PREPARATION AND EVALUATION OF
STAPHYLOCOCCUS AUREUS VACCINES
FOR THE CONTROL OF MASTITIS IN
DAIRY BUFFALOES (*Bubalus bubalis*)

Abdul Shakoor

A thesis submitted in partial fulfillment of the
requirement for the degree of

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IN

Veterinary Clinical Medicine and Surgery

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In The Name of Allah, The Compassionate, The Merciful.
To

The Controller of Examinations,
University of Agriculture,
Faisalabad, Pakistan.

We, the Supervisory Committee, certify that the contents and form of thesis submitted by Abdul Shakoor (80-ag-274) have been found satisfactory and recommend that it be processed for evaluation by the External Examiner(s) for the award of the degree.

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DEDICATION

I dedicate this humble effort to the small holder Buffalo Farmers of the Punjab, Pakistan.
“Punjab might have turned socialist but for the existence of buffalo which provides sustenance to millions of landless poorer section of our society”.

(Khan and Rehman, 1982)
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Abdul Shakoor
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE NO.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td></td>
<td>LIST OF PLATES</td>
<td>viii</td>
</tr>
<tr>
<td></td>
<td>LIST OF APPENDIX</td>
<td>ix</td>
</tr>
<tr>
<td>1</td>
<td>INTRODUCTION</td>
<td>01</td>
</tr>
<tr>
<td>2</td>
<td>REVIEW OF LITERATURE</td>
<td>06</td>
</tr>
<tr>
<td>3</td>
<td>MATERIALS AND METHODS</td>
<td>66</td>
</tr>
<tr>
<td>4</td>
<td>RESULTS</td>
<td>89</td>
</tr>
<tr>
<td>5</td>
<td>DISCUSSION</td>
<td>171</td>
</tr>
<tr>
<td>6</td>
<td>SUMMARY</td>
<td>207</td>
</tr>
<tr>
<td>7</td>
<td>REFERENCES</td>
<td>211</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Findings of some Pakistani studies conducted during 1966-2002 on prevalence of common mastitis pathogens of buffaloes.</td>
<td>05</td>
</tr>
<tr>
<td>3.1</td>
<td>Protocol of antigen administration in rabbits to evaluate antigenicity of selected isolate of <em>S. aureus</em>.</td>
<td>69</td>
</tr>
<tr>
<td>3.2</td>
<td>Protocol for the preparation of hyperimmune serum in rabbits and buffaloes calves.</td>
<td>70</td>
</tr>
<tr>
<td>3.3</td>
<td>Distribution of Rabbits inoculated with different mastitis vaccines</td>
<td>75</td>
</tr>
<tr>
<td>3.4</td>
<td>Distribution of Buffaloes inoculated with Different mastitis vaccines.</td>
<td>76</td>
</tr>
<tr>
<td>3.5</td>
<td>Groups of buffalo, types of <em>S. aureus</em> vaccine, dose and route of administration, duration of study and sampling points for field evaluation of live attenuated <em>S. aureus</em> and dextran sulphate adjuvanted <em>S. aureus</em> mastitis vaccines.</td>
<td>77</td>
</tr>
<tr>
<td>3.6</td>
<td>Protocol of DNCB test in pregnant buffaloes vaccinated with different <em>S. aureus</em> mastitis vaccines.</td>
<td>81</td>
</tr>
<tr>
<td>4.1</td>
<td>California Mastitis Test-based Quarter point prevalence (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine (DSAV).</td>
<td>92</td>
</tr>
<tr>
<td>4.2</td>
<td>Surf Field Mastitis Test-based quarter point prevalence rate (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine (DSAV).</td>
<td>97</td>
</tr>
<tr>
<td>4.3</td>
<td>Milk yield (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine (DSAV).</td>
<td>98</td>
</tr>
<tr>
<td>4.4</td>
<td>Milk protein concentration (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine (DSAV).</td>
<td>100</td>
</tr>
<tr>
<td>4.5</td>
<td>Milk fat percentage (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period either with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine (DSAV).</td>
<td>101</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td>Milk somatic cell count (Mean ±SD; $10^7$/mL of milk) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvated <em>S. aureus</em> vaccine (DSAV).</td>
<td>102</td>
</tr>
<tr>
<td>4.7</td>
<td>Geometric mean milk whey IHA antibody titers in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvated <em>S. aureus</em> vaccine (DSAV).</td>
<td>105</td>
</tr>
<tr>
<td>4.8</td>
<td>Geometric mean serum IHA antibody titers in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvated <em>S. aureus</em> vaccine (DSAV).</td>
<td>110</td>
</tr>
<tr>
<td>4.9</td>
<td>Quarter-based incidence rate (%) of <em>S. aureus</em> mastitis in 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>112</td>
</tr>
<tr>
<td>4.10</td>
<td>Quarter-based prevalence (%) of <em>S. aureus</em> mastitis in 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>118</td>
</tr>
<tr>
<td>4.11</td>
<td>Milk yield (Mean±SD; L/24 hr) of 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>121</td>
</tr>
<tr>
<td>4.12</td>
<td>Milk protein (Mean±SD; %) in 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>123</td>
</tr>
<tr>
<td>4.13</td>
<td>Milk Fat (Mean±SD; %) in 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>125</td>
</tr>
<tr>
<td>4.14</td>
<td>Mean post challenge somatic cell count recorded at two days’ intervals among different groups of buffaloes vaccinated twice during dry period with four different <em>S. aureus</em> vaccines.</td>
<td>129</td>
</tr>
<tr>
<td>4.15</td>
<td>Somatic cell count (Mean±SD; $\times 10^3$) in quarter milk samples of 4 groups of buffaloes vaccinated twice during the dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>132</td>
</tr>
<tr>
<td>4.16</td>
<td>Mean skin thickness (mm±SE) at various time intervals in response to DNCB test in group B1 vaccinated with live attenuated <em>S. aureus</em> mastitis vaccine.</td>
<td>134</td>
</tr>
<tr>
<td>4.17</td>
<td>Whey IHA antibody titers (GMT) against <em>S. aureus</em> in four different groups of buffaloes vaccinated twice during dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>137</td>
</tr>
<tr>
<td>Table No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>4.18</td>
<td>Serum IHA antibody titres (GMT) against vaccinal <em>S. aureus</em> in 4 groups of buffaloes vaccinated twice during dry period with 4 different <em>S. aureus</em> vaccines.</td>
<td>139</td>
</tr>
<tr>
<td>4.19</td>
<td>Viability studies of <em>S. aureus</em> maintained in different media at temperature below 10°C.</td>
<td>141</td>
</tr>
<tr>
<td>4.20</td>
<td>Results of IHA antibodies against <em>S. aureus</em> recorded after 7 days post inoculation in rabbits to mastitis vaccine stored at 37°C for nine months period.</td>
<td>144</td>
</tr>
<tr>
<td>4.21</td>
<td>Results of IHA test recorded to elaborate antigenic Stability of monovalent mastitis vaccine stored at 25.0±2°C for nine months period.</td>
<td>147</td>
</tr>
<tr>
<td>4.22</td>
<td>Milk yield (Mean±SD) in two group of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine DSAV.</td>
<td>149</td>
</tr>
<tr>
<td>4.23</td>
<td>Surf Field Mastitis Test-based quarter point prevalence rate (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine DSAV.</td>
<td>152</td>
</tr>
<tr>
<td>4.24</td>
<td>California Mastitis Test-based quarter point prevalence rate (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus</em> vaccine (LSAV) and dextran sulphate adjuvanted <em>S. aureus</em> vaccine DSAV.</td>
<td>154</td>
</tr>
<tr>
<td>4.25</td>
<td>Quarter point prevalence (%) of <em>S. aureus</em> intramammary infection in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus vaccine</em> (LSAV) and dextran sulphate adjuvanted <em>S. aureus vaccine</em> (DSAV).</td>
<td>157</td>
</tr>
<tr>
<td>4.26</td>
<td>Cumulative incidence of <em>S. aureus</em> intramammary infection over a six month period in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus vaccine</em> (LSAV) and dextran sulphate adjuvanted <em>S. aureus vaccine</em> (DSAV).</td>
<td>159</td>
</tr>
<tr>
<td>4.27</td>
<td>Cumulative severity score of <em>S. aureus</em> clinical mastitis over a six month period in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated <em>S. aureus vaccine</em> (LSAV) and dextran sulphate adjuvanted <em>S. aureus vaccine</em> (DSAV).</td>
<td>162</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.28</td>
<td>Cost-benefit ratio in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated ( S. aureus ) vaccine (LSAV) and dextran sulphate adjuvated ( S. aureus ) vaccine (DSAV) vis-à-vis unvaccinated control.</td>
<td>163</td>
</tr>
<tr>
<td>4.29</td>
<td>Day 180 preventative efficacy of dextran sulphate adjuvanted ( S. aureus ) vaccine (DSAV) in a group of 50 lactating buffaloes (200 quarters).</td>
<td>165</td>
</tr>
<tr>
<td>4.30</td>
<td>Day 180 preventative efficacy of live attenuated ( S. aureus ) vaccine (LSAV) in a group of 50 lactating buffaloes (200 quarters).</td>
<td>166</td>
</tr>
<tr>
<td>4.31</td>
<td>Day 120 preventative efficacy of dextran sulphate adjuvanted ( S. aureus ) vaccine (DSAV) in a group of 50 lactating buffaloes (200 quarters).</td>
<td>167</td>
</tr>
<tr>
<td>4.32</td>
<td>Day 120 preventative efficacy of live attenuated ( S. aureus ) vaccine (LSAV) in a group of 50 lactating buffaloes (200 quarters).</td>
<td>168</td>
</tr>
<tr>
<td>4.33</td>
<td>Pre vaccination (day 0) and post-vaccination (day 180) colony count of ( S. aureus ) in two groups of buffaloes vaccinated twice during postpartum period with live attenuated ( S. aureus ) vaccine (LSAV) and dextran sulphate adjuvanted ( S. aureus ) vaccine (DSAV).</td>
<td>170</td>
</tr>
<tr>
<td>Fig. No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>4.1</td>
<td>COMPARATIVE SERUM I:HA ANTIBODY TITRES AGAINST S. aureus IN DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH 4 DIFFERENT VACCINES</td>
<td>94</td>
</tr>
<tr>
<td>4.2</td>
<td>GEOMETRIC MEAN I:HA SERUM ANTIBODY TITRES IN RABBITS VACCINATED WITH S. aureus VACCINES</td>
<td>95</td>
</tr>
<tr>
<td>4.3</td>
<td>GEOMETRIC MEAN I:HA ANTIBODY TITRES (GMT) AGAINST THREE DIFFERENT CONCENTRATIONS OF S. aureus AT DIFFERENT DAYS POST VACCINATION</td>
<td>104</td>
</tr>
<tr>
<td>4.4</td>
<td>GEOMETRIC MEAN I:HA ANTIBODY TITRES (GMT) AGAINST S. aureus AT DIFFERENT POST PRIMARY AND SECONDARY INOCULATION IN RABBITS.</td>
<td>106</td>
</tr>
<tr>
<td>4.5</td>
<td>COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES OF UNVACCINATED CONTROL GROUP</td>
<td>108</td>
</tr>
<tr>
<td>4.6</td>
<td>QUARTER-BASED INCIDENCE RATE (%) OF S. aureus MASTISITIS IN 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT VACCINES</td>
<td>109</td>
</tr>
<tr>
<td>4.7</td>
<td>COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES VACCINATED WITH OIL ADJUVANTED S. aureus VACCINE (OSAV)</td>
<td>113</td>
</tr>
<tr>
<td>4.8</td>
<td>COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES VACCINATED WITH DEXTRAN SULPHATE ADJUVANTED S. aureus MASTIS VACCINE (DASV)</td>
<td>114</td>
</tr>
<tr>
<td>4.9</td>
<td>COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES VACCINATED WITH SIMPLE BACTERIN S. aureus VACCINE (SSAV)</td>
<td>115</td>
</tr>
<tr>
<td>4.10</td>
<td>COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES VACCINATED WITH LIVE ATTENUATED S. aureus VACCINE (LSAV)</td>
<td>116</td>
</tr>
<tr>
<td>4.11</td>
<td>CUMULATIVE SERUM MEAN TITRES (GMT) OF 4 DIFFERENT S. aureus VACCINES IN BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD</td>
<td>117</td>
</tr>
<tr>
<td>4.12</td>
<td>MEAN MILK YIELD (L/24) OF 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH 4 DIFFERENT S. aureus VACCINES</td>
<td>119</td>
</tr>
<tr>
<td>4.13</td>
<td>COMPARATIVE MILK YIELD (L/24) OF 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES</td>
<td>122</td>
</tr>
<tr>
<td>4.14</td>
<td>GROUP MEAN MILK PROTEIN CONCENTRATION (%) DURING 120 DAYS POST CALVING PERIOD IN 4 GROUPS OF BUFFALOES VACCINATED TWICE WITH 4 DIFFERENT S. aureus VACCINES</td>
<td>124</td>
</tr>
<tr>
<td>4.15</td>
<td>COMPARATIVE PROTEINS % RECORDED AT MONTHLY INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES</td>
<td>126</td>
</tr>
<tr>
<td>4.16</td>
<td>COMPARATIVE FAT (%) RECORDED AT MONTHLY INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES</td>
<td>127</td>
</tr>
<tr>
<td>Fig. No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>4.17</td>
<td>COMPARATIVE MEAN POST CHALLENGE SOMATIC CELL COUNT RECORDED AT TWO DAYS' INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES</td>
<td>130</td>
</tr>
<tr>
<td>4.18</td>
<td>COMPARATIVE MEAN SOMATIC CELL COUNT RECORDED AT MONTHLY INTERVALS IN DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES</td>
<td>131</td>
</tr>
<tr>
<td>4.19</td>
<td>QUARTER-BASED PREVALENCE (%) OF S. aureus IN 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT VACCINES</td>
<td>133</td>
</tr>
<tr>
<td>4.20</td>
<td>COMPARATIVE WHEY IHA ANTIBODY TITRES (GMT) OF 4 DIFFERENCE S. aureus VACCINES IN BUFFALOES VACCINATED TWICE DURING DRY PERIOD</td>
<td>135</td>
</tr>
<tr>
<td>4.21</td>
<td>COMPARATIVE PREVACCINATION AND POST VACCINATION COLONY COUNT OF S. aureus IN TWO GROUPS OF BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE</td>
<td>138</td>
</tr>
<tr>
<td>4.22</td>
<td>CUMULATIVE INCIDENCE OF S. aureus INTRAMAMMARY INFECTION OVER A SIX MONTH PERIOD IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV)</td>
<td>140</td>
</tr>
<tr>
<td>4.23</td>
<td>COMPARATIVE QUARTER POINT PREVALENCE (%) OF S. aureus INTRAMAMMARY INFECTION IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV)</td>
<td>143</td>
</tr>
<tr>
<td>4.24</td>
<td>COMPARATIVE CALIFORNIA MASTITIS TEST-BASED QUARTER POINT PREVALENCE (%) OF MASTITIS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV)</td>
<td>145</td>
</tr>
<tr>
<td>4.25</td>
<td>COMPARATIVE PREVACCINATION AND POST VACCINATION COLONY COUNT OF S. aureus IN TWO GROUPS OF BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE</td>
<td>148</td>
</tr>
<tr>
<td>4.26</td>
<td>CUMULATIVE INCIDENCE OF S. aureus INTRAMAMMARY INFECTION OVER A SIX MONTH PERIOD IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV)</td>
<td>150</td>
</tr>
<tr>
<td>Fig. No.</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>---------</td>
<td>-------</td>
<td>----------</td>
</tr>
<tr>
<td>4.27</td>
<td>COMPARATIVE QUARTER POINT PREVALENCE (%) OF <em>S. aureus</em> INTRAMAMMARY INFECTION IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED <em>S. aureus</em> VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTE <em>S. aureus</em> VACCINE (DASV).</td>
<td>153</td>
</tr>
<tr>
<td>4.28</td>
<td>COMPARATIVE CALIFORNIA MASTITIS TEST-BASED QUARTER POINT PREVALENCE (%) OF MASTITIS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED <em>S. aureus</em> VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTE <em>S. aureus</em> VACCINE (DASV).</td>
<td>155</td>
</tr>
<tr>
<td>4.29</td>
<td>COMPARATIVE SURF FIELD MASTITIS TEST-BASED QUARTER POINT PREVALENCE RATE (%) OF MASTITIS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED <em>S. aureus</em> VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTE <em>S. aureus</em> VACCINE (DASV).</td>
<td>158</td>
</tr>
<tr>
<td>4.30</td>
<td>COMPARATIVE MILK YIELD (MEAN ± SD) IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED <em>S. aureus</em> VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTE <em>S. aureus</em> VACCINE (DASV).</td>
<td>160</td>
</tr>
<tr>
<td>4.31</td>
<td>COMPARATIVE MILK PROTEIN CONCENTRATION (MEAN ± SD) IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED <em>S. aureus</em> VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTE <em>S. aureus</em> VACCINE (DASV).</td>
<td>164</td>
</tr>
</tbody>
</table>
## LIST OF PLATES

