B) and nine (11.1%) isolates were of SCCmec type IV (2B&5), sixteen (19.8%) isolates carried SCCmec type V (5C2) and two (2.5%) SCCmec type III (3A).

In contrary to present study, Havaei et al. (2015) reported SCCmec type IV was the most frequent SCCmec types in bovine mastitis cases and all 10 mecA-positive strains harboured SCCmec IV. Similarly, Nam et al. (2005) showed all 14 MRSA isolates, belong to SCCmec type IV. Findings of other researchers were (Witte et al., 2007 and Van Den Eede et al., 2009) also supported the present finding.

In contrary to present finding, Nadig et al. (2006) isolated SCCmec type III cassettes with the exception of two isolates from Nagpur, which had type II cassettes. Pu et al. (2009) typed 22 MRSA positive isolates, of these 3 isolates were SCCmec type IVa and 19 isolates were type II, but similar to present study they reported SCCmec type IV is predominately associated with CA-MRSA. Shabir et al. (2010) typed Methicillin-resistant Staphylococcus aureus (MRSA) isolates and revealed 59.65% (34/57) isolates carries SCCmec type III/IIIa and 40.35% (23/57) carries SCCmec type IV. Among these SCCmec type, only SCCmec type III and subtype IIIa were identified in the isolates from India. Romeeza et al. (2010) reported 60% (21/35) isolates possess SCCmec type IA while 40% (14/35) isolates possess SCCmec type IIC. Alli et al. (2015) studied SCCmec typing of S. aureus isolates obtained from clinical specimen from human. Out of 66 mecA positive isolates, 24 (36.4%) carried the SCCmec type II element, 4 (6.1%) with SCCmec type III, 10 (15.2%) with SCCmec IV and 28 (42.4%) harboured SCCmec type V. Mert et al. (2011) isolated 357 staphylococci, including 313 CoNS (261 MRCoNS, 52 Methicillin-susceptible coagulase negative staphylococci (MSCoNS) and 44 were S. aureus (22 MRSA and 22 Methicillin-susceptible S. aureus). Out of these, 22 MRSA and 261 MRCoNS were tested for presence of SCCmec types. All the 22 MRSA isolates carried SCCmec type III cassettes, Whereas 261 MRCoNS isolates having SCCmec types I, II, III, IV, V and some combinations of these types.
Results and Discussion...

were detected in 61 (23.3%), 0 (0%), 31 (11.8%), 65 (24.9%), 37 (14.1%) and 46 (17.6%) isolates, respectively which is not in accordance with the reported percentage of SCC\textit{mec} types of this present study.

Similarly, various studies has been conducted by various scientist for the identification of various SCC\textit{mec} types among \textit{Staphylococcus} spp. of animal and human beings and revealed varying level of occurrence of this types among this species (Moroney \textit{et al.}, 2007; Lulitanond \textit{et al.}, 2010; Jimenez \textit{et al.}, 2011; Asghar, 2014; Havaei \textit{et al.}, 2015; Hu \textit{et al.}, 2015; Fateh Rahimi and Sharmin Karimi 2016; Khaji and Shahreza, 2017).

\textbf{PLATE 4.12:} SCC\textit{mec} type specific PCR amplification products (SCC\textit{mec} type I-V) from animal MRS isolates.

1 = \textit{β} F1/\textit{α} 3 R1 primer targeting ccrA2-B gene complex amplify 937 bp.

2 = ccrCF/ccrCR1 primer targeting \textit{ccrC} gene complex amplify 518 bp.

3 = 1272F1/1272R1 primer targeting IS1272 gene complex amplify 415 bp.

4 = 5RmecAF1/5R431R1 primer targeting \textit{mecA}–IS431 gene complex amplify 359 bp.

L = 50 bp DNA ladder

Sample ID. 20 – SCC\textit{mec} type I

Sample ID. 86, 54A – SCC\textit{mec} type V

Sample ID. P3 – SCC\textit{mec} type III
PLATE 4.13: SCCmec type specific PCR amplification products (SCCmec type I-V) from human MRS isolates.