<table>
<thead>
<tr>
<th>PLATE No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sealed packings of 4 different <em>S. aureus</em> mastitis vaccines (from L to R; LSAV, PSAV, DASAV &amp; OSAV).</td>
<td>73</td>
</tr>
<tr>
<td>2.</td>
<td>Post milking teat dip challenge with live inoculum of selected <em>S. aureus</em>.</td>
<td>78</td>
</tr>
<tr>
<td>3.</td>
<td>Collection of blood samples from vaccinated buffaloes.</td>
<td>80</td>
</tr>
<tr>
<td>4.</td>
<td>DNCB test kit showing its basis essentials.</td>
<td>82</td>
</tr>
<tr>
<td>5.</td>
<td>Areas marked on the neck of buffaloes to perform DNCB test.</td>
<td>83</td>
</tr>
<tr>
<td>6.</td>
<td>A glimpse of milk somatic cell count (SCC) after being treated with a modified Newman-Lempert’s Staining Technique.</td>
<td>85</td>
</tr>
<tr>
<td>7.</td>
<td>A typical α-β <em>S. aureus</em> selected isolate on 5% blood agar plate.</td>
<td>90</td>
</tr>
<tr>
<td>8.</td>
<td>Seven digit API Staph. biochemical profile of selected <em>S. aureus</em> isolate.</td>
<td>91</td>
</tr>
</tbody>
</table>
# LIST OF APPENDIX

<table>
<thead>
<tr>
<th>Appendix No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Geometric mean serum antibody titer in rabbits vaccinated with 4 different <em>S. aureus</em> vaccine for a period of 2 months.</td>
</tr>
<tr>
<td>II</td>
<td>Newman-Lempert’s Staining Technique for Somatic Cell Count</td>
</tr>
<tr>
<td>III</td>
<td>Guidelines for significance of colony number of specific organisms isolated pure with other colony type (based on 0.01 ml quarter sample streaked on blood agar).</td>
</tr>
<tr>
<td>IV</td>
<td>Anthony’s Method of Capsule Staining</td>
</tr>
<tr>
<td>V</td>
<td>Hiss’s Method of Capsule Staining</td>
</tr>
<tr>
<td>VI</td>
<td>India Ink Method of Capsule Staining</td>
</tr>
<tr>
<td>VII</td>
<td>Attenuation of <em>S. aureus</em> isolate</td>
</tr>
<tr>
<td>VIII</td>
<td>Composition of live attenuated <em>S. aureus</em> vaccine</td>
</tr>
<tr>
<td>IX</td>
<td>Composition of oil-adjuvanted <em>S. aureus</em> vaccine (OSAV)</td>
</tr>
<tr>
<td>X</td>
<td>Composition of dextran sulphate-adjuvanted <em>S. aureus</em> vaccine (DSAV)</td>
</tr>
<tr>
<td>XI</td>
<td>Composition of plain bactrin <em>S. aureus</em> vaccine (PSAV)</td>
</tr>
<tr>
<td>XII</td>
<td>General Plan for Haemagglutination Test</td>
</tr>
<tr>
<td>XIII</td>
<td>Serum antibody response to different concentrations of <em>S. aureus</em> at different days in rabbits</td>
</tr>
</tbody>
</table>
CHAPTER - 1

INTRODUCTION

Mastitis (inflammation of the milk-producing organ of mammals) is the most costly disease of the dairy industry (DeGraves and Fetrow, 1993; Kossaibati et al., 1998) and is a worldwide problem among dairy animals (Colditz and Watson, 1985; Lightner et al., 1988). Field surveys of major livestock diseases in Pakistan have indicated that mastitis is one of the most important health problems of cattle and buffaloes (Cady et al., 1983; Ajmal, 1990; Egenolf, 1990; Khan et al., 1991). Mastitis reduces the milk yield of affected animals (Cady et al., 1983; Shah, 1991; DeGrave and Fetrow, 1991). It causes a host of problems to milk processors and milk producers, impairs the quality of the milk produced and poses a public health risk to consumers (Saeman et al., 1987; Murphy et al., 1988; Srinivasan and Singh, 1988; Barbano, 1989; Smith and Hogan, 1996; Smith and Hogan, 1999a; Smith and Hogan, 1999b; Philpot, 2003; Ullah, 2004). In Pakistan, statistics of current losses associated with this disease are not available although, it was estimated in 1978 that in Punjab alone, the total losses caused by clinical mastitis amounted to Rs. 240 millions per annum (Chaudhry and Khan, 1978). It is pertinent to mention that this survey did not take into account the losses caused by sub-clinical mastitis, the form of the disease 15-40 times more frequent than the clinical one. In a study of the Nili-Ravi buffalo of Pakistan, mastitis affected buffaloes had on an average a 57 day shorter lactation period and produced 438 kg less milk than the healthy ones (Cady et al., 1983).

A wide variety of micro-organisms have been associated with mastitis (Bramley and Dodd, 1984; Kapur et al., 1992; Allore, 1993). Of these, Staphylococcus aureus is probably the most important throughout the world despite advances in therapeutics, modern milking techniques, and considerable improvements in farm hygiene and management (Srinivasan and Singh, 1988; Philpot and Nickerson, 1991; Kapur et al., 1992; Giraudo et al., 1997; Adesiyun and Romain, 1998; Owens et al., 2001; Eckersal et al., 2001). Typically most S. aureus infections are chronic and sub-clinical with periodic flare-ups of clinical symptoms. As per National Mastitis Council Inc. of USA between 70 to 80% of all losses
caused by mastitis are associated with sub-clinical mastitis (Philpot and Nickerson, 1991; Radostits et al., 2000). In Pakistan too, mastitis has been ranked as number one disease among the major diseases of dairy animals in Pakistan (Akhtar, 1995) and S. aureus reportedly is the most important mastitis pathogen as can be seen in Table 1.1 depicting the findings of the studies conducted between 1966 and 2002.

*Staphylococcus aureus* intramammary infections are difficult to cure and eliminate from infected animals. While reviewing the role of antibiotics in mastitis therapy, Sandholm et al. (1990) concluded that the bacteriologic cure rate for *S. aureus* mastitis treated during lactation by antibiotics effective *in vitro* is about 50% when the follow-up bacteriologic assessment is made 2–3 weeks after therapy. Others (Wilson and Sears, 1996) have documented a cure rate of even less than 10% and do not consider it economically worthwhile to treat *S. aureus* infection with antibiotics during lactation. The poor cure rate in *S. aureus* mastitis is attributable to poor penetration of antibiotics into the areas of scarring and inflammation, inactivation of antibiotics by bacteria, milk and serum components, intracellular location of the microbe, metabolically inactive organisms, bacterial L-form, resistance to antibiotics and improper treatment procedures (Sandholm et al., 1990; Nickerson and Owens, 1994).

Buffaloes are recognized as the world’s second most important milk producing species (McDowell et al., 1995). The water buffalo is a neglected bovine animal with a notable and so far unexploited potential, especially for milk and meat production (Acharya, 1992; Cockrill, 1974, 1977a; Mahadevan, 1978). There is growing interest, in both the advanced and the developing countries, in the full exploitation of the domestic water buffalo, as is shown by the expanding international trade in breeding stock and the establishment of many new herds in countries where the buffalo is a newcomer (Cockrill, 1977b).

Buffalo is justifiably the mainstay of dairying in Pakistan. About 20 million heads of dairy buffaloes in Pakistan (which constitute about 8.5% of the total world buffalo population) contribute about 25% of the global milk production by this species (Anwar, 1995; Moioli, 1996). In Pakistan about 74.2% of the total milk production (28.62 million tons) is contributed by buffaloes. Major portion of the milk produced in Pakistan comes
from 6.26 million small farmers who maintain less than six dairy animals per household. About 55% of these small farmers i.e. 3.47 million are landless and keep only one or two dairy animals (Livestock Censuses, 1996). The average per caput availability of milk is about 118 kg/year, which is the highest in developing countries of the region. The supply of milk is constrained by urban demand on milk and milk products, which is increasing at the rate of 5–7% per annum. In order to bridge the gap between supply and demand of milk, it is imperative to control such production limiting diseases as mastitis.

In common with the pattern in most developing countries, dairy farming in Pakistan is characterized by household production using family labour. A common practice in rural household is for each family to keep 3–5 heads of buffaloes primarily for house family use (Jost, 1980; Chaudhry, 1984). The animals are predominantly hand milked and contagious mastitis caused by *S. aureus* is most prevalent in Pakistan as per findings of the studies undertaken thus far in Pakistan (Ahmad, 1966; Ghumman, 1967; Ahmad 1968; Amin, 1973; Hashmi, 1978; Sahi, 1983; Shireen, 1984; Iqbal, 1991; Qamar, 1992; Fazal-ur-Rehman, 1995; Razzaq, 1998; Memon et al., 1999) (Table-1.1). Reviewing the incidence of mastitis in buffaloes and cattle from India and Pakistan, Allores (1993) also concluded that contagious organisms are responsible for most of the clinical cases and *S. aureus* is at the top of the list in both cattle and buffalo. She also recommended that researchers must step ahead in order to find out its permanent remedial and preventative solution instead of recording its incidence and prevalence over and over again.

In countries where dairying is well-developed (e.g. Europe, North America, Australia and New Zealand), post-milking teat dipping and dry period antibiotic therapy are the most important components of mastitis control strategy. Teat dipping entails dipping of all teats of all animals immediately after milking in an antiseptic solution. This practice has to be carried out twice daily throughout the lactation. Pakistani farmers being uneducated, non-progressive and generally non-commercial are not inclined to this stringent mastitis control strategy. Therefore, standard mastitis control practices in Pakistan are difficult to put into practice. Not only that, at present not a single especially formulated teat dip preparation is available in Pakistan. Dry period antibiotic therapy is not being practiced even on the organized dairy farms in Pakistan. Against this backdrop the contrivance of vaccination holds the promise of a suitable alternative/adjunct mastitis control strategy. It is being
practiced in countries like USA and Australia with considerable success because firstly it is a single shot or a few shots at the most per year and secondly, it is cost-effective. Development of improved and new vaccines for important diseases of dairy animals has been identified as one of the important animal health imperatives in Pakistan (Afzal and Usmani, 2002). A perusal of literature, however, indicated that attempt to investigate the control of *S. aureus* mastitis in buffalo through vaccination thus far is limited to a solitary report by Pal and Pathak (1977).

It is important to accept upfront that vaccination alone cannot answer all the economic and animal welfare problems posed by mastitis. Hygiene, management and therapy all have important roles to play in controlling the disease. In contrast to previous eras, mastitis vaccines especially against *S. aureus* have now reached a level of efficacy that should make them an integral part of mastitis control (Watson *et al.*, 1993).

Keeping in view the dairy farming scenario prevailing in Pakistan, the present study was planned to prepare and compare the efficacies of four *S. aureus* vaccines (viz. live attenuated vaccine, plain bacterin, dextran sulphate adjuvanted bacterin and oil-adjuvanted bacterin) under laboratory and field conditions in order to find out the most suitable vaccine for the control of mastitis in buffaloes.
Table 1.1: Findings of some Pakistani studies conducted during 1966–2002 on prevalence of common mastitis pathogens of buffaloes.

<table>
<thead>
<tr>
<th>Worker(s)</th>
<th>Animals</th>
<th>S. aureus</th>
<th>Str. agalactiae</th>
<th>E. coli</th>
<th>Mixed¹</th>
<th>Others (C. pyogenes, Pseud. aeruginosa, Mycobac, Yeasts, Klebsiella, C. bovis, Cl. vulgaris, B. cereus, A. aerogenes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ahmad (1966)</td>
<td>Buffaloes</td>
<td>44.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ghumman (1967)</td>
<td>Buffaloes</td>
<td>91.20</td>
<td>0.80</td>
<td>1.60</td>
<td>-</td>
<td>0.8²</td>
</tr>
<tr>
<td>Ahmad (1968)</td>
<td>Buffaloes</td>
<td>84.00</td>
<td>2.00</td>
<td>4.00</td>
<td>10.00¹</td>
<td>-</td>
</tr>
<tr>
<td>Hashmi (1978)</td>
<td>Buffaloes and Cows</td>
<td>43.09</td>
<td>36.82</td>
<td>12.55</td>
<td>-</td>
<td>4.2¹, 2.9², 0.4³</td>
</tr>
<tr>
<td>Hashmi &amp; Munir (1981)</td>
<td>Buffaloes</td>
<td>34.55</td>
<td>34.44</td>
<td>20.00</td>
<td>-</td>
<td>2.0²</td>
</tr>
<tr>
<td>Anwar &amp; Chaudhari (1983)</td>
<td>Buffaloes</td>
<td>40.00</td>
<td>45.06</td>
<td>25.00</td>
<td>-</td>
<td>5.0²</td>
</tr>
<tr>
<td>Sahi (1983)</td>
<td>Buffaloes</td>
<td>45.45</td>
<td>33.57</td>
<td>12.59</td>
<td>-</td>
<td>4.2¹, 1.4², 0.7²</td>
</tr>
<tr>
<td>Hussain et al. (1984)</td>
<td>Buffaloes</td>
<td>28.26</td>
<td>40.20</td>
<td>19.66</td>
<td>8.69</td>
<td>17.33¹, 0.26², 7.2²</td>
</tr>
<tr>
<td>Razzaq (1998)</td>
<td>Buffaloes</td>
<td>53.33</td>
<td>40.00</td>
<td>6.66</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Memon et al. (1999)</td>
<td>Buffaloes</td>
<td>38.00</td>
<td>8.00</td>
<td>11.00</td>
<td>13.00³</td>
<td>11.00⁵</td>
</tr>
<tr>
<td>Ahmad (2001)</td>
<td>Buffaloes</td>
<td>33.99</td>
<td>35.46</td>
<td>27.09</td>
<td>-</td>
<td>1.48¹, 1.97²</td>
</tr>
<tr>
<td>Akram (2002)</td>
<td>Buffaloes</td>
<td>40.32</td>
<td>14.52</td>
<td>17.74</td>
<td>4.84², 3.23³, 4.84³</td>
<td></td>
</tr>
<tr>
<td>Khan (2002)</td>
<td>Buffaloes</td>
<td>45.00</td>
<td>23.00</td>
<td>18.00</td>
<td>-</td>
<td>14.00¹</td>
</tr>
<tr>
<td><strong>Mean Percentages</strong></td>
<td></td>
<td>50</td>
<td>23.12</td>
<td>13.97</td>
<td>13.57</td>
<td>10.16</td>
</tr>
</tbody>
</table>
CHAPTER - 2

REVIEW OF LITERATURE

Staphylococci have been found associated with disease ever since the Scottish surgeon, Sir Alexander Ogston (1880) discovered round microorganisms in infected tissues. He introduced the term “staphylococcus” (Greek staphyle, bunch of grapes; kokkos, berry), referring to the microscopic picture of stained organism. The importance of the microorganism as pathogen for animals has been known for more than 100 years. In 1887, Nocard isolated staphylococci from mastitis in sheep and in 1890 Guillebeau suggested that these organisms were responsible for mastitis in cattle (Jonsson and Wadstrom, 1993).

Mastitis probably has been observed ever since man first domesticated cow. It is hard to imagine a single herd anywhere in the world (regardless of size) to be truly free from mastitis (Philpot and Nickerson, 1991).

This section of the manuscript reviews the informations on economic losses due to mastitis bubaline mastitis and associated microorganisms, significance of *S. aureus* in mastitis and the need for an immunological control, Virulence factors of *S. aureus* and their role in immunization, immunology of udder, intraspecific cross reactivity in staphylococci associated with mastitis, important bottlenecks in producing an effective *S. aureus* mastitis vaccine, special cultural conditions for expression of immunologically important virulence factors, monovalent and bivalent vaccines, live attenuated vaccines, adjuvanted vaccines, toxoids, *S. aureus* vaccine efficacy, effect of vaccination on the level, duration and severity of intramammary infection, need for a polyvalent vaccine.

2.1. Economic Losses Due to Mastitis

Mastitis is the largest single factor contributing to economic losses caused by infectious disease to the dairy producers worldwide. Mastitis in ruminant causes huge economic losses. In the United States the estimated loss to the economy is more than US $ 2 billion per annum (Jasper *et al.*, 1982) and the worldwide cost of this disease is
reported to be US $35 billion per annum (Mayer, 1990). Every case of clinical mastitis in a cow in California is estimated to cost at least US $200 (Bennett, 1990). In Australia there are about 2.5 million dairy cows and mastitis costs the Australian Dairy Industry more than $A100 million per year.

The cost of mastitis to the UK Dairy Industry is current by estimated at £93 million per year (British Mastitis Conference, 1997). It is estimated that India alone loses 53 crores of rupees a year due to mastitis (Dhanda and Sathi, 1963) as cited by Sharma and Misra (1966). In Argentina milk production losses due to mastitis reach $221 million annually (Anonymous, 1983).

In Pakistan statistics of current losses associated with this disease are not available, although it was estimated in 1978 that in Punjab alone, the total losses caused by clinical mastitis amounted to Rs. 240 millions per annum (Chaudhry and Khan, 1978).

2.2. **Bubaline (Buffalo) Mastitis and Associated Microorganisms**

Domesticated buffalo provide products and have other traits that fill market niches in over 50 countries; milk rich in fat, protein and total solids, desirable flavored processed products, acceptable meat and hides and are proven beasts of draft or cartage. Major habitats are East Asia, Pacific rim countries lying between the 30° North-South latitudes (Warm climate Zone). There are nucleus groups in 8 countries of Latin America and 7 Europe (Cockrill, 1977a). Buffaloes are recognized as the world’s second most important milk producing species (MacDowell et al., 1995).

Buffalo diseases are generally the same as those of cattle, since the buffalo (**Bubalis bubalis**) is closely related in phylogeny to cattle. Buffaloes are susceptible to most of the organisms causing mastitis in cattle (Adlakha and Sharma, 1992). Mastitis is the most important disease of milch animals because it affects the physical, chemical, bacteriological, technological, organoleptic and other qualities of milk. It was wrongly presumed that buffaloes were more resistant to mastitis. The research findings have proved beyond doubt that buffalo is as susceptible to mastitis as cow (Srinivasan and Singh, 1988). Contrary to it, Wanasinghe (1985) reported that the buffalo was more resistant to udder infections than cattle. Therefore it is possible that the quantity and / or quality of somatic cell which appear in buffalo milk as a response to mammary infections
may be different to that of cattle (Silva and Silva, 1994). The incidence of clinical mastitis varies from 1 to 10 percent at various places in India (Kalra and Dhanda, 1964; Rao and Ramaiah, 1982; Kumar, 1988), whereas the sub-clinical mastitis varies from 10 to 50 percent in cows and 5 to 20 percent in buffaloes (Singh, 1974; Chander and Buxi, 1975; Sharma and Rai, 1977; Kumar, 1988). Thus from available literature, it is clear that incidence of mastitis is lesser in buffaloes than in cows, under similar managerial conditions. As such there might be some genetic and anatomical factors operating in that which might be providing higher resistance against mastitis in buffalo than in cow (Uppal et al., 1994). Krishaswamy et al. (1965) studied histological difference in the teats of lactating cow and buffalo at the region of teat sphincter and reported that rich muscle fibers and vascular tissue in the buffalo teat may be the factors responsible for lesser intramammary infection in buffaloes. However, no detailed study on buffalo’s teat is available in the literature so far.

Dairy industry of Pakistan comprises buffalo and cattle. Buffalo is the main dairy animal that accounts for roughly 75% of all milk produced in the country (Sarwar et al., 2002). Nili-Ravi breed of buffalo is the principal breed in Pakistan (Sarwar and Ishaq, 1957; Cockrill, 1974; McDowell, et al., 1995) and is among the highest milk producing breeds of buffalo (Cockrill, 1974; Cockrill, 1977a; Ahmad and Saji, 1997). Factors affecting milk yield in buffalo are similar to those in cattle (Cady et al., 1983). Among these mastitis is at the top. It is caused by a variety of microorganisms as reported by many workers (Potter and Babiuk, 1993; Hwang et al., 2000). Mastitis is the most important disease of buffalo, because it poses innumerable problems to milk production, milk processing, quality of the product and public health risks to consumers resulting in staggering economic loss to the dairy industry. Mastitis affects the physical, chemical, bacteriological, technological, organoleptic and other qualities of milk. It was wrongly presumed that buffaloes are resistant to mastitis. The research findings have proved beyond doubt that buffalo is as susceptible to mastitis as cow (Srinivasan and Singh, 1988). The causative organisms associated with mastitis in buffalo in India include *Staphylococci*, *Streptococci*, *E. coli*, *Pseudomonas* spp., *Corynebacterium*, *Mycoplasma*, *Str. dysgalactiae*, *Mycobacterium tuberculosis*, *Pseudomonas. vulgaris*, *Nocardia*, *Saccharomyces*, *Torulopsis, Bacillus* spp., *Cryptococcus* spp., *Aspergillus, Rhizopus* and
Penicillium, etc. Among the pathogens causing bovine mastitis, *S. aureus* is a predominant organism (Srinivasan and Singh, 1988; Allore, 1993; Kapur et al., 1992).

Studies conducted on etiologic agents of bubaline mastitis in Pakistan have documented the association of *Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus epidermidis, Staphylococcus capotus, unidentified staphylococci, Streptococcus dysagalactiae, Streptococcus agalactiae, Streptococcus pyogenes, Corynebacterium bovis, Corynebacterium pyogenes*, other Diphtheroids, *E. coli, Bacillus spp, yeast, Prothotheica, Pseudomonas aeruginosa, Mycobacterium tuberculosis* with the disease (Ahmad, 1966; Ghuman, 1967; Ahmad, 1968; Hashmi, 1978; Hashmi and Munir, 1981; Anwer and Chaudhari, 1983; Sahi, 1983; Hussain et al., 1984; Shireen, 1984; Qamar, 1992; Allore, 1993; Fazal-ur-Rehman, 1995; Muhammad et al., 1995; Muhammad et al., 1997; Razzaq, 1998; Memmon et al., 1999; Ahmad, 2001; Akram, 2002; Khan, 2002).

A vast majority of these studies would implicate the contagious mastitis pathogens (*S. aureus* and *Strep. agalactiae*) as the most frequent etiologic agents of mastitis in buffalo.

2.3. Significance of *S. aureus* in Mastitis and the Need for an Immunological Control

*Staphylococcus aureus* is an important cause of udder infections in dairy herds (Berkema et al., 1998; Dingwell et al., 2003; Fox and Gay, 1993; Sargeant et al., 1998; Wilson et al., 1997). Intramammary infection (IMI) with *S. aureus* may result in clinical or sub-clinical mastitis and is usually associated with increased somatic cell count (SCC). *S. aureus* remains a problem in a variety of locations and under different management styles. Nine percent of cows in New York State (US) were *S. aureus* culture-positive in at least one of the quarters (Wilson et al., 1997). Keefe et al. (1997) reported that 70% of 30 randomly selected Prince Edward Island (Canada) dairy herds had at least one cow infected with *S. aureus* on a single herd culture. In the Ontario Sentinel Project, bulk milk samples from 58 of 59 herds in Ontario were culture-positive for *S. aureus* (Kelton et al., 1999a), while 92% of the herds had at least one cow culture-positive for *S. aureus* (Kelton et al., 1999b). The pathogen is contagious and spreads easily within dairy herds.
When multiple cows in a herd are infected, bulk tank somatic cell count (BTSCC) increases and legal limits for BTSCC may be violated or thresholds for premium bonus may not be met (Adkinson et al., 2001; Dekkers et al., 1996; Schukken et al., 1993). Therefore, control of *S. aureus* mastitis remains important and necessary (Berkema et al., 2004).

Owing to small sizes of herds, barring an extremely miniscule number, dairy cows and buffaloes in Pakistan are hand milked. Contagious mastitis pathogens in particular *S. aureus* are reportedly a problem in hand milked dairy herds (Oliver et al., 1975). In countries having well-developed dairying, post-milking teat dipping and dry cow therapy are the most important components of mastitis control strategy. Teat dipping entails (total or selective) dipping of all teats of all animals immediately after milking in an antiseptic solution. This practice has to be carried out twice daily throughout the lactation. Pakistani farmers being uneducated, non-progressive and by and large non-commercial are not inclined to this stringent mastitis control strategy. Therefore, standard mastitis control practices (e.g. 5 point plan recommended by National Mastitis Council, USA, 1999) in Pakistan are difficult to put into practice. Furthermore, at present not a single especially formulated teat dip preparation is available in the country. Same is true about the dry cow therapy. Against this backdrop, the contrivance of vaccination against *S. aureus* mastitis holds the promise of a suitable alternative/adjunct mastitis control strategy because it is a single shot or a few shots at the most per year and secondly, it is cost-effective (Amorena, et al., 1996). Buffalo diseases are generally the same as those of cattle. The buffalo is probably equally susceptible to the majority of infectious diseases of cattle (Adlakha and Sharma, 1992). Buffaloes are susceptible to most of the organisms causing mastitis in cattle.

As discussed above, *S. aureus* is the most common contagious pathogen causing mastitis in dairy buffaloes (Ahmad, 1966; Ghuman, 1967; Ahmad, 1968; Hashmi, 1978; Hashmi and Munir, 1981; Anwer and Chaudhari, 1983; Sahi, 1983; Hussain et al., 1984; Shireen, 1984; Qamar, 1992; Allore, 1993; Fazal-ur-Rehman, 1995; Muhammad et al., 1995; Muhammad et al., 1997; Razzaq, 1998; Memmon et al., 1999; Ahmad, 2001; Akram, 2002; Khan, 2002). The intramammary infections caused by *S. aureus* are responsible for a considerable economic loss from reduced milk production and
derioration of milk quality. Eradication of *S. aureus* from dairy herds is very difficult because the organism is a common inhabitant of the teat skin. Bacteriological cure rates after antibiotic treatment seldom exceed 50% because of the poor penetration of antibiotics into areas of scarring and inflammation, inactivation of the antibiotics by milk and serum component, intracellular location of organisms, and formation of bacterial L-forms during treatment. Infections commonly persist throughout the lifetime of the cow. Mastitis is the most common reason for antibiotic use in dairy buffaloes, and recently, concern has been expressed about the presence of antibiotic residues in milk. The ubiquitous nature of Staphylococci coupled with their resistance to antibiotics and the absence of suitable vaccine prophylaxis severely limits the staphylococcal control programme (Jonsson and Wadstrom, 1993). The ultimate control of mastitis relies on the prevention rather than treatment and new strategies for this needed (Tollersrud et al., 2000; Pyorala, 2002).

Although studies cited above indicated that *S. aureus* is the predominant mastitis pathogen but in addition to it, *Streptococcus agalactiae* (Giraudo and Busso, 1980; Smith and Hogan, 1995; Watts, 1988), *E. coli* (Giraudo and Busso, 1980) and *Strep. uberis* (Bramley, 1982; Kruze and Bramely, 1982; Hogan et al., 1989) also account for a considerable proportion of clinical and sub-clinical mastitis.

2.4. Virulence Factors of *S. aureus* and Their Role in Immunization

*S. aureus* produces a wide range of enzymes (coagulase, hyaluronidase, nuclease, lipases, proteases), several toxins (α-, β-, γ- and δ-leukocidin) and other virulence determinants (e.g. capsular polysaccharides, fibronectin binding proteins etc. (Marks and Vaughan, 1950; Jackson and Mayman, 1958; Madoff and Weinstein, 1961; Bernheimer and Schwartz, 1963; Jocobs et al., 1964; Bernheimer, 1965; Manoher et al., 1966; John and Robinson, 1968; Kreger et al., 1971; van der Vijver et al., 1975; Arbuthrott et al., 1976; Adlam et al., 1977; Goudsward et al., 1978; Elisa and Koffer, 1980; King and Wilkinson, 1981; Adegoke and Ejo, 1981; Watson, 1982; Karakawa and Vann, 1982; Wilkinson, 1983; Mollby, 1983; Tabassum and Ajmal, 1983; Arvidson, 1983; Gudding et al., 1984; Johnsson et al., 1985; Johne et al., 1989; Bramley et al., 1989; Lindahl et al., 1990; Memo et al., 1991; Nelson et al., 1991; Kenny et al., 1992; Fattom et al., 1993;
Expression of some virulence determinants of *S. aureus* is a function of medium in which it is growing. Watson (1982) conducted a study on the virulence of *S. aureus*
grown in vitro or in vivo. Paired comparisons were made of various strains of S. aureus grown in broth inside dialysis sacs anchored in the peritoneal cavities of sheep (in vivo culture) and in a variety of bacteriological media in the laboratory (in vitro culture). The organisms grown in vivo possessed enhanced virulence compared with in vitro grown organisms, when injected intradermally in sheep, or intraperitoneally in mice and when infused into lactating mammary glands of ewes. Growth under in vivo conditions conferred on the bacteria an increased resistance to phagocytosis by ovine neutrophils. The bacteria grown under in vivo conditions possessed an additional cell-associated component as determined by immunodiffusion tests and optical density profiles of gel infiltration eluates; however, this substance was not visible in electron micrographs in the form of a capsule (Watson, 1982).

Capsular polysaccharide can inhibit phagocytosis by neutrophils (Pourtrel et al., 1988). An earlier study (Paape and Wergin, 1979) reported that encapsulated S. aureus isolated from milk of cows with mastitis inhibited phagocytosis by neutrophils and capsule production was enhanced by serial passage through bovine mammary glands. The capsule polysaccharide has been considered as a potential candidate for a vaccine to minimize mastitis in cows (Opdebeek et al., 1988).

Amorena et al. (1994) evaluated the use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. Experiments on the development of a vaccine against staphylococcal mastitis were carried out in ewes. The vaccine (Spanish patent no. 9200223) had the following components: (i) inactivated formalinized bacteria (S. aureus and a coagulase-negative staphylococcal species, Staphylococcus simulans) and S. aureus toxoid in presence of an adjuvant (dextran sulphate, MW 500,000); and (ii) S. aureus exopolysaccharide included within liposomes. High serum antibody titres were obtained against whole cells from S. aureus, S. simulans, S. hyicus and S. epidermidis strains. However, there was no response to cells from S. warneri and S. chromogenes strains. An immune response (serum IgG) against the inoculated exopolysaccharide was obtained when > or = 20 micrograms of exopolysaccharide were included in liposomes and when > or = 20 mg of exopolysaccharide were adjuvanted with dextran sulphate instead of liposomes. The incidence of S. simulans sub-clinical mastitis and of S. aureus acute mastitis was
significantly lower in vaccinated animals than in unvaccinated controls. Specifically, on challenge with *S. simulans*, two out of 14 glands became infected among the vaccinated animals and nine out of ten glands in the unvaccinated group (p < 0.001). On challenge with *S. aureus*, no protection was detected when component (ii) was omitted from the vaccine; nine out of ten animals developed mastitis (two mild, two moderate and five severe).

Fibronectin-binding proteins (FnBP) are thorough to mediate attachment of *S. aureus* to host cells which may initiate disease (Christensen *et al.*, 1985). Most isolated *S. aureus* specifically bind to extracellular matrix fibronectin (Kuypers and Proctor, 1989). FnBP has shown to mediate binding of *S. aureus* to soluble fibronectin (Mamo *et al.*, 1988). It has been reported that FnBP promotes adhesion of *S. aureus* to host tissue and may act as virulence factor. Thus, FnBP on *S. aureus* involved in the pathogenesis could be important in the development of subunit vaccine against bovine mastitis (Lopes *et al.*, 1985). These three virulence factors were protective when used in the form of subunit vaccines especially in rabbits (Park *et al.*, 1999).

Toxins, especially alpha toxin, play an important part in the development of staphylococcal mastitis. It is produced as a terminal event in the pathogenesis of the disease (Anderson, 1978). Alpha toxin is produced and released only towards the end of logarithmic bacterial growth in the mammary gland that is, at a time when mastitis already exists. Anti-alpha toxin neutralizes alpha toxin. It can, therefore, ameliorate the terminal pathological changes in the disease. It has little or no effect on the establishment and early development of mastitis. This view is supported by the observations in older cows, that have higher level of serum anti-alpha toxin, the incidence of acute clinical episodes is lower (Anderson, 1978). Alpha toxin should be incorporated in any vaccine against *S. aureus* mastitis to prevent acute clinical flare-ups.