1 = β F1/α 3 R1 primer targeting ccrA2-B gene complex amplify 937 bp.
2 = ccrCF/ccrCR1 primer targeting ccrC gene complex amplify 518 bp.
3 = 1272F1/1272R1 primer targeting IS1272 gene complex amplify 415 bp.
4 = 5RmecAF1/5R431R1 primer targeting mecA–IS431 gene complex amplify 359bp.
L = 50 bp DNA ladder.

Sample ID. H9 – SCCmec type I  
Sample ID. H79 – Untypable  
Sample ID. H49 – SCCmec type IV

Sample ID. H63 – SCCmec type V  
Sample ID. H68 – SCCmec type III  
Sample ID. C – Control
4.6 CORRELATION STUDY OF SCCmec TYPES WITH VIRULENCE GENES (TOXIC GENES)

Correlation of virulence gene with different SCCmec type was studied in current investigation. Out of total 56 MRS isolates from animal and humans, 10 isolates yielded PVL positive, PVL were predominantly associated with SCCmec type IV and V (CA-MRS, 80%), whereas 2 (20%) isolates were SCCmec type I (HA-MRS). Similarly, 9 isolates yielded hla positive, hla were predominantly associated with SCCmec type IV and V (CA-MRS, 66.67%), whereas 33.33% (HA-MRS) associated with SCCmec type I and SCCmec type III. Seven isolates were positive for icaA gene, which was predominantly associated with SCCmec type I and III (HA-MRS, 57.14%), whereas 42.86% (CA-MRS) isolates associated with SCCmec type IV and V. One isolates from untypable staphylococci were icaA positive.

Overall prevalence of virulence genes is more common in CA-MRS isolates as compare to HA-MRS during the study.

The various reports are conflicting as to whether there is a relation between SCCmec type IV and Panton-Valentine leukocidin (PVL). PVL is a S. aureus-specific exotoxin, which is encoded by two co-transcribed genes, designated lukF-PV and lukS-PV and is associated with skin and soft tissue infections and severe necrotizing pneumonia, similarly other researcher reported that CA-MRSA was characterised by SCCmec type IV and that PVL was a stable genetic marker for CA-MRSA (Vandenesch et al., 2003) as it was found that 80% of PVL toxic gene associated with CA-MRS during current finding. The relationship between CA-MRSA, SCCmec type IV and PVL was confirmed in a study by Shukla et al. (2004) in the USA. However, another study by O’Brien et al. (2004) in Australia did not find a relationship between CA-MRSA, SCCmec type IV and PVL. Further studies showed PVL positive CA-MRSA strains harbouring SCCmec type I and III in the Netherlands (Wannet et al., 2005) and PVL-positive HA-MRSA strains in Algeria (Ramdani-Bouguesa et al., 2006) as it observed in current study also. In general, 40 to 90% of the MRSA strains that harbour SCCmec type IV carry PVL and less than 5% of the MRSA strains harbouring SCCmec type I to III carry PVL (Deresinski, 2005) which supported the present findings. Further studies are needed to investigate the possible relation between SCCmec type IV, V and PVL in CA-MRSA strains.
Similarly, in accordance with present study, Asghar, 2014 reported statistically significant correlation between the presence of the PVL gene and SCCmec type IV. Whereas, higher prevalence of virulence gene were reported in CA-MRS by Vandenesch et al. (2003), they collected 117 different isolates of CA-MRSA (SCCmec type IV) and all were found positive for PVL gene locus. Moroney et al. (2007) revealed 41.67% (25/60) CA-MRSA. Out of these 25 S. aureus, 23 isolates were SCCmec type IV and were PVL positive (92%) and 2 isolates were type IV were PVL negative (8%). Jimenez et al. (2011) identified the genes encoding virulence factors. Among these, SCCmec type IVc isolates frequently carried the PVL encoding genes and harboured virulence determinants resembling susceptible strains while SCCmec type I isolates were often negative. Hu et al. (2015) studied association of SCCmec type with PVL gene and reported highest correlation of SCCmec type (60.90%, 14/23) with PVL gene followed by SCCmec V (50%, 4/8). Bhatta et al. (2016) revealed the majority of the community acquired MRSA (90.4 %) were PVL positive, while PVL was detected only 4 (7.1 %) hospital associated MRSA strains. Liu et al, (2016) reported 21(10.3%) isolates were CA-MRSA isolates. All CA-MRSA isolates belonged to SCCmec IV and V, accounting for 47.6% and 52.4%, respectively. Whereas, CA-MRSA isolates were found to be positive for one or more virulence genes, 28.6% of isolates carried PVL, seb, sek, seq, hla, hlb, hld and hlg-2.