A trial was made by Ikramm (2004) for production, partial purification of *S. aureus* alpha toxin along with its antigenic response in rabbits. A total of 44 rabbits were divided into three groups i.e A, B and C. Group-A was further divided into 5 subgroups of 4 rabbits each (A1 thru A5). In group-A five subgroups were administered with different amounts of partially purified *S. aureus* alpha toxoid alone i.e. 1:10, 1:20, 1:40,
1:80, 1:160 haemolytic units (HU) respectively. Similarly group-B was further divided into 5 subgroups (B1 thru B5). Group-B was administered with different doses of Dextran sulphate adjuvanted alpha toxoid of \textit{S. aureus}. The group-C was kept as control. The analysis of purified alpha toxin was done by haemolytic activity and dermonecrotic activity in rabbits. The different levels of alpha toxin were determined by haemolytic units. Blood sampling was done at weekly intervals to collect serum from all subgroups for a period of three weeks in order to measure the antigenic response using the toxin neutralization test (Alpha haemolytic titre). There was production of alpha-haemolytic antibodies in both cases i.e. simple toxoid and adjuvanted toxoid. Alpha hemolytic antibodies titre was directly proportional to the concentration of toxoid dilution. As 1:10 HU showed highest alpha-hemolytic antibodies titre as compared to the remaining i.e. 1:20, 1:40, 1:80, and 1:160 HU. Dextran sulphate adjuvanted toxoid showed overall better result than simple toxoid at all concentration of toxoid in rabbits. Subsequently, similar type of study was also made by Ihsanullah (2004) about the beta toxin of \textit{S. aureus} in rabbits and similar trend of results was recorded.

2.5. Immune and other Defense Mechanisms of Bovine of Udder

Mammary gland is a complex, modified skin gland that provides neonatal offspring with milk for nourishment and disease resistance. Specific and innate immune factors associated with mammary gland tissues and secretion also play a vital role in protecting the gland from infectious diseases. Through genetic selection and technological advances in milk removal, the bovine mammary gland yields far more milk than is needed to nourish the newborn calf. This excess is the basis of the dairy industry (Sordillo \textit{et al.}, 1997).

Mammary gland is protected by a variety of defense mechanisms, which can be separated into two distinct categories: (i) Those related to innate immunity and (ii) specific immunity. Innate immunity (nonspecific responsiveness) is the predominant defense during the early stages of infection. Nonspecific responses are present or are activated quickly at the site of infection by numerous stimuli; however, they are not augmented by repeated exposure to the same insult. Nonspecific or innate responses of the mammary gland are mediated by the physical barrier of the teat end, macrophages,
neutrophil, Natural killer-like cells, and by certain soluble factors. Conversely, the specific or acquired immune systems recognize specific determinants of a pathogen that facilitate selective elimination. Recognition of pathogenic factors is mediated by antibody molecules, macrophages and several lymphoid populations. Because of the “memory” of certain lymphocytes, specific immune responses can be augmented by repeated exposure to a pathogen.

Mastitis occurs when bacteria gain entrance to the mammary gland via the teat canal. For this reason, the teat end is considered to be the first line of defense against pathogens. The teat end contains sphincter muscles that maintain the tight closure between milking and hinder bacterial penetration. Increased patency of these muscles is directly related to an increased incidence of mastitis (Murphy et al., 1953). The incidence of mastitis is lesser in buffaloes than in cows, under similar management conditions. As such there might be some genetic and anatomical factors operating in teat which might be providing higher resistance against mastitis in buffalo than in cow (Uppal et al., 1994). The histological differences in the teats of lactating cow and buffalo at the region of teat sphincter indicated that rich muscle fibers and vascular tissue in the buffalo teat may be the factors responsible for lesser intramammary infection in buffaloes (Krishnaswamy et al., 1965)

Teat canal is lined with keratin, which is crucial to the maintenance of the barrier function of the teat end, and removal of keratin has been correlated to an increased susceptibility to bacterial invasion and colonization (Bramley and Dodd, 1984; Capuco et al, 1992). Teat keratin is a waxy material that is derived from stratified squamous epithelium. The keratin structure enables the trapping of invading bacteria, thus hindering their migration into the gland cistern (Habbit et al., 1969). Within the keratin lining, antimicrobial agents have been identified. The esterified and nonesterified fatty acids present in teat keratin, such as myristic acid, palmitoleic acid, and linoleic acid, are bacteriostatic (Treece et al, 1966). Additionally, cationic protein in the teat canal can bind electrostatically to mastitis pathogens, which alters the bacterial cell wall, thus rendering them more susceptible to osmotic pressure. The inability to maintain osmolarity causes lysis and death of the invading pathogens (Murphy and Stuart, 1953; Treece et al., 1966).
Bacterial pathogens that are able to traverse the teat end opening must then escape the antibacterial activities of the mammary gland microenvironment in order to establish disease. The activities of resident and newly recruited leukocytes during the early stages of pathogenesis play a pivotal role in the establishment of intramammary infection (IMI). Milk somatic cell count (SCC) consist of several types, including neutrophils, macrophages, lymphocytes, and a smaller percentage of epithelial cells (Schukken et al., 2003). In the healthy lactating mammary gland, total SCC are often $<10^5$/ml of milk. During a bacterial IMI, however, total SCC can increase to $>10^6$/ml of milk within just a few hours (Paape et al., 1981; Persson et al., 1992). Studies (Hill, 1981; Grommers et al., 1989) have shown that the severity and duration of mastitis is critically related to the promptness of the leukocyte migratory response and the bactericidal activity of SCC at the site of infection. Some bacteria release metabolic by-products, enterotoxins, or cell-wall components as they colonize and grow in the mammary gland. These bacterial factors either directly or indirectly serve as chemoattractants for leukocytes. If somatic cells move rapidly from the blood stream and are able to eliminate the inflammatory stimuli (bacteria), then recruitment of leukocytes ceases, and SCC returns to healthful levels. If bacteria are able to survive, this immediate host response, then the inflammation continues, resulting in SCC migration between adjacent mammary secretory cells towards the alveolar lumen (Capuco et al., 1986). Prolonged diapedesis of leukocytes causes damage to mammary parenchyma tissue, resulting in decreased production of milk (Harmon and Heal, 1982; Sordillo and Nickerson, 1988). The duration and severity of the inflammatory response have a major impact on the quantity and quality of the milk produced (Sordillo et al., 1987; Sordillo et al., 1989).

Neutrophils are the predominant cell type found in mammary tissues and in mammary secretions during early inflammation and can constitute $>90\%$ of total mammary gland leukocytes (Paape et al., 1981; Sordillo et al., 1987; Sordillo et al., 1989). These nonspecific cells travel from the blood to the mammary gland in response to a variety of inflammatory mediators, such as cytokines, complement, and prostaglandins (Persson et al., 1993; Brunnmann and Graudie, 1994). Once at the site of infection, neutrophils phagocytose and kill bacterial pathogens. Neutrophils exert their bactericidal effect through a respiratory burst that produces hydroxyl and oxygen radicals, which are
key components of the oxygen-dependent killing mechanism. Bacteria are killed by the action of superoxide ions, hypochlorite, and hydrogen peroxide. During phagocytosis, bacteria also may be exposed to several oxygen-independent reactants such as peroxidase, lysozyme, various hydrolytic enzymes, and lactoferrin. In addition to their phagocytic capabilities, neutrophils are a source of small antibacterial peptides called defensins, which are able to kill a variety of the pathogens that cause mastitis (Selsted et al., 1993).

Macrophages are the predominant cell type found in the milk and tissues of healthy involuted and lactating mammary glands (Jonsen and Eberhart, 1981; Lee et al., 1980; Sordillo and Nickerson, 1988; Sordillo et al., 1998). Like neutrophils, macrophages are active mammary gland phagocytic cells that are capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson, 1988). The phagocytic rate of macrophages can be increased substantially in the presence of opsonic antibody for specific pathogens. Because of the indiscriminate ingestion of fat, casein, and milk components, mammary gland neutrophils and macrophages are less effective at phagacytosis than are blood leukocytes (Paape et al., 1981; Weber et al., 1983; Sordillo and Babiuk, 1991). The phagocytic and bactericidal activities of these cells are especially diminished during the periparturient period (Paape et al., 1981; Weber et al., 1983).

In addition to their role in early nonspecific defenses, macrophages also play a key role in antigen processing and presentation (Fitzpatrick et al., 1992; Politis et al., 1992). Antigens from ingested bacteria are processed within macrophages and appear on the membrane in association with major histocompatibility complex (MHC) class II antigens. These MHC class II antigens are polymorphic membrane molecules that are required for the recognition of foreign antigens.

Generation of effective specific immunity involves both antigen-presenting cells and lymphocytes. Lymphocytes are the only cells of the immune system that recognize antigens through membrane receptors that are specific for invading pathogens. There are two distinct subsets of lymphocytes, which differ in function and protein products, T and B lymphocytes. The T lymphocytes can be further subdivided into αβT lymphocytes,
which include CD4+ (T helper) lymphocytes and CD8+ (T cytotoxic or T-suppressor) lymphocytes, and γδ T lymphocytes. Depending upon the stage of lactation and tissue location, the percentages of these cells can vary significantly (Sordillo et al., 1997).

The primary role of B lymphocytes is to produce antibodies against invading pathogens. Unlike macrophages and neutrophils, B lymphocytes utilize their cell surface receptors to recognize specific pathogens. The B lymphocyte can internalize, process and present antigen in the context of MHC class II molecules to T-helper lymphocytes. Upon presentation of the processed antigen to T-helper lymphocytes, IL-2 is secreted by the T lymphocyte, which in turn induces proliferation and differentiation of the B lymphocyte into plasma cells that produce antibody or memory cells. Under certain conditions, differentiation of B lymphocytes can be directly stimulated by an antigen such as lipopolysaccharide. Unlike T lymphocytes, the percentages of B lymphocytes remain fairly constant between stages of lactation (Shafer et al., 1996).

Soluble factors are associated with defense functions of the mammary gland in concert with cellular defenses in milk and tissue; each system modifies the effector functions of the other. These soluble factors can be divided into innate and specific components. Immunoglobulins function as the soluble effector of specific or humoral immune responses. These proteins are produced by antigen-activated B lymphocytes that subsequently proliferate and differentiate into antibody secreting plasma cells. Antibodies in lacteal secretions are synthesized locally or are selectively transported or transudated from serum (Bastida, 1992; Sordillo and Nickerson, 1988). Four classes/isotypes of Ig are known to influence mammary gland defense against bacteria causing mastitis: IgG1, IgG2, IgA, and IgM. Each of these differs in physiochemical and biological properties (Gershwin et al., 1995; Musoke et al., 1987).

The concentration of each Ig class/isotype in mammary secretion varies depending on stage of lactation and infection status of the mammary gland. In healthy glands, the concentration of Ig is low during lactation but slowly increases during the nonlactating periods and reaches peak concentrations during colostrogenesis (Sordillo et al., 1987). High concentrations of Ig also occur in the mammary gland during inflammation. Total antibody concentration in normal milk is low (less than 1 mg/ml),
but during inflammation, concentrations approach 80 mg/ml in colostrum and secretions from infected glands. The main function of antibodies in milk is to coat or label bacteria so they can be recognized more easily for engulfment by neutrophils, a process known as opsonization. Antibodies also serve as antitoxins, which inactivates the poisons produced by bacteria in the udder (Nickerson, 1990).

The concentration of Ig in the gland is dependent upon the degree of permeability of secretory tissue and number of Ig-producing cells that are present in the mammary gland (Sordillo et al., 1987). Although, IgG₁ is the predominant isotype in healthy bovine lacteeal secretion, neutrophils can transport IgG₂ to the mammary gland as they emigrate to the site of inflammation (Musoke et al., 1987; Gershwin et al., 1995).

Research has shown that IgG₁, IgG₂, and IgM can act as bacterial opsonins that enhance phagocytosis of neutrophils and macrophages. These antibodies can bind bacterial pathogens directly or with the C3b component of complement (Howard et al., 1980). Neutrophils and macrophages can bind antibody-C3b-bacteria complexes via their Fc receptors and subsequently more effectively phagocytize the invading bacteria. In contrast, IgA appears to contribute to agglutination, preventing bacterial colonization and toxin neutralization (Musoke et al., 1987).

The mammary gland also contains nonspecific bacteriostatic components that work independently and in concert with Ig and cellular factors to provide protection to the mammary gland. These factors include lactoferrin, complement, lysozyme, and the lactoperoxidase-thiocyanate-hydrogen peroxide system.

Lactoferrin is an iron-binding protein produced by epithelial cells and leukocytes and in the presence of bicarbonate, sequesters free ferric ions present in milk. Lactoferrin is bacteriostatic by its ability to prevent growth of bacteria, such as Staphylococci and Coliforms, which have iron requirements (Bishop et al., 1976; Bullen et al., 1978). Withholding iron from bacteria may enhance killing by phagocytes by preventing the production of dismutase, a bacterial enzyme that inactivates superoxide radicals. Lactoferrin also may be active in modulation and regulation of macrophages, lymphocytes, and neutrophil functions (Smith and Oliver, 1981; Smith and Todhunter, 1982). In ruminants, lactoferrin and specific IgG₁ antibodies act synergistically to inhibit
the growth of *E. coli* and *Klebsiella pneumoniae* (Oliver and Bushe, 1987). However, the bacteriostatic activity of lactoferrin can be abolished in the presence of citrate, a buffer produced by the epithelial cells that chelates iron into a form that is readily usable by bacteria. Some bacteria, such as *Streptococcus agalactiae*, may be able to utilize lactoferrin as iron source by binding lactoferrin via surface receptors (Rainard, 1992). In the healthy mammary gland, the concentration of lactoferrin is low but increases during involution and inflammation (Sordillo et al., 1987). Because of the high concentration of citrate and the low concentration of lactoferrin produced during lactation, the foremost role of lactoferrin apparently is in defense of the involuted mammary gland, particularly against coliforms (Smith and Oliver, 1981; Muhammad et al., 1994).

Complement is a collection of proteins that is present in serum and milk, which functions in concert with a specific antibody to cause lysis of invading bacteria. Concentrations of complement are highest in colostrum, inflamed mammary glands, and during involution. In contrast, concentrations of complement are lowest during lactation. Therefore, because of its intermittent presence in milk, complement is thought to play only a minor bactericidal role in the mammary gland (Reiter, 1978; Reiter and Oram, 1980), however, complement-sensitive organisms, including some strains of *E. coli* are killed by the alternative complement pathway.

Lysozyme is a bactericidal protein that is present in milk and that functions by cleaving peptidoglycans from the cell wall of Gram-positive bacteria as well as the outer membrane of Gram-negative bacteria (Reiter, 1978). It may enhance the binding of lactoferrin to bacterial cell walls (Schambacher and Smith, 1975). Lysozyme limits chemotaxis and toxic oxygen production by neutrophils (Gordon et al., 1979). Because ruminant milk contains only a small concentration of lysozyme that is 300 times less than human milk (Chandran et al., 1964), this system may offer little protection to the bovine mammary gland.

The enzyme lactoperoxidase, in the presence of thiocyanate and hydrogen peroxide, is bacteriostatic for Gram-positive bacteria such as *S. aureus* and Streptococci and bactericidal for Gram-negative bacteria such as coliforms (Outteridge and Lee, 1988). However, several factors can vary the effectiveness of this system in the
mammary gland epithelial cells. Lactoperoxidase is produced in small concentration by mammary gland. The levels of thiocyanate in the mammary gland are dependent on the plane of nutrition.

The lactoperoxidase-thiocyanate-hydrogen peroxidase system exert its antibacterial properties through the production of hypothiocyanate, a reactive metabolite from the oxidation of thiocyanate (Richie et al., 1982). Myeloperoxidase produced by neutrophils also catalyzes the same reaction and additionally catalyzes the oxidation of chloride, the product of which provides the bacteriocidal activity of this system. In hamans, myeloperoxidase is entirely responsible for the antibacterial activity of this system (Moldoveanu et al., 1982). However, the low oxygen tension of the mammary gland can inhibit the production of hydrogen peroxide, thus limiting the effectiveness of this antimicrobial system against the pathogens that cause mastitis.