In contrary to present study, Machuca et al. (2013) studied correlation between the molecular types identified and the profile of virulence factors, they reported virulence genes were associated with specific molecular type’s sek and PVL were predominantly associated with SCCmec I and SCCmec IVc. No significant association between a molecular type and the virulence factors was found. Taherirad et al. (2016) found frequencies of Panton-Valentine leucocidin (PVL) and biofilm production gene (icaA) were significantly lower in the type III strains compared to the other SCCmec types. De Almeida et al. (2013) observed 77.3% and 82.8% of hla and lukE-D genes among S. aureus isolates, respectively. The hlb gene, which encodes alpha hemolysin, was identified only in isolates from subclinical mastitis, accounting for 27.5% of total isolates. The presence and possible expression of this gene may explain the relation between S. aureus isolates carrying hlb gene and the occurrence of chronic cases, because this gene can promote the escape of bacteria from the host immune system and
assist in its process of obtaining nutrients (Huseby et al., 2007), helping the survival of the pathogen.

4.7 METHICILLIN-RESISTANT STAPHYLOCOCCI AND ITS ZOONOTIC AND HUMANOSIS IMPORTANCE

During the study, the isolates from animal (Milk, pus/exudate) yielded various SCCmec type (SCCmec type I, III, IV and V), similarly the same SCCmec types were isolated from the human nasal swab of labors/worker/personnel who remain in close contact with this animals. During the current study, same SCCmec types prevail in animal and human beings which attribute to transmission of Methicillin-resistant staphylococci from animal to human or vice versa during the study indicating potential zoonotic pathogen prevalence in farm and their workers during the study.

Simultaneously, we found that the MRSA strains harboured the high and variable prevalence of SCCmec alleles which is an additional pathogenic factor for infections. SCCmec IV and V were the most prevalent alleles in our findings. Community Acquired MRSA (CA-MRSA) is mainly considered by SCCmec IV and V alleles, while Healthcare HA-MRSA are recognized by SCCmec I, II and III alleles (De Lencastre et al., 2007). During the current study, MRS strains were mainly associated with CA-MRSA. In the other hand, detection of SCCmec types IV and V suggested the emergence of CA-MRSA strains in this geographical area and occurrence of SCCmec I and II alleles indicated a possible transmission of MRSA from humans to animals. Further studies are needed to establish clonal relation of MRS from animal and farm workers with advance molecular techniques (Pulsed field gel electrophoresis, Staphylococcal Protein A analysis, multi locus sequence typing). The result of these studies may shed light on the clonility and transmission of resistance strain between human and animal. High prevalence of SCCmec types IV and V were also detected by various authors (Nemeghaire et al., 2014, Haran et al., 2012 and Havaei et al., 2015).

MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans (Spoor et al., 2013). MRSA colonization in cattle may be an occupational risk to the people in close contact with MRSA infected cattle such as veterinarians, farmers, milkers and people working at slaughterhouses (Paterson et al., 2012). Transmission of animal MRSA to veterinary personnel has been found and it is
Results and Discussion

more common for large animal personnel than small animal personnel (Wulf et al., 2008; Hanselman et al., 2006).

Although, MRSA has been reported as transmissible diseases of zoonosis as well as humanosis importance, the direction and routes of transmission are superficially understood. Some authors have reported bidirectional transmission of MRSA (AVMA, 2014). Animal to human transmission occurs through direct contact, environmental contamination and through handling of infected animal product (Nunang and Young, 2007) whereas human to animal transmission is still unclear (Weese, 2010).