The role of cytokines in the pathophysiology of bovine mastitis has been the subject of many studies. Cytokines are naturally produced proteins that play an important role in essentially all aspects of host defense by regulating the activity of cells that participate in specific and non-specific immunity. The term cytokine describes a heterogeneous group of proteins produced by a spectrum of both immune and nonimmune cells under diverse circumstances. The immunomodulatory capacity of the cytokine network is complex. Individual cytokines can interact with other cytokines synergistically, additively, or antagonistically on multiple cell targets (Lawman et al., 1989). Cytokines are often referred to as hormones because they are usually produced transiently and locally with potent biological activity at extremely low doses. Because of their extreme potency, elevated levels of certain cytokines can be detrimental to the host as well. To date, >30 cytokines have been identified, purified, and characterized by their regulatory activities. As large quantities of recombinant cytokines become available for research, more information is being generated concerning the potential immunotherapeutic application of cytokines for the control of bovine mastitis. The major groups of cytokines studied to date include interleukin (IL), colony-stimulating factor (CSF), interferon (IFN), and tumour necrosis factor (TNF). IL-2 is the most extensively characterized of all bovine cytokines (Sordillo et al., 1997). Recent studies showed that colostrum samples that were obtained during the final week of gestation had low IL-2
activity, which correlates with diminished immune cell function and increased susceptibility to mastitis during this period (Sordillo et al., 1991). The possibility of enhancing bovine mammary gland defenses with IL-2 to increase resistance to mastitis has received considerable research attention.

2.6. Intraspecific (within the species) and interspecific Cross Reactivity in Staphylococci Associated with Mastitis

During the development of any vaccine, an important consideration has been the potential for the vaccine to protect the target animals from the great number of strains of specific organism and closely related microorganism which may cause the disease. Vaccine – challenge experiments have shown that the vaccine induces excellent protection from clinical mastitis following intramammary challenge with several heterologous strains in lactating cows and ewes (Watson, 1988). However, it is logistically very difficult to test more than a few challenge strains using this approach. Therefore an in vitro method was developed by Watson and Franklin (1988) for assessing the degree of immunological cross reactivity (for pseudocapsular antigens) between the vaccine strain of *S. aureus* and field isolates from cases of bovine mastitis. This assay was used to quantify the cross-reactivity of >150 isolates from Australia, New Zealand, Norway, the U.K. and the U.S.A. There was a large variation among strains in cross reactivity of their pseudocapsules with those of reference strain. For 104 Australian isolates, the range of cross-reactivity indices (CRI) was 6.1-63.2% (on a scale of 0-100%, with 0% being complete identity and 100% being nil identity); for 61 overseas strains the range of CRI values was 25.7-72.1%. The data indicated that pseudocapsule antigens of Australian strains were antigenically more closely related to those of the reference strain than were pseudocapsule antigens of strains from the 4 other countries.

Experiments were carried out in ewes using a new vaccine developed for the prevention of mastitis caused by *S. aureus*. The vaccine comprised three major components: (i) killed *S. aureus* cells which had been cultured to induce synthesis of pseudocapsule; (ii) toxoided staphylococcal beta haemolysin and (iii) the adjuvant dextran sulphate. Ewes systemically vaccinated twice during pregnancy developed significantly elevated circulating levels of IgG1 and IgG2 anti-pseudocapsule antibody,
as well as increased serum titres of anti-beta haemolysin. Five different strains of *S. aureus* were used to challenge both vaccinated and control ewes by the intramammary route during the ensuing lactation. The incidence of acute gangrenous mastitis and nonacute, clinical mastitis was significantly lower in vaccinated than in control groups after challenge with each strain. Vaccinated ewes produced significantly more milk than control ewes after challenge with four of the five strains of *S. aureus* (Watson, 1988).

Czechoslovakian workers, Rysannek *et al.* (1988) conducted a study on a *S. aureus* bacterin prepared from a virulent strain of organism together with alpha, beta haemolysin and aluminum hydroxide as an adjuvant. This bacterin was injected twice subcutaneously in the vicinity of supramammary lymph nodes at an interval of 18 days at the end of lactation. It gave protection against experimental infection with a heterogeneous strain of *S. aureus* and a reduced incidence of *S. aureus* mastitis was noted.

A study was carried out on the cross-reactivity of different *S. aureus* isolates recovered from mastitic buffaloes belonging to the 4 different districts (Faisalabad, Jhang, Tobataksing and Sargodha) of Punjab province of Pakistan. Antisera were raised against the collected isolates in rabbits following the standard method. Then the cross reactivity of isolates against the selected vaccinal *S. aureus* isolate was checked using direct slide agglutination test. There was 100 % cross reactivity indicating a potential of vaccine effectiveness in the field (Naeem, 2004).

As far as could be ascertained, no study has yet examined the cross-reactivity between *S. aureus* and around 30 other species of genus Staphylococcus which have reportedly been associated with mastitis in different parts of the world.

2.7. Important Bottlenecks in Production of Effective Mastitis Vaccine

There are several barricades which needs to be overcome in order to prepare an effective *S. aureus* vaccine. These include disparity of antigenic types, identification of virulence factors, lack of proper inoculation route and time schedule in relation to lactation cycle. In addition to it, several bacterial species responsible for causing mastitis are among the basic facts that prevent the formulation of an efficacious mastitis vaccine. An other more fundamental problem relevant to staphylococcal mastitis immunization is
the mammary gland's invasion by pathogenic Staphylococci leading to inflammatory response. (an increased milk somatic cell count together with excretion of Staphylococci is a criteria of mastitis) which is a normal udder defence. An immunization can only function by enhancing this mechanism. It is therefore, inevitable that the reaction in the immunized gland to invasion by Staphylococci constitute mastitis. Only when protection can be achieved by eliciting an inflammatory response which is insufficient to constitute sub-clinical mastitis, will immunization against bovine mastitis have succeeded (Anderson, 1978). The lack of effective vaccines can be attributed to incomplete knowledge of the pathogenesis of staphylococcal mastitis and of the defence mechanisms of the ruminant udder (Rainard and Poutrel, 1991; Watson, 1992; Fitzpatrick, 2000).

Yancey (1999), while reviewing vaccines and diagnostic methods for bovine mastitis concluded that a number of problems were associated with vaccination of dairy cows against mastitis. One of these was that the number of mastitis pathogens was numerous and heterogeneous. Vaccine development efforts thus far have concentrated mainly on the major mastitis pathogens. While at least one S. aureus bacterin has been commercially available for a number of years, within the next decade, additional efficacious vaccines for several of the most common agents of mastitis are likely.

Effective immunization against mastitis has been a goal of mastitis researchers for many years. Several authors have reviewed the problems associated with vaccination against mastitis (Anderson, 1978; Nickerson, 1999; Yancey, 1993). The nature of the disease creates a number of unique challenges for the production of successful immunity against mastitis (Yancey, 1993). Mastitis is defined as inflammation of the mammary gland, yet the purpose of vaccination is to enhance the immune response. In case of mastitis, an enhanced immune response is not always considered beneficial. One component of the immune response is the migration of large numbers of white blood cells (in the udder called somatic cells) to the infected gland. The presence of somatic cells in the milk is not considered a positive outcome as somatic cells are evidence of mastitis and reduce milk quality. Effective immunization is difficult because of the very nature of milk (Yancey, 1993). The volume of milk present in the gland dilutes the number of immune cells available to fight infection and milk components such as fat and casein reduce the bactericidal abilities of the infection fighting immune cells. Additionally, a dairy animal is exposed to numerous organisms that
have the potential to cause mastitis and milk is an excellent substrate for bacterial growth. The definition of a successful mastitis vaccine may vary depending upon the herd situation. Farmers may expect mastitis vaccines to reduce the severity and frequency of mastitis, prevent new infections and eliminate existing infections (Yancey, 1993). While these expectations seem reasonable, it is unlikely that any one vaccine will be able to achieve all of these objectives. Furthermore, the evaluation of mastitis vaccines is complicated by the underlying biology of the various mastitis pathogens. One of the most frustrating mastitis pathogens is *S. aureus*. This organism is a highly successful mastitis pathogen in that it has evolved to produce infections of long duration with limited clinical signs. Most infections with this pathogen are sub-clinical in nature and are detected by the production of poor quality milk. While clinical mastitis may occur sporadically, affected animals rarely become seriously ill and the major economic effect of this disease is reduced milk yield and quality premiums received by the producer. Animals are at risk for this organism throughout lactation and often becoming infected after prolonged periods of exposure. Unless a vaccine can prevent new infections throughout lactation and dramatically reduce the SCC of affected animals, it may be difficult for a producer to recognize the benefit of using a *S. aureus* vaccine (Ruegg, 2001).

The pseudocapsule of *S. aureus* increases the resistance of this microorganism to phagocytosis (Foster, 1991; Rainard and Poutrel, 1991; Watson *et al.*, 1993). This is expressed during growth in *vivo* (Watson, 1982) or in simulated conditions (Watson and Watson, 1989). However, its production in *vitro* is influenced by the composition of growth medium (Jhone *et al.*, 1989; Mamo *et al.*, 1987; Norcross and Opdebeeck, 1983; Rainard and Poutrel, 1991; Rather *et al.*, 1986). Cultivation methods to achieve an optimum production of *S. aureus* capsular exopolysaccharides (CPS) are still the subject of debate, as is also the exact structure of this pseudocapsule (Foster, 1991; Mamo *et al.*, 1991; Norcross and Opdebeeck, 1983; Rather *et al.*, 1986; Watson and Watson, 1989). However, several researchers (Norcross, 1991; Rainard and Poutrel, 1991; Watson *et al.*, 1993) agree that this pseudocapsule should be included in a *S. aureus* mastitis vaccine (Nordhaug *et al.*, 1994b).

Several research workers (Watson, 1981; Watson, 1984; Nansen, 1972; Mathison *et al.*, 1984) supported the proposition that IgG₂ antibody is critically important for immunity to staphylococcal mastitis in ruminants. IgG₂ appears to mediate protection by
opsonizing Staphylococci for phagocytosis by neutrophils (Watson, 1976). Neutrophil-mediated phagocytosis is the major mechanism for controlling bacterial infections in the ruminant mammary gland (Jain, 1976) and ruminent neutrophils have membrane receptors for IgG2 and C3b but not for other immunoglobulin isotypes (Watson, 1975; McGuire et al., 1979; Grewell et al., 1978). It is now known that live S. aureus vaccines stimulate substantial IgG2 responses (Kennedy and Watson, 1982) whereas killed S. aureus vaccines given with Freund's adjuvant promoted synthesis of the IgG1 subclass (Kennedy and Watson, 1982). Because of the obvious disadvantages of using a live S. aureus vaccine, an immunological adjuvant was sought which may predict a strong IgG2 anti-bacterial response when given to ruminants in combination with killed S. aureus vaccines. Dextran sulphate showed promise in this respect (Watson, 1987).

A substantial and durable IgG2 anti-CPS response as well as modest but sustained increase in circulating level of alpha toxin antibody is essential. An Earlier study (Cox, 1997) had shown that it was extremely difficult to achieve IgG2 anti-S. aureus responses with killed vaccines. Production of an effective S. aureus mastitis vaccine has presented several challenges because of involvement of several virulence factors in the immunity against this organism. Relyveld (1984) has shown that crucial virulence factor appears to be the CPS which is expressed by the organism when grown in whey-supplemented medium. Thus, it appears that vaccine capable of conferring protection from staphylococcal mastitis needs to contain capsular polysaccharide (CPS). Furthermore, mastitis vaccination challenge study (Han et al., 1998a; Han, et al., 1998b) has shown that increased levels of circulating alpha toxin antibody were also necessary for protection from highly toxigenic strains.

2.8. Special Cultural Conditions for Expression of Immunologically Important Virulence Factors

Although a few strains of S. aureus produce a true capsule (Watson and Watson, 1989), in general the organism is not encapsulated. However, when growing under in vivo conditions in a variety of lesions, S. aureus produces an extracellular glycocalyx comprised largely of hydrated polysaccharides (Watson, 1982; Mayberry-Carson et al., 1984). The glycocalyx enhances virulence of the organism by impairing complement and antibody-mediated opsonization and inhibiting phagocytosis (Karakawa and Young,
This pseudocapsule acts as an important virulence determinant for *S. aureus* in the ruminant mammary gland by impeding neutrophil-mediated phagocytosis of the organism (Watson, 1982). Expression of glycocalyx usually ceases when *S. aureus* is cultured under *in vitro* conditions in conventional bacteriological media (Watson, 1982). For the development of a successful vaccine, a crucial requirement is incorporation of bacterial cells shrouded with pseudocapsule. Pseudocapsule synthesis is induced by growing the organisms under simulated *in vivo* cultural conditions i.e. in dilute milk whey (Watson, 1987).

Effort was made by Watson (1982) to determine the source of virulence (capsule or pseudocapsule) of *S. aureus* by growing *in vitro* or *in vivo*. Paired comparisons were made of various strains of *S. aureus* grown in broth inside dialysis sacs anchored in the peritoneal cavities of sheep (*in vivo* culture) and in a variety of bacteriological media (Standard nutrient broth, brain-heart infusion broth, tryptone-soya broth, or Muller-Hinton broth, 5 per cent sheep blood agar, and mannitol salt agar (Oxoid)) in the laboratory (*in vitro* culture). The organisms grown *in vivo* possessed enhanced virulence compared with *in vitro* grown organisms, when injected intradermally in sheep, when injected intraperitoneally in mice and when infused into lactating mammary glands of ewes. Growth under *in vivo* conditions conferred on the bacteria an increased resistance to phagocytosis by ovine neutrophils. The bacteria grown under *in vivo* conditions possessed an additional cell-associated components as determined by immunodiffusion tests and optical density profiles of gel filtration eluates: however, this substance was not visible in electron micrographs in the form of a capsule.

Watson and Watson (1989) conducted a study on the expression of a pseudocapsule by *S. aureus* by growing it in a variety of cultural conditions. Strains of *S. aureus* from cases of mastitis in ruminants were cultured in various media and the cells examined electron microscopically for a pseudocapsule. Organisms grown inside the udder, or in nutrient broth supplemented with ovine, bovine or caprine milk whey ("udder broth") produced a large, well-defined pseudocapsule outside the cell wall, but such cells had no true capsule when tested by the India ink method. Modified Staphylococcus 110 medium induced secretion by the organisms of copious extracellular slime but did not induce the pseudocapsule. Organisms grown in the presence of milk whey showed a
strong propensity to autoagglutinate and produced pseudocapsular material which contained antigens in common with Staphylococci grown \textit{in vivo}. Supplementing media with lactose, dextrose or casein failed to modify the cell surface of \textit{S. aureus} in a manner analogous to that induced by ruminant milk whey.

\textit{Staphylococcus aureus} cultured under \textit{in vivo} conditions expresses additional cell-surface antigens when compared with \textit{in vitro}-grown organism of the same strain (Watson and Prideaux, 1979). \textit{In vivo} grown \textit{S. aureus} are more virulent/pathogenic than their \textit{in vitro}-grown counterparts (Watson, 1982). The \textit{in vivo} antigens are associated with a pseudocapsule (or glycocalyx). The pseudocapsule has been shown to be present on \textit{S. aureus} in milk of cows suffering clinical staphylococcal mastitis (Watson, 1989). By growing \textit{S. aureus} under simulated udder condition (i.e. in nutrient broth complemented by bovine milk whey) to express production of pseudocapsule, one can circumvent the need for \textit{in vivo} culture to express this immunologically important component of \textit{S. aureus} for vaccine production.

Another critical hurdle in developing a practical vaccine was finding an immunological adjuvant which, when combined with \textit{S. aureus} bacterin, would preferentially stimulate synthesis of IgG\textsubscript{2} anti-staphylococcal antibody. It has now been established that dextran sulphate has these properties (Kirlin and Watson, 1987).

2.9. Vaccines Trials

Since the inception of vaccination as a means of controlling infectious diseases in livestock and humans, attempts have been made to vaccinate animals against mastitis. Generally these attempts have not met with desirable success for a variety of reasons including but not limited to the following (Colditz and Watson, 1985): (1) Mastitis may be caused by a very wide range of microbes (mainly bacteria). (2) Many of the attempts to vaccinate were empirical in nature or naively followed protocols that had been successful with other diseases (e.g. systemic immunization with toxoids). (3) Little information was available on the immunology of the mammary gland and the role of virulence factors of pathogens in the production of immunity. In recent times, rapidly accumulating new information on bacterial pathogenesis, the inflammatory response, and
expression of immunity on secretory epithelia has provided opportunities and impetus for
the development of vaccines to control mastitis in ruminants. (Watson et al., 1993)

As pointed out earlier, mastitis is a disease complex in which a variety of bacterial
species may be involved. It follows that the problem of immunity is complex. It is
unlikely that a single vaccine could protect against all forms of mastitis. Notwithstanding
this, Derbyshire (1962) expressed the optimism that for certain specific infections, an
efficient vaccine would be of considerable value in future.