On the other hand, the strains originating from livestock are often divergent from human strains and the infection with this type of LA-MRSA could be considered zoonosis and in this case MRSA would be an emergent zoonotic agent (Morgan, 2008). Bovine and human MRSA strains indistinguishable by phenotyping and genotyping methods have been found providing evidence for MRSA transmission between human and cattle (Hata et al., 2010). MRSA infected cattle acts as a reservoir and later transmit the infections to other animals and humans. Using various molecular tools, other authors have studied the similarity between human and bovine isolates. Juhasz-Kaszanyitzky et al. (2007) isolated MRSA from cows with subclinical mastitis and from a person who worked with these animals and reported that bovine and human strains were indistinguishable by phenotyping and genotyping methods. The human MRSA 252 strain uniquely shares multiple DNA sequence blocks with S. aureus of animal reported by Brody et al. (2008). Hata et al. (2010) evaluated their relationship between bovine and human MRSA isolates. Of this, 3 of the bovine isolates showed identical genotypes to the human isolates.
CHAPTER-V
SUMMARY AND CONCLUSIONS

*Staphylococcus aureus* is well-known epidemic nosocomial pathogen causing considerable human morbidity and mortality worldwide, simultaneously it is a leading cause of infections of some economically important livestock species and, as a prominent bacterial cause of contagious bovine mastitis, a major economic burden for the dairy cattle industry. Among the staphylococci group, *Staphylococcus aureus* strains are more dangerous to dairy animals and are of greatest attention for scientific community worldwide as they possess properties like capabilities to produce enzymes, toxins and intrinsic virulence factors which cause invasion & tissue damage to mammary gland, and capability to resist phagocytosis (protein-A). Some strains of the same bacteria having an ability to resist antibiotic therapy due to production of beta lactamases a group of enzymes that inactivate penicillin and closely related antibiotics which designated as Methicillin-resistant staphylococci.

Resistance to methicillin and other β-lactam antibiotics is caused by the *mecA* gene, which is situated on a mobile genetic element, the Staphylococcal Cassette Chromosome *mec* (SCCmec). Initially, MRSA strains were encountered only in the hospitals, but in the late 1990s first virulent community-acquired MRSA (CA-MRSA) clones, characterized by the presence of the toxin Panton-Valentine leukocidin (*PVL*) appeared rapidly and unexpectedly. They quickly spread worldwide, initially only in the community, but later on also in the healthcare facilities, displacing in some countries typical HA-MRSA. For this reason, nowadays, distinction between CA-MRSA and mostly multi resistant HA-MRSA become challenging.

Five main SCCmec types have been identified globally. SCCmec types I, II and III are mainly associated with healthcare associated MRSA (HA-MRSA) strains, whereas SCCmec type IV and V is associated with community associated MRSA (CA-MRSA) strains, as well as with the Paediatric clone MRSA strains.

The literature revealed that there are lots of reports on staphylococcal cassette chromosome *mec* (SCCmec) types in Methicillin-resistant staphylococci of human origin across the globe including India, but in compare to human, few reports on SCCmec typing have been published from animals across the globe and still very few
Summary and Conclusions...

from India. The identification and molecular SCCmec typing and epidemiological studies have been conducted from pig and human, but scanty of references available regarding animal and human SCCmec types, epidemiological studies and their relationship in the world and in India.

The research work gave overall picture of staphylococcus SCCmec types and virulence factors (toxic genes) prevalent in this region in animals and closely associated human beings to this animals, that had facilitate the study of epidemiological correlation between human and animals [(hospital associated methicillin resistant staphylococci (HA-MRS) or community associated methicillin resistant staphylococci (CA-MRS)] which helps to adopt effective control strategy. Along with the study of virulence and toxic genes, there correlation with the SCCmec types had been established during this work. The complete spectrum of antibiotic resistance in staphylococcus has been deciphered insight into pathogenic potentials. The research work had given highlight of overall evolution of staphylococcus as pathogen through the SCCmec typing.