2.9.1. Monovalent and Bivalent Vaccines

Conventionally prepared S. aureus bactrins, with or without toxoids and with or
without adjuvants, stimulate very poor IgG2 antibody responses and provide little or no
protection from Staphylococcal mastitis (Watson, 1989; McDowell and Watson, 1974;
Watson and Kennedy, 1981). Mellenberger (1977) has reviewed the role of vaccination
against mastitis. According to the author, challenge experiments with mice, goats, and dairy
cows have indicated that vaccination during lactation and during the non-lactating state will
decrease the rate of new infections by Strep. agalactiae and S. aureus and lessen the severity
of clinical attacks. In contrast, field studies with commercial dairy herds have shown that
immunization with bacterin-toxoid vaccines does not decrease the new infection rate caused
by Streptococcal and Staphylococcal bacteria. Despite an increase in serum antibody titre,
milk whey contains less than 2% as much staphylococcal antitoxin as blood serum since
degree of permeability between mammary tissue and blood vessels determines the quantity
of milk antitoxin. The antitoxin titre of milk does not increase following a subcutaneous or
intramuscular injection of S. aureus antigen because an irritation or bacterial invasion of
mammary epithelium is required before substantial quantities of circulating antibodies pass
into mammary tissue or milk during lactation.

A study was conducted by Watson and Kennedy (1981) on immunization against
experimental Staphylococcal mastitis in sheep by observing an effect of challenge with a
heterologous strain of S. aureus. Ewes were immunized in late pregnancy with killed S.
aureus vaccines prepared from organisms grown either under in vitro (vaccine T) or in
vivo (Vaccine V) cultural conditions; other ewes were immunized with a live S. aureus
vaccine and a further group remained non-vaccinated controls. On criteria of milk
production data, bacteriological status of milk and clinical signs of acute mastitis, it was apparent that animals which had been immunized with the live vaccine were better protected from challenge than those immunized with either killed vaccines.

An investigation was carried out on the effect of immunization on the early influx of neutrophils during staphylococcal mastitis in ewes. The neutrophil influx into mammary secretions was studied in unimmunised, and in systemically and locally immunized, lactating and non-lactating ewes experimentally infected with S. aureus. Systemic immunization was affected by subcutaneous injection of live bacteria or by intramuscular injection of killed bacteria in Freund’s incomplete adjuvant. The results suggested that differences in the rate of influx of neutrophils into infected mammary glands of immunized and unimmunised ewes could be attributed to immunological enhancement of neutrophil recruitment or to limitation of toxic damage to tissues with consequently diminished neutrophil invasion (Colditz and Watson, 1982).

Kennedy and Watson (1982) conducted a study on cellular basis for differences in humoral immune responses of sheep immunized with live or killed S. aureus vaccines. The IgG₂-containing cell response was significantly greater than the IgG₁-containing cell response in abscesses and nodes. In contrast, the IgG₁-containing cell response predominated over the IgG₂-containing cell response in granulomas and draining nodes. There were generally greater numbers of cells containing anti-staphylococcal antibody in abscesses/granulomas. Similarly, Watson and Campbell (1979) conducted a study on the vaccination against experimental staphylococcosis in sheep evaluating the observations on bacteriology and pathology following challenge. Sheep were vaccinated with a killed S. aureus vaccine (2 doses) which had been cultured in vitro (Group 1), a killed S. aureus vaccine (2 doses) cultured in vivo (Group 2) or a single dose of a live vaccine (Group 3). Other sheep were used as non-vaccinated controls. All sheep were challenged by intravenous injection of $2.6 \times 10^{11}$ washed, viable S. aureus organisms, the vaccinated animals being given the challenge inoculums at various intervals after vaccination. The control sheep survived for 29 hours (mean) after challenge. Animals given killed vaccines survived longer, (particularly Group 2) if challenged less than 40 days post-vaccination, compared with those challenged more than 40 days post-vaccination. Animals in Group 3 survived longer if challenged after 40 days post-vaccination than
those in Groups 1 or 2. There were no significant differences between the treatment groups for numbers of *S. aureus* recovered from blood in the 3 hours period following challenge. Histological and bacteriological examination showed that the kidneys were more severely affected by the challenge inoculum than heart, spleen, liver or lungs. The kidneys showed both toxigenic and lymphoreticular reactions and large numbers of Staphylococci were recovered from kidneys than other organs.

Conflicting reports have appeared in literature concerning the efficacies of bacterin. Brock *et al.* (1975) conducted a study to determine the effect of intramuscular and intramammary vaccination of cows on antibody levels and resistance to intramammary infection by *S. aureus*. Cows were vaccinated simultaneously by the intramuscular and intramammary route with formulated *S. aureus* cells of strains BB, Mexi and 3528. Vaccination resulted in slight increases in serum agglutinin titres, but the levels of the agglutinins in milk and colostrum were not higher in vaccinated cows than in the unvaccinated controls. Vaccination did not result in higher levels of IgM, IgG1, IgG2 or IgA immunoglobulins in serum, colostrum or milk as compared with controls. Vaccinated cows, particularly those in which strain 3528 was used, showed some resistance to infection following challenge with low numbers of viable *S. aureus* Mexi, but there was no resistance to infection when similar numbers of the virulent BB strain were used for challenge. It was concluded that vaccination is unlikely to prevent infection of the bovine udder by *S. aureus*.

Yoshida *et al.* (1984) conducted a study on a staphylococcal capsular vaccine for preventing mastitis in two herds in Georgia, USA. The vaccine consisted of heat-killed capsular type A and B *S. aureus* strains. It was administered intramuscularly to 97 and 125 cows in the herds. An equal number of animals were kept as controls. Two weeks after primary vaccination, a booster injection was given. No side effects were observed, in herd 1, leukocyte content of milk decreased markedly one week after the booster injection. Significant resistance to infection was maintained for 4 months after vaccination and loss of milk was about half that of untreated controls. On the other herd, incidence of mastitis, numbers of infected quarters and leukocyte counts decreased as early as one week after primary vaccination and resistance to infection continued for 6 months.
An optimal concentration of antigen is required for eliciting a desirable antibody response. Opdebeek and Norcross (1982) conducted an experimental study on the antibody response in lactee secretions of cows after immunization with various concentrations of staphylococcal and streptococcal antigens. A total of 55 lactating Holstein cows were randomly allocated to 6 groups. Five of these groups (No. 2 through 6) were inoculated on 2 occasions in the region of the external inguinal lymph node with various concentrations of 3 bacterial antigens. Saline solution was administered to group 6 as a control. The antigen preparations consisted of a *S. aureus* bacterin, a *Strep. agalactiae* bacterin, and staphylococcal alpha-toxoid. These antigens were administered as a composite preparation suspended in saline solution. The concentration of each of the 3 vaccine components which was required to stimulate a maximal immune response in the lactating gland appears to have been established.

Routes of administration of an antigenic preparation also affects the level of immunity produced. Nickerson *et al.* (1985) designed a study to assess the extent of the enhancement of the cellular immune response of the bovine udder by local and systemic immunization against staphylococcal mastitis in 30 first-lactation Jersey cows. Ten cows were inoculated with a *S. aureus* protein A vaccine in the supramammary lymph node area and 10 with a commercial inactivated vaccine intramuscularly. A group of 10 was left untreated control. Over three lactations, numbers of intramammary *S. aureus* infections were similar in the three groups, but the vaccinated groups had higher spontaneous cure rates (83% and 73%) than the control (47%). At the end of the trial, cytological analysis of infiltrating leukocytes in teat and tissue of infected and uninfected quarters gave higher counts of lymphocytes, plasma cells and total leukocytes in vaccinated cows.

Nickerson *et al.* (1992) evaluated the effect of a *S. aureus* bacterin on serum antibody, new infection and mammary histology in non-lactating dairy cows. The influence of this *S. aureus* mastitis vaccine on immunologic status and rate of new IMI was evaluated. At drying off, cows were vaccinated, either intramuscularly or subcutaneously in the area of the supramammary lymph node, or left as unvaccinated controls; vaccinates received booster injections at 6th week. Serum antibody concentrations, bacteriologic status, and SCC of quarter milk samples were determined.
Four weeks after revaccination, cows were challenged by intramammary infusion of *S. aureus* and then killed 24 to 72 h later. Mean serum antistaphylococcal antibody titre of vaccinated cows during the trial was 4.7-fold that of controls. Challenge resulted in IMI rates of 92, 36, and 60% for control cows, cows vaccinated intramuscularly, and cows vaccinated in the area of the supramammary lymph node. Vaccination by either route had no influence on mammary parenchymal tissue components compared with controls; however, leukocyte infiltration was greater in quarters from cows vaccinated in the area of the supramammary lymph node than in quarters from unvaccinated controls. Plasma cell populations producing IgG1, IgG2, IgA, and IgM were the greatest in quarters of cows vaccinated in the area of the supramammary lymph node followed by those in quarters of cows vaccinated intramuscularly and control cows.

An experimental challenge study on the evaluation of protein A and a commercial bacterin as vaccines against *S. aureus* mastitis was undertaken by Pankey et al. (1985). Protein A and a commercial staphylococcal bacterin were evaluated by experimental challenge with *S. aureus* (ATCC 29740). Thirty cows in first lactation were allowed to 3 treatment groups, viz., protein A, bacterin, and non-vaccinated controls. Studies were through three lactations and included bacteriological and cytological analyses of quarter milk samples. Rate of intramammary infection with *S. aureus* was similar for vaccinated and unvaccinated cows. Rates of spontaneous cure within each lactation were significantly higher for vaccinated cows. For all three lactations, spontaneous cure rates were 83, 73, and 47% for protein A, bacterin, and control cows, respectively. Somatic cell counts were significantly lower for vaccinated cows for quarters infected with *S. aureus*, but no differences were demonstrated for milk production by lactation. Incidence of clinical mastitis was higher in unvaccinated cows, but a few experimental animals developed clinical mastitis for a valid comparison.

An experimental investigation was made by Watson (1986) on a killed *S. aureus* mastitis vaccine which was prepared by growing the organism in a nutrient medium containing milk components (preferably sterilized whey). He concluded that the vaccine should preferably also include a toxoid component (predominantly beta-haemolysin excreted by the *S. aureus*) and an adjuvant, like, dextran sulphate to stimulate production of IgG2 antibodies.
A trial was conducted on the ovine opsonins for *S. aureus* cell wall and pseudocapsule. Blood serum, milk whey and purified IgG₁ and IgG₂ antibody preparations from sheep immunized with live or killed *S. aureus* vaccines were assayed for opsonising activity against *S. aureus* using ovine mammary neutrophils as effector cells. Serum had much higher levels of opsonins than whey; whey did not inhibit the potent opsonising activity of hyperimmune serum. IgG₁ antibodies against cell wall antigens of *in vitro*-grown *S. aureus* and pseudocapsular antigens of *in vivo*-grown *S. aureus* had low opsonising activity. IgG₂ antibody against *in vitro*-grown *S. aureus* surface antigens had significantly greater opsonising activity than IgG₁ of this specificity. The most powerful opsonising activity was associated with IgG₂ antibody against the pseudocapsular antigens produced by *S. aureus* grown under *in vivo* or simulated *in vivo* cultural conditions. Australian workers (Watson and Schwartzkoff, 1990) conducted a field trial in 5 commercial dairy herds using a patented staphylococcal mastitis vaccine comprising killed *S. aureus* bacteria (cultured under simulated *in vivo* conditions), staphylococcal toxoids and a composite immunological adjuvant. The vaccine or placebo (5 ml) was given by deep intramuscular injection into the gluteal muscles. The priming dose was given approximately eight weeks pre-calving and the booster dose in the contralateral gluteal muscles approximately four weeks pre-calving. It was concluded that this vaccine had a high level of efficacy. There was a considerable variation between herds in terms of prevalence of mastitis but among the five herds involved in the trial, clinical mastitis caused by *S. aureus* was reduced by 45–52% in vaccinates, compared with controls. Levels of sub-clinical mastitis were reduced by 18% in vaccinates compared with controls. New sub-clinical infections with *S. aureus* were reduced by 25% in vaccinates, compared with controls. These important reductions in mastitis were achieved in the first lactation following commencement of vaccination. It was also suggested that continuing a vaccination programme over a number of years would progressively reduce the infection status of the herd and provide a cumulative improvement in herd health (Watson, 1989).

Nickerson (1991) determined the efficacy of a *S. aureus* bacterin for the prevention of new intramammary infection in non-lactating dairy cows. Serum analysis for anti-staphylococcal antibodies demonstrated that titres of vaccinated cows were approximately 4.7-fold those of unvaccinated controls and remained elevated throughout a trial period of
10 weeks. Results showed that vaccination provided protection against *S. aureus* challenge in dry cows. Watson (1992) cited a review by Foster (1991) on some of the recent advances in the knowledge of *S. aureus* antigens. The reviewer concluded that results of strategic research in this area led to the development of a vaccine which was effective in controlling staphylococcal mastitis in ruminants.

Gilbert *et al.* (1994) conducted a study on immunogenicity of *S. aureus* type 5 capsular polysaccharide-ovalbumin conjugate. Six dairy cows were immunized subcutaneously with purified type 5 capsular polysaccharide (CP5) of *S. aureus* or CP5-ovalbumin conjugate in Freund’s incomplete adjuvant. The CP5 antibody titres were measured in sera and the isotypes identified by ELISA. At the doses tested, the purified CP5 did not induce a humoral response in the cows. Immunization of 2 cows with the CP5-ovalbumin conjugate elicited a CP5 antibody response mainly of the IgG2 isotype, after 4 weeks. A second injection of conjugate, 3 months after the first, resulted in a rapid and lasting anti-CP5 response without exceeding the first antibody peak value. Intramammary infusion of purified CP5 did not induce an inflammatory response in the milk of immunized cows. A marked recruitment of cells occurred in the milk of the sensitized cows after intramammary infusion of ovalbumin. It was concluded that injection of CP5-protein carrier conjugate in cows produced both antibody response against CP5 and carrier-specific recruitment of cells in milk of immunized animals. Likewise, Amorena, *et al.* (1996) conducted a study on the evaluation of vaccines against staphylococcal mastitis in dairy cattle under commercial farm condition. A preliminary study was carried out to evaluate the effect of a staphylococcal mastitis vaccines on SCC, and milk production, under natural conditions in commercial farms of cows. Compared with the control group, the vaccinated group had an increase of 17.8% in the proportion of animals which presented a lower SCC in the 5 months following vaccination than in the 5 month before vaccination. In the 5 months following vaccination, vaccinated animals had a lower SCC (20.6% reduction) and higher milk production (6.1% increment) in relation to the control group, suggesting that the economic benefit of the vaccine may be 13 times higher than the cost.

A field trial of a staphylococcal mastitis vaccine was conducted by Watson *et al.* (1996). Approximately half the animals were vaccinated and the remainder were non-
vaccinated controls. The vaccine was given twice during the last 10 weeks of pregnancy. Effects of vaccination were assessed during the ensuing lactation on the basis of frequency of clinical and sub-clinical mastitis and microbiological investigations of the milk. A total of 273 cases of clinical mastitis was recorded. *S. aureus* was isolated from 112 of these, 45 cases in vaccinates and 67 cases in controls; the difference was not statistically significant. One herd was notable in having a high incidence of clinical staphylococcal mastitis. This herd accounted for 15.8% of the animals in the field trial, but 54.5% of cases of clinical staphylococcal mastitis. For this herd, vaccinated animals had significantly lower incidence of clinical staphylococcal mastitis and prevalence of sub-clinical mastitis, relative to controls. An unexpected feature of the trial as a whole was the low incidence of clinical mastitis from which *S. aureus* was isolated in pure culture (26.3% of cases) and the high incidence of clinical *Strep. uberis* mastitis (22.7% of cases). This trial showed that the vaccine was effective in reducing the incidence of clinical mastitis and prevalence of sub-clinical mastitis in a herd that had a serious staphylococcal problem.