A total of 202 animal samples (167 milk sample and 35 pus/exudate samples from cattle and buffalo) were collected and examined from dairy cattle and buffaloes. Simultaneously, 100 nasal swabs were collected from the closely associated laborer, personnel and farm workers. Simultaneously, the isolates of staphylococci previously been recovered from subclinical and clinical mastitic milk from bovines and human nasal swabs which were maintained at the Department of Veterinary Microbiology, Junagadh Agricultural University, were included in the study for SCCmec typing and to study the prevailed of virulence genes. Out of total 202 and 100 human nasal swabs, 86 (42.57%) and 62 (62%) isolates were identified as Staphylococcus spp. based on biochemical and molecular based identification, respectively.

During the study, a multiplex PCR was carried out for identification of methicillin resistant Staphylococcus aureus by amplifying various genes viz. 16S rRNA, Coa, Nuc and meca from animal and human isolates. Staphylococcal isolates recovered from these milk, pus/exudate from animal and nasal swabs from the humans were characterized for pathogenicity or virulence factors using biochemical and molecular based detection associated with pathogenicity of staphylococci and were also subjected to antibiotic sensitivity test for determination of antibiotic sensitivity pattern.
Based on amplification of all four genes by multiplex PCR, Out of total 86 and 62 staphylococci isolates, 74 and 50 isolates were Coagulase Negative Staphylococci (CoNS), 12 (from each) were Coagulase Positive Staphylococci (CoPS) and 9 and 20 isolates were identified as Methicillin-Resistant Staphylococci (MRS) from animal and human, respectively. Out of total 9 and 20 MRS isolates, 8 and 18 were Methicillin-Resistant Coagulase Negative Staphylococci (MRCoNS), 1 and 2 isolates were Methicillin-Resistant Coagulase Positive Staphylococci (MRCoPS) from animal and humans, respectively. One isolate was identified as Methicillin-Resistant Coagulase Negative Staphylococcus aureus (MRCoNSA) from the animal and 2 isolates were identified as MRSA from humans. Of these 2 isolates, one isolate was Methicillin-Resistant Coagulase Negative Staphylococcus aureus (MRCoNSA) and one isolate was Methicillin-Resistant Coagulase Positive Staphylococcus aureus (MRCoPSA).

During the current study, total 235 Staphylococcus spp. were isolated and studied. Of these, 148 Staphylococcus spp. (86 from animal and 62 from human nasal swab) were from the present study and 87 Staphylococcus spp. (47 from animal and 40 from human nasal swab) from departmental isolates were included for the study of virulence genes and SCCmec typing.

A total 133 staphylococcal isolates were identified from animals and 102 were from human nasal swabs. Of these, 16 (12.03%) from animal and 40 (39.21%) isolates from human were having meca gene which were classified as Methicillin-resistant staphylococci. Among these staphylococcal isolates, 3 (2.26%) different isolates were found positive for all these virulence genes i.e. PVL, hla and icaA from animal isolates, whereas 7 (6.86%), 6 (5.88%) and 4 (3.92%) isolates were found positive for PVL, hla and icaA gene from human isolates, respectively.

The overall percentage of virulence genes among animal and human were studied, which revealed meca gene accounting for the highest proportion (19.59%), followed by PVL gene (4.26%), hla gene (3.83%) while icaA gene was detected only in 1.70% of isolates.

The available data is limited regarding subtyping of MRSA SCCmec elements (I to XI) in animal and human cases under subtropical conditions including India. The SCCmec types I, II, and III are found predominantly in Hospital Acquired Methicillin-resistant Staphylococci (HA-MRS) isolates, whereas SCCmec type IV and V are typically found in Community Acquired Methicillin-resistant Staphylococci (CA-
MRS) strains and lack other multidrug resistance genes. While SCCmec element typing has become essential for the characterization of MRS clones in epidemiological studies.