Calzolari *et al.* (1997) conducted a field trial for the evaluation of a vaccine against bovine mastitis in heifers. A vaccine against bovine mastitis was developed. The vaccine was based on inactivate, highly encapsulated *S. aureus* cells; a crude extract of *S. aureus* exopolysaccharides; and inactivated unencapsulated *S. aureus* and *Streptococcus spp.* cells. The results of this trial revealed significantly fewer intramammary infections caused by *S. aureus* at various levels of severity (clinical, sub-clinical, and latent) in cows that were vaccinate. The odds ratios of all types of intramammary infections caused by *S. aureus* for dairies A and B, which were determined by a logistic mode, were 1.84 and 1.89, respectively, for quarters of vaccinated cows and quarters of controls cows. The colony counts for *S. aureus* in milk from infected quarters of vaccinated cows were significantly lower than those in milk from infected quarters of control cows. Also, the somatic cell counts per millilitre in milk from vaccinated cows were significantly decreased when the initial somatic cell count was <500,000 cells/mL at the start of the trial. The vaccine had no observable effect on fat production in milk or on streptococcal infections. In another trial, these workers (Giraudo *et al.*, 1997) tested the same vaccine on 30 heifers during a 7-month trial period. The frequencies of intramammary infections caused by *S. aureus* were reduced from 18.8% for heifers in the control group to 6.7 and
6.0% for heifers in the prepartum and postpartum groups, respectively. This protective effect was maintained for at least 6 months. The relative risk of mastitis caused by *S. aureus* was 0.31 (P=0.0001) and 0.28 (0.0001) for heifers in the prepartum and postpartum groups, respectively, compared with that for heifers in the control group. The results of the trial indicated the effectiveness of the vaccine in decreasing the incidence of intramammary infections caused by *S. aureus*.

Nickerson *et al.* (1997) carried out a study on 70 dairy heifers to evaluate a polyvalent commercial *S. aureus* vaccine (Lysigen®; Boehringer Ingelheim Animal Health, Inc., St. Joseph, Missouri, USA) in order to determine the possibility of reduction of mastitis at the time of freshening. Animals were divided into two groups of 35 each. One group was vaccinated @ 5 ml per animal intramuscularly and the other group was kept unvaccinated control. Sera were collected for subsequent analysis of antistaphylococcal antibody titres. It was found that the rate of new infection was lower in vaccinates compared with that in controls (8.9 vs 16.2%). Likewise, at freshening, the percentage of quarters infected with *S. aureus* was lower in vaccinates compared with that in controls (3.6 vs. 10.3%). This study demonstrated very clearly a positive effect of vaccination in preventing new infections and reducing the chronicity of infection in dairy heifers.

Korean workers (Hwang *et al.*, 2000) conducted a study to evaluate clinical effects of autogenous toxoid-bacterin treatment of *S. aureus* sub-clinical mastitis in lactating cows, 22 cows which had at least on *S. aureus* infected quarter were selected from among cows at a *S. aureus* prevalent dairy farm. Eleven cows were injected with their own autogenous toxoid-bacterin and the others were maintained as non-injected control. In the toxoid-bacterin injected group, 27% of infected quarters were cured during the 12-week trial, compared to 5% in the control group. New intramammary infections with *S. aureus* were only detected in 3 quarters of the control group. Mean IgG antibody titre against *S. aureus* somatic antigens and alpha-toxin in serum and milk were significantly increased in the toxoid-bacterin injected group (p<0.05) and remained higher than those of the control group which showed no significant changes (p<0.05). In contrast to the control group, from 3 weeks after the second injection of the toxoid-bacterin injected group, mean *S. aureus* cfu/mL in milk samples from injected quarters
with *S. aureus* was significantly decreased until the end of the study (p<0.05). In the toxoid-bacterin injected group, significant decreases of mean somatic cell count (SCC) were detected from milk samples from infected quarters with *S. aureus* from week 7 to week 10 (p<0.05). The data showed that autogenous toxoid-bacterin treatment against *S. aureus* sub-clinical mastitis in lactating cows may increase the cure rate of the infections, reduce the severity of the infections and also prevent occurrence of the new infection.

2.9.2. Live Attenuated *S. aureus* Mastitis Vaccines

A feature of the scientific literature on Staphylococcal mastitis vaccines is the relatively low efficacy achieved with bacterin and toxoid vaccines, used with or without adjuvants (Anderson, 1978). In contrast, there are just a few reports of subcutaneous immunization with live *S. aureus* vaccines and the data suggested that this approach to vaccination offered considerable promise (Brider, 1907; Derbyshire, 1961). Studies carried out in Australia using sheep have confirmed that live *S. aureus* vaccines given subcutaneously provided significantly greater protection from experimental Staphylococcal mastitis than did conventionally prepared killed *S. aureus* vaccines (Watson and Kennedy, 1981). Several immunological and inflammatory mechanisms appeared to be responsible for the immunity which was observed following vaccination with a live *S. aureus* vaccines.

Immunizing ewes with live *S. aureus* vaccines results in mammary neutrophils from these animals having enhanced phagocytic capacity compared with neutrophils from nonimmunized ewes or ewes immunized with killed staphylococcal vaccines (Watson, 1975). This enhanced phagocytic capacity is specific for *S. aureus* and is attributable to cytophilic IgG2 antibody on the neutrophil membrane (Watson, 1976). Live staphylococcal vaccines promote the synthesis of IgG2 antibody whereas killed staphylococcal vaccines induce much greater synthesis of IgG1 than IgG2 (Kennedy and Watson, 1982; Watson, 1987).

Systemic immunization of ewes with live *S. aureus* results in an enhanced inflammatory response in the mammary gland following intramammary infection with Staphylococci (Colditz and Watson, 1982). Most of these early inflammatory cells were neutrophils and the identification of this mechanism had broad implications for mastitis.
vaccines as Hill (1981) showed that the severity of experimental coliform mastitis in cows depended on the rate at which neutrophils were mobilized from blood into the gland, as well as the presence of opsonins in mammary secretion. In case of ewes immunized with live S. aureus vaccine, the arrival of neutrophils at the infected focus occurred earlier, the neutrophil influx was of greater magnitude in the first 6 hours postinfection, and these freshly recruited neutrophils carried cytophilic IgG2 opsonins on their membrane.

It is now established that fresh clinical isolates of S. aureus possess a pseudocapsule (glycocalyx) that may be lost on subculture in vitro (Costerton et al, 1981). In vivo growth induces expression of the pseudocapsule (Speers and Nade, 1985; Watson and Prideaux, 1979) and it was found that S. aureus in milk collected from cows with naturally acquired, clinical, staphylococcal mastitis had a thick pseudocapsule shrouding the cell wall (Watson and Watson, 1989). S. aureus grown under in vivo cultural conditions (in dialysis sacs implanted in the peritoneal cavity of sheep) were more virulent than when grown under in vitro conditions (Watson, 1982). This increase in virulence/pathogenicity was associated with the expression of the pseudocapsule and key virulence antigens ("in vivo" antigens), which seemed to be embedded in the pseudocapsule. These antigens were antiphagocytic but animals immunized with a live S. aureus vaccine developed significant levels of IgG2 antibody against them and were relatively resistant to experimental staphylococcal mastitis (Watson and Kennedy, 1981).

Many of the early experimental S. aureus vaccines simply were toxoided culture filtrates supernates (Minett, 1939) and there is no doubt that they provided some degree of protection from the more acute forms of the disease, presumably by neutralizing toxins in the udder, thereby limiting local tissue damage and reducing toxemia. It was clearly found that attenuated, live vaccine (produced by reducing haemolysin expression) provided significant protection against experimental challenge with S. aureus strains of low toxigenicity (Watson, 1984). However, the live attenuated vaccine did not protect ewes which were challenged with strains of S. aureus, which produced high titres of exotoxins. These results suggested that antitoxic immunity was an important component in staphylococcal mastitis vaccines, especially as a means of limiting tissue damage when antibacterial mechanisms failed to eliminate all cocci (Watson et al., 1993).
It has been known for many years that live S. aureus vaccines, given subcutaneously, provide a considerable degree of protection from staphylococcal mastitis (Bidre, 1907; Derbyshire, 1961). This was confirmed by Watson and Lee (1978) and Watson (1984). Ruminant neutrophils bear membrane receptors for the Fc portion of IgG2 molecules (Watson, 1976). Following immunization with live S. aureus vaccine, ruminants mount a strong IgG2 anti-S. aureus antibody response (Watson, 1987). The IgG2 in plasma and on neutrophil membranes is in dynamic equilibrium. Neutrophils collected from ewes that were immunized with a live S. aureus vaccine had enhanced capacity to phagocytose and kill S. aureus in in vitro assays, compared with neutrophils from non-immunized or those immunized with a conventionally prepared, killed S. aureus vaccine (Watson, 1975). This enhanced phagocytosis was entirely attributable to the presence of cytophilic IgG2 antibody on the neutrophil membranes (Watson, 1976; Watson, 1975).

A study was made on the role of humoral and cellular mediators in enhanced mammary inflammatory reactions to Staphylococcal infection in systemically immunized ewes. Prior systemic immunization with live S. aureus vaccine enhances the early recruitment of neutrophils into non-lactating mammary glands infected with Staphylococci. The study investigated the role of humoral and cultural mediators in this phenomenon. These findings suggested that qualitative but not quantitative characteristics of mammary leucocytes influenced the inflammatory response to infection in systemically immunized ewes (Colditz and Watson, 1982).

A study was carried out by Kennedy and Watson (1982) on the cellular basis for differences in humoral immune responses of sheep immunized with living or killed S. aureus vaccines. The IgG2-containing cell response was significantly greater than the IgG1-containing cell response in abscesses and nodes in Group L. In contrast, the IgG1-containing cell response predominated over the IgG2-containing cell response in granulomas and draining nodes. There were generally greater numbers of cells containing anti-staphylococcal antibody in abscesses/granulomas and nodes.

An Australian worker (Watson, 1981) conducted a study on immunologically specific resistance to infection with a particular reference to staphylococcal mastitis and
suggested that the protective immune response following immunization with live staphylococcal vaccines may be associated with an additional antigen produced by metabolic processes within the bacteria. He was of the view that antigenic composition of \textit{S. aureus} may be exploited to develop a better vaccine against staphylococcal mastitis.

The effect of immunization on the early influx of neutrophils during staphylococcal mastitis in ewes was studied. The neutrophil influx into mammary secretions was studied in unimmunised, and in systemically and locally immunized, lactating and non-lactating ewes experimentally infected with \textit{S. aureus}. Systemic immunization was affected by subcutaneous injection of live bacteria or by intramuscular injection of killed bacteria in Freund’s incomplete adjuvant. The results suggested that differences in the rate of influx of neutrophils into infected mammary glands of immunized and unimmunised ewes could be attributed to immunological enhancement of neutrophil recruitment or to limitation of toxic damage to tissues with consequently diminished neutrophil invasion (Colditz and Watson, 1982).

Watson and Lee (1978) conducted an experiment on immunity to experimental staphylococcal mastitis for the comparison of live and killed vaccines in sheep. Eleven pregnant Merino ewes were immunized with either a killed \textit{S. aureus} cell-toxoid vaccine (intramuscularly) or a living culture of the same organism (subcutaneously). A further 3 animals were used as non-immunised controls. There were no significant differences between the vaccinated groups for agglutinating antibody to staphylococci or for anti-\(\alpha\)-haemolysin in either serum or whey. Three weeks after lambing the ewes were challenged by intramammary infusion of virulent staphylococci. All animals developed an acute mastitis with significant decreases in milk yields being recorded 48 hours post-challenge. Seven days after challenge the mean milk production of ewes given the live vaccine had recovered to within 5\% of the pre-challenge mean yield. However, milk productions of controls and ewes given the killed vaccine had further decreased and were significantly lower than for animals vaccinated with live staphylococci. There were no significant differences between the two vaccinated groups for numbers of bacteria or leucocytes in milk samples collected after challenge.
An evaluation study of an attenuated live staphylococcal mastitis vaccine was made in 4 lactating heifers, which were immunized in late pregnancy with 2 doses of attenuated, live *S. aureus* and challenged during early lactation by intramammary infusion of approx. 100 organisms of the same attenuated strain into one quarter. At challenge, IgG1 and IgG2 antibodies against *S. aureus* surface antigens were significantly greater in blood serum of vaccinated heifers than in controls. The challenge dose of *S. aureus* did not produce prolonged clinical signs of acute mastitis in any of the heifers. Results of clinical assessment, bacteriology and measurements of milk production suggested that vaccinated heifers had a higher resistance to the challenge dose than controls (Watson, 1984).

The efficacy of live commercial vaccine against *S. aureus* mastitis was evaluated by Calvinho et al. (1989) in two matched groups of 10 each of Holand – Argentino cows (one vaccinated group and one non-vaccinated control group). Evaluation was made by clinical observation, the California Mastitis Test and bacteriological culture of milk over a 6-month period. Although the teats of both groups were dipped in a culture of *S. aureus* three weeks after vaccination of the first group, to give an additional challenge, no significant differences could be found between the vaccinated and the non-vaccinated animals.

Another study was undertaken to evaluate the immune response of experimental live attenuated *S. aureus* vaccine in 30 rabbits dividing into 3 groups (A, B and C). The concentration of bacterial cells was determined by viable count and adjusted to 10⁶ cells/mL. Group A was inoculated with single dose @ 0.2mL/animal subcutaneously, while twice administration at fortnightly was made in group B and the group C served as control. Serum samples were collected at weekly interval till 6 weeks. Antibody titre was measured by agglutination tests. Antibody response highest at day 21 in groups A and B and was same at day 28 in group B (Farooq et al., 2004).

2.9.3. *Adjuvanted S. aureus* Vaccines

Adjuvants are modulators of the immune system and include a diverse range of substances. The initial definition of an adjuvant by Ramon (1926) was a substance that, when used in combination with a specific antigen, enhanced levels of immunity beyond those developed with the vaccine alone. This definition has now been widened (Jolles and
Paraf, 1973) to include any substance that acts: (i) on a hapton or antigen enhancing its antigenic properties, or (ii) on the cells involved in the immune response.

Adjuvants or immune stimulators can be classified into 5 different groups, viz chemically defined bacterial or fungal products, biological products of the immune system, synthetic biological analogues and chemical preparations (Calcium phosphate, Dextran sulphate) (Vanselow, 1987).

2.9.3.1. Dextran Sulphate (DXS) Adjuvanted S. aureus Vaccines

Polyanions such as dextran sulphate are strong adjuvants which appear to act directly on macrophages, enhancing antibody response as well as delayed hypersensitivity (Waksman, 1979; McCarthy et al., 1977). Dextran sulphate activates macrophages to be cytotoxic and cytoplastic (Baldwin and Bayers, 1979). It also induces an increase in cyclic GMP in conjunction with B cells activation (Hadden, 1979).

Several workers conducted studies to find a suitable adjuvant to be used in bacterin to initiate the IgG₂ subclass of antibodies responsible for destruction of S. aureus. A study was undertaken in ewes on vaccination against experimental staphylococcal mastitis. Experiments were carried out in ewes using a new vaccine developed for the prevention of mastitis caused by S. aureus. The vaccine comprised three major components: (i) killed S. aureus cells which had been cultured to induce synthesis of pseudocapsule; (ii) toxoided staphylococcal beta haemolysin and (iii) the adjuvant dextran sulphate. Ewes systemically vaccinated twice during pregnancy developed significantly elevated circulating levels of IgG₁ and IgG₂ anti-pseudocapsule antibody, as well as increased serum titres of anti-beta haemolysin. Five different strains of S. aureus were used to challenge both vaccinated and control ewes by the intramammary route during the ensuing lactation. The incidence of acute gangrenous mastitis and nonacute, clinical mastitis was significantly lower in vaccinated than in control groups after challenge with each strain. Vaccinated ewes produced significantly more milk than control ewes after challenge with four of the five strains of S. aureus (Watson, 1988).

Davidson (1987) discussed the development and mechanisms of killed and live vaccines against S. aureus mastitis. He concluded that live vaccines trigger production of
specific antibodies that enhance ability of neutrophils to destroy \textit{S. aureus}, whereas killed vaccines did not stimulate production of specific antibodies. Live \textit{S. aureus} vaccine produced IgG$_2$, while bacterin instigated the production of IgG$_1$. IgG$_2$ is much more helpful for phagocytosing \textit{S. aureus} by combining with neutrophils. When dextran sulphate was used as an adjuvant with bacterin, it also enhanced the IgG$_2$ production (Watson, 1987).