A total 133 and 102 isolates were identified as *Staphylococcus* spp. using PCR. Of these, 16 and 40 isolates from animal and humans were identified as Methicillin-resistant *Staphylococci*, respectively. Of these 16 animal MRS isolates, 14 isolates having one of the SCCmec types (SCCmec type I, 2; SCCmec type II, 0; SCCmec type III, 1; SCCmec type IV, 5; SCCmec type V, 6), whereas 2 isolates were Untypable, similarly from 40 human MRS isolates, 28 isolates having one of the SCCmec types (SCCmec type I, 7; SCCmec type II, 0; SCCmec type III, 3; SCCmec type IV, 9; SCCmec type V, 9), whereas 12 isolates were Untypable. The 18.75% (3/16) isolates were classified as hospital associated methicillin-resistant staphylococci (HA-MRS) (SCCmec type I, 2; SCCmec type III, 1), whereas 68.75% (11/16) isolates were classified as community associated methicillin resistant staphylococci (CA-MRS) (SCCmec type IV, 5; SCCmec type V, 6) and 12.5% (2/16) isolates remained untypable from animal MRS isolates. Similarly, study revealed 25% (10/40) isolates were classified as hospital associated methicillin resistant staphylococci (HA-MRS) (SCCmec type I, 7; SCCmec type III, 3), whereas 45% (18/40) isolates were classified as community associated methicillin resistant staphylococci (CA-MRS) (SCCmec type IV, 9; SCCmec type V, 9) and 30% (12/40) isolates remained untypable from human MRS isolates. The overall percentage of CA-MRS (63.04%) was higher as compare to HA-MRS (28.26%) among both the species.

Based on the results obtained during present study, following conclusions can be drawn.

1. In the present investigation it was observed that prevalence of *Staphylococcus* spp. was 42.57% from animal and 62% from human samples.
2. The overall percentage of Methicillin-resistance staphylococcus were found lower in animal (12.03%) as compared to human (39.21%) isolates.
3. The higher rates of methicillin, gentamicin, ofloxacin and levofloxacin sensitivity were observed in human as compared to MRS isolates of animal origin, whereas higher susceptibility to amikacin and rifampicin followed by oxytetracyclin and chloramphenicol were observed in MRS isolates of animal origin, indicating heavy use of higher antibiotics in human population compared to animal.
4. The antibiotic resistance pattern showed by animal and human isolates were observed to be different, indicating different usage pattern of antibiotics in human and veterinary practice.

5. The percentage of virulence genes \((PVL, icaA, hla)\) among Methicillin-resistance staphylococcus were found nearly similar in animal and human isolates.

6. The percentage of methicillin resistance genes among 235 staphylococcal isolates from animal and human were 23.83% (56). Among this 56 MRS isolates, 4.68%, 3.83% and 2.99% isolates acquired \(PVL, hla\) and \(icaA\) gene, respectively.

7. The 89.67% correlation of phenotypic oxacillin susceptibility test with \(mecA\) gene amplification among MRS isolates from animal and human were recorded.

8. The \(SCCmec\) type V revealed to be predominant type followed by type IV, type I and type III from animal isolates, whereas \(SCCmec\) type IV and V revealed to be predominant \(SCCmec\) types followed by type I, type III from human isolates.

9. The 2 MRS isolates from animal and 12 MRS from human were Untypable and type II was absent.

10. One MRSA from animal was belong to type \(SCCmec\) V, whereas 2 human MRSA isolates were belongs to I & IV.

11. The \(PVL\) and \(hla\) genes were predominantly associated with CA-MRS as compare to HA-MRS, whereas \(icaA\) gene was predominantly associated with HA-MRS compared to CA-MRS.

12. The overall prevalence of virulence genes was more common in CA-MRS isolates as compared to HA-MRS.

13. The similar \(SCCmec\) types in both the groups (Animal and human) suggests co-circulation of MRS isolates between human and animal population which was indication of possible lateral gene transfer between the staphylococcal isolates in this region.

14. Most of the reported \(SCCmec\) types (except \(SCCmec\) type II) were found in this region. Higher number of CA-MRS associated types IV, V and a marked number of these in animal isolates suggested the possible transfer of resistance from human to animal isolates.


Bibliography


Bibliography


Kondo, Y.; Ito, T.; Ma, X. X.; Watanabe, S.; Kreiswirth, B.N.; Etienne, J. and Hiramatsu, K. 2007. Combination of multiplex PCRs for staphylococcal cassette chromosome mec type assignment: rapid identification system for mec,


Bibliography


Sun, J.; Yang, M.; Sreevatsan, S.; Bender, J. B.; Singer, R. S.; Knutson, T. P.; Marthaler, D. G. and Davies, P. R. 2017. Longitudinal study of *Staphylococcus*


Bibliography


## APPENDIX-A

### Composition of Culture Media

**Nutrient broth**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Meat extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
</tbody>
</table>

**Brain heart infusion broth**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digested of animal tissue</td>
<td>10.0</td>
</tr>
<tr>
<td>Calf brain infusion</td>
<td>12.5</td>
</tr>
<tr>
<td>Beef heart infusion</td>
<td>5.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.0</td>
</tr>
<tr>
<td>Disodium phosphate</td>
<td>2.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

**Nutrient agar**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.4 ± 0.2.</td>
</tr>
</tbody>
</table>
### Blood agar base

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptic digest of animal</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5</td>
</tr>
<tr>
<td>Agar agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Defibrinated blood</td>
<td>Defibrinated blood 70 ml</td>
</tr>
</tbody>
</table>

### Mannitol Salt Agar

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>75.0g</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>10.0g</td>
</tr>
<tr>
<td>Pancreatic Digest of Caesin</td>
<td>5.0g</td>
</tr>
<tr>
<td>Peptic Digest of Animal Tissue</td>
<td>5.0g</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>1.0g</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>0.025g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0g</td>
</tr>
</tbody>
</table>

### Mueller-Hinton agar

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef infusion</td>
<td>3.0</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>17.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

### HiCrome MeReSa Agar

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein enzymic hydrolysate</td>
<td>13.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5</td>
</tr>
<tr>
<td>Beef extract</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>40.0</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>5.0</td>
</tr>
<tr>
<td>Chromogenic mixture</td>
<td>5.3</td>
</tr>
</tbody>
</table>
APPENDIX-B

Composition of chemicals and reagents

**Gram Stain**

**Crystal Violet**

**Solution A**
- Crystal Violet: 2.0g (90% dye content)
- Ethyl Alcohol: 20.0ml (95%)

**Solution B**
- Ammonium Oxalate: 0.8g
- Distilled Water: 80.0ml

(Note: Mix Solutions A and B)

**Gram's Iodine**
- Iodine: 1.0g
- Potassium Iodide: 2.0g
- Distilled Water: 300.0 ml

**Ethyl Alcohol (95%)**
- Ethyl Alcohol (100%): 95.0 ml
- Distilled Water: 5.0ml

**Safranin**
- Safranin 0: 0.25ml
- Ethyl Alcohol (95%): 10.0ml
- Distilled Water: 100.0ml

**Catalase Test reagent**
- Hydrogen Peroxide: 3% (H2O2)

**DNA Extraction reagent**
- NTE buffer (0.1M NaCl, 20 mM Tris-HCl [pH 7.4], 1mM EDTA [pH 7.5])
- 0.5% SDS
100 μg of proteinase K/ml
phenol-chloroform-isoamylalcohol (25:24:1)
chilled 100% ethanol
7.5M Ammonium acetate.
70% ethanol
TE buffer (10 mM Tris HCl – 5 mM EDTA, pH 7.8)

**Agarose Gel Loading Buffer (6X)**
Bromophenol blue 0.25% (w/v)
Xylene cyanol FF 0.25% (w/v)
Ficoll 15% (w/v)
(Type 400; Pharmacia)
Dissolved in appropriate volume of deionized water.

**Ethidium Bromide (1%)**
Ethidium bromide 10 mg
Distilled water 1.0 ml

**Electrophoresis Buffer (TBE 10x)**
Tris Base 108 g(890 mM)
Boric Acid 55 g(890 mM)
EDTA (pH 8.0) 40 ml(20 mM)