A study on the serological response of sheep to live and killed \textit{S. aureus} vaccines was carried out. Adult sheep were immunized intramuscularly with a killed \textit{in vivo}-grown \textit{S. aureus} vaccine combined either with dextran sulphate or with Freund's incomplete adjuvant. Another group received an attenuated live \textit{S. aureus} vaccine intracutaneously. All groups had large IgG$_2$ antibody responses, but the response for this isotype had waned by 14 weeks after primary vaccination. Examination of the ratios of IgG$_2$ antibody to IgG$_1$ antibody suggested that dextran sulphate may be a useful adjuvant for preferentially stimulating synthesis of IgG$_2$ antibody against Staphylococcal pseudocapsular antigens (Watson, 1987). Likewise, a research was made on the effect of dextran sulphate on IgG subclass of antibody in efferent popliteal lymph of sheep. IgG$_1$ and IgG$_2$ participation in anti-hapten, anti-carrier and immunoglobulin-containing cell (Ig-cc) responses was studied in sheep immunized with killed \textit{S. aureus}-Dinitrophenyl (DNP) conjugates. Total IgG$_1$ and IgG$_2$ anti-Staphylococcal and anti-DNP antibody output in lymph was determined using enzyme-linked immunosorbent assays (ELISA’s). DXS markedly increased antibody responses to \textit{S. aureus}-DNP in the popliteal lymph node of sheep and was shown to strongly enhance immunological memory. The ratios of IgG$_2$-cc:IgG$_1$-cc and IgG$_2$:IgG$_1$ anti-staphylococcal antibody in lymph-draining popliteal lymph nodes of sheep stimulated with DXS and \textit{S. aureus}-DNP was significantly greater than the same ratios in animals given antigen alone. The IgG$_2$ subclass-specific immunomodulatory effects of DXS were exerted whether the adjuvant was given with primary and /or secondary inoculation. There was no difference in the ratio of IgG$_2$:IgG$_1$ anti-DNP antibody in lymph from animals given \textit{S. aureus} DNP with and without DXS (Kirlin and Watson, 1987).

Watson (1992) conducted a trial on vaccination against experimental \textit{S. aureus} mastitis in 26 dairy heifers in Australia. The organism in the vaccines were cultured under simulated \textit{in vivo} conditions to induce expression of a pseudocapsule. Dextran sulphate
(responsible for the synthesis of IgG₂ antibody) was included in the vaccine as the primary adjuvant. Vaccinated heifers developed very high levels of both IgG₁ and IgG₂ anti-pseudocapsule antibody in serum and were more resistant to clinical mastitis following challenge than were controls. The vaccinates had a significantly greater milk production. The most promising vaccine had dextran sulphate combined with mineral oil as the adjuvant and was injected intramuscularly.

A subsequent study at the same Australia laboratory (Watson and Davies, 1993) examined the influence of adjuvants on the immune response of sheep to a novel S. aureus vaccine. Sheep were immunized twice with two S. aureus vaccines which contained either killed bacterial cells shrouded with pseudocapsules or toxoided β haemolysin, together with various adjuvants. Circulating antibody responses were monitored using an ELISA (anti-pseudo-capsule responses) and an anti-β haemolysin assay. Combining a killed cell/pseudocapsule/dextran sulphate (DXS) vaccine and the toxoid vaccine did not cause any diminution of antibody responses compared with separate injection of the two preparations. Addition of calcium phosphate to DXS as an adjuvant for the combined vaccine did not extend the duration of anti-pseudocapsule responses compared with those obtained with dextran sulphate alone. Nor was there any benefit in terms of durability of the response by increasing the amount of DXS: doses of DXS of 10-20 mg/kg live weight provoked significantly higher peak responses but caused acute clinical reactions at the vaccination site and did not prolong the antibody response. In contrast, the combined vaccine given with DXS and emulsified in Freund's Incomplete Adjuvant (FIA) resulted in a large anti-pseudocapsule response with elevated levels of antibody being sustained for at least one year; there was a significant IgG₂ anti-pseudocapsule response in animals receiving the vaccine with DXS and FIA. In the above experiments, 10^10 pseudocapsule-shrouded bacterial cells were used in the vaccine. Reducing the concentration of cells to 10^9 caused a slightly reduced anti-pseudocapsule response (not significant) whereas increasing the concentration to 10^11 did not increase the response.

Amorena et al. (1994) evaluated the use of liposome-immunopotentiated exopolysaccharide as a component of an ovine mastitis staphylococcal vaccine. Experiments on the development of a vaccine against staphylococcal mastitis were
carried out in ewes. The vaccine (Spanish patent no. 9200223) had the following components: (i) inactivated formalinized bacteria (*S. aureus* and a coagulase-negative staphyloocceal species, *Staphylococcus simulans*) and *S. aureus* toxoid in presence of an adjuvant (dextran sulphate, MW 500,000); and (ii) *S. aureus* exopolysaccharide included within liposomes. High serum antibody titres were obtained against whole cells from *S. aureus*, *S. simulans*, *S. hyicus* and *S. epidermidis* strains. However, there was no response to cells from *S. warneri* and *S. chromogenes* strains. An immune response (serum IgG) against the inoculated exopolysaccharide was obtained when \( > \) or \( = 20 \) micrograms of exopolysaccharide were included in liposomes and when \( > \) or \( = 20 \) mg of exopolysaccharide were adjuvanted with dextran sulphate instead of liposomes. The incidence of *S. simulans* sub-clinical mastitis and of *S. aureus* acute mastitis was significantly lower in vaccinated animals than in unvaccinated controls. Specifically, on challenge with *S. simulans*, two out of 14 glands became infected among the vaccinated animals and nine out of ten glands in the unvaccinated group \( (p < 0.001) \). On challenge with *S. aureus*, no protection was detected when component (ii) was omitted from the vaccine; nine out of ten animals developed mastitis (two mild, two moderate and five severe).

Australian workers (Watson *et al.*, 1996) conducted a field trial of a staphylococcal mastitis vaccine in dairy herds in relevance to clinical, sub-clinical and microbiological assessments. The main objective of this trial was to assess the efficacy of a new staphylococcal mastitis vaccine (Special features of the vaccine were use of strain of *S. aureus* that produced profuse pseudo-capsule when cultured in medium containing milk whey; toxoided, concentrated culture filtrate containing both hemolysin, derived from two strains of *S. aureus*; use of a mineral oil/dextran sulphate adjuvant to preferentially stimulate synthesis of IgG2 antibody) under commercial dairying conditions. A field trial involving 1819 cows and heifers was conducted on seven dairy herds in Victoria. The trial was done blind; approximately half the animals were vaccinated and the remainder were untreated controls. The vaccine was given twice during the last 10 weeks of pregnancy. Effects of vaccination were assessed, during the ensuing lactation, on the basis of clinical and sub-clinical mastitis and microbiological investigations of the milk. A total of 273 cases of clinical mastitis were recorded. *S.
was isolated from 112 of these, 45 cases in vaccinates and 67 cases in controls; the difference was not statistically significant. One herd was notable in having a high incidence of clinical staphylococcal mastitis. This herd accounted for 15.8% of the animals in the field trial but 54.5% of cases of clinical staphylococcal mastitis. For this herd, vaccinated animals had significantly lower incidence of clinical staphylococcal mastitis and prevalence of sub-clinical mastitis, relative to controls. An unexpected feature of the trial as a whole was the low incidence of clinical mastitis from which S. aureus was isolated in pure culture (26.3% of cases) and the high incidence of clinical Strep. uberis mastitis (22.7% of cases). In conclusion, the trial showed that the vaccine was efficacious in reducing the incidence of clinical mastitis and prevalence of sub-clinical mastitis in a herd that had a serious staphylococcal mastitis problem.

2.9.3.2. Oil-Adjuvanted S. aureus Vaccines

A depot theory was proposed by Glenney et al. (1931) which stated that repository adjuvants (principally aluminium salts and water-in-oil emulsions) held the antigen at its site of deposition, delaying its adsorption; subsequently released antigen induced a secondary response. Nakashima et al. (1981) demonstrated the augmentation by adjuvants of antigen retention at the site of injection and the draining lymph node (Vanselow, 1987). Keeping this in view, various workers conducted a variety of studies on mastitis vaccines to prolong the immune response.

Nordhaug et al. (1994a) conducted a field trial with an experimental vaccine against S. aureus mastitis in cattle. A S. aureus vaccine containing whole, inactivated bacteria with pseudocapsule and alpha and beta toxoids with a mineral oil as adjuvant, was used in this field trial. Heifers were injected in the area of the supramammary lymph nodes with vaccine or placebo twice before calving and observed and sampled throughout their first lactation. Antibody response toward the pseudocapsule and the alpha toxin was significant in serum from the vaccinated animals. These antibody concentrations were significantly higher in serum and milk during the entire lactation compared with those of the controls. The antibody response to the beta toxin was moderate in serum from vaccinated cows; differences in antibody concentrations in milk were not significant between groups. The antibody response to the pseudocapsule consisted of the IgG1 and
IgG2 isotypes, but, in milk, only the concentration of IgG1 was significantly increased in the vaccinated animals during the lactation compared with the control animals.

A subsequent report by Nordhaug et al. (1994b) dealt with an field trial with and experimental vaccine against S. aureus mastitis in cattle keeping in view the clinical parameters. A total of 108 heifers were included in a placebo-controlled multicenter study on the use of an experimental S. aureus mastitis vaccine containing whole, inactivated bacteria with pseudocapsule, alpha and beta toxoids, and a mineral oil as adjuvant. The heifers were injected in the area of the supramammary lymph nodes twice before calving and were observed and sampled throughout the first lactation. None of the vaccinated cows suffered from clinical S. aureus mastitis, and only 8.6% suffered from sub-clinical S. aureus mastitis, but a total of 16.0% of the control cows suffered from clinical or sub-clinical S. aureus mastitis. Mean SCC in vaccinated and control cows were the same throughout the lactation. Local swellings at the injection site were palpable in a substantial proportion of the vaccinated cows. In the statistical analyses, when cow was used as the unit of concern, no significant differences occurred between groups. However, when all parameters on udder health were considered together, the results indicated a potential protective effect of this vaccine during the entire lactation.

Australian investigators (Watson et al., 1996) conducted a field trial of a staphylococcal mastitis vaccine in dairy herds based on clinical, sub-clinical and microbiological assessments. The main objective of this trial was to assess the efficacy of a new staphylococcal mastitis vaccine (Special features of the vaccine were use of strain of S. aureus that produced profuse pseudo-capsule when cultured in medium containing milk whey; toxoided, concentrated culture filtrate containing both hemolysin, derived from two strains of S. aureus; use of a mineral oil/ dextran sulphate adjuvant to preferentially stimulate synthesis of IgG2 antibody) under commercial dairying conditions. A field trial involving 1819 cows and heifers was conducted on seven dairy herds in Victoria. The trial was done blind; approximately half the animals were vaccinated and the remainder were untreated controls. The vaccine was given twice during the last 10 weeks of pregnancy. Effects of vaccination were assessed, during the ensuing lactation, on the basis of clinical and sub-clinical mastitis and microbiological investigations of the milk. A total of 273 cases of clinical mastitis were recorded. S.
**S. aureus** was isolated from 112 of these, 45 cases in vaccinates and 67 cases in controls; the difference was not statistically significant. One herd was notable in having a high incidence of clinical staphylococcal mastitis. This herd accounted for 15.8% of the animals in the field trial but 54.5% of cases of clinical staphylococcal mastitis. For this herd, vaccinated animals had significantly lower incidence of clinical staphylococcal mastitis and prevalence of sub-clinical mastitis, relative to controls. An unexpected feature of the trial as a whole was the low incidence of clinical mastitis from which **S. aureus** was isolated in pure culture (26.3% of cases) and the high incidence of clinical **S. uberis** mastitis (22.7% of cases). The investigators concluded that the vaccine was efficacious in reducing the incidence of clinical mastitis and prevalence of sub-clinical mastitis in a herd that had a serious staphylococcal mastitis problem.

A double-blind, placebo-controlled study was conducted in sheep by **Tollersrud et al.** (2002) to compare two vaccines using different adjuvants with regard to their ability to stimulate antibody production against the alpha- and beta-toxins and the exopolysaccharide of **S. aureus**. The vaccines contained identical antigens, consisting of inactivated whole bacteria of two strains of **S. aureus** in addition to alpha- and beta-toxoid. One vaccine contained mineral oil, while the other used a water-soluble acrylic acid polymer resin (Carbopol) as adjuvant. Saline served as the placebo. One hundred and forty ewes were vaccinated twice before lambing, by subcutaneous injection with vaccine or placebo in the region of the supramammary lymph node, and were observed and sampled over a period of 6 months. The vaccine containing mineral oil as adjuvant induced significantly greater immune responses to the alpha- and beta-toxins than did the vaccine containing Carbopol. The latter vaccine induced higher levels of antibodies to exopolysaccharide. The degree of local adverse reactions did not differ between the two groups. The results indicated differences between the oil-adjuvanted and Carbopol-adjuvanted vaccines with regard to their ability to stimulate antibody production against **S. aureus** protein antigens in sheep (Tollersrud et al., 2002).

### 2.9.3.3. Other Adjuvants and Biological Response Modifiers

Several workers investigated **S. aureus** bacterins incorporating adjuvants other than oil and dextran sulphate. The resumes of such studies are given as under in a
succinct manner. Cameron et al. (1979) conducted a trial on the composition and evaluation of the efficacy of *S. aureus* vaccine. An alum-precipitated *S. aureus* vaccine composed of a formalin-inactivated whole culture of a strain which produces Smith surface antigen and combined with the whole culture of a highly toxigenic strain, was found to afford a good immunity to staphylococcal skin infection in rabbits. Three injections of the vaccine provided immunity which lasted for at least 6 months against a primarily pyogenic strain of *S. aureus* and for at least 3 months against a toxigenic strain. From experiments using vaccines prepared from cells or toxoid only, it was deduced that, although there is a measure of strain specific immunity, a good heterologous immunity can be established with a combined product provided that it contained adequate quantities of toxoid. The use of such a vaccine as a potential aid in the control of bovine staphylococcal mastitis was discussed.

A study was made by Watson (1987) on the serological response of sheep to live and killed *S. aureus* vaccines. Adult sheep were immunized intramuscularly with a killed *in vivo*-grown *S. aureus* vaccine combined either with dextran sulphate (group DD) or with Freund’s incomplete adjuvant (group FF). Another group (LL) received an attenuated live *S. aureus* vaccine intracutaneously. The animals were given a primary vaccination followed two weeks later by a booster vaccination. A fourth group of sheep (LD) was primed with the live vaccine and given a booster vaccination with the killed vaccine combined with dextran sulphate. ELISA was used to quantify blood serum levels of IgM, IgG1 and IgG2 antibody directed against the pseudocapsular antigens of *S. aureus* grown under in vivo conditions. Groups LD, DD, and FF had sharp increase in mean levels of IgM antibody in the first few weeks after vaccination with another large increase in mean values for group FF at 12 weeks after primary vaccination. Group LL showed virtually no increase in levels of IgG1 antibody; the other three groups had maximum mean values for IgG1 antibody at 5 weeks (FF) and 8 weeks (LD and DD) after primary vaccination. All groups had large IgG2 antibody responses (the largest for group LD), but the response for this isotype had waned by 14 weeks after primary vaccination. Examination of the ratios of IgG2 antibody to IgG1 antibody suggested that dextran sulphate may be a useful adjuvant for preferentially stimulating synthesis of IgG2 antibody against Staphylococcal pseudocapsular antigens.
Czechoslovakian workers, Rysanek et al. (1988) conducted a study on a *S. aureus* bacterin prepared from a virulent strain of organism together with alpha, beta haemolysin and aluminum hydroxide as an adjuvant. This bacterin was injected twice subcutaneously in the vicinity of supramammary lymph nodes at an interval of 18 days at the end of lactation. It gave protection against experimental infection with a heterogeneous strain of *S. aureus* and a reduced incidence of *S. aureus* mastitis was noted.

Some plants derived adjuvants have also been investigated. Chinese workers (Hu et al., 2003) investigated the adjuvant effect of ginseng extracts was studied in cattle on the immune responses to immunize against *S. aureus*. A crude ginseng extract (GS) and the purified ginsenoside R(b1) (R(b1)) were evaluated for their adjuvant effects in dairy cattle at immunisation with ovalbumin (OVA) and/or a *S. aureus* bacterin used for prevention of bovine mastitis. To evaluate a suitable dose of GS as an adjuvant, 36 lactating cows were randomly divided into six groups. The cows were inoculated twice intramuscularly with a 2-week interval, with saline solution, OVA in saline, or OVA in combination with 4, 16 or 64 mg GS, or A! (OH) (3). The level of specific antibodies to OVA in serum and milk whey was measured before immunisations and 1-5 weeks after the second immunisation. The antibody response in serum was significantly higher in animals immunised with OVA and GS than in animals immunised with OVA alone. A significant increase in milk antibody titres compared with OVA only was only found 2 weeks after the second immunisation in the group immunised with OVA and 4 mg GS. In the second part of the study, 18 heifers were randomly divided into three groups and were immunised twice intramuscularly with a two week interval, with the *S. aureus* bacterin (control), or with the bacterin in combination with 4 mg GS or 1mg R(b1). The specific antibody response to *S. aureus* and the lymphocyte proliferation after stimulation with PWM, concanavalin A (Con A) or a specific *S. aureus* antigen was evaluated in blood samples taken before and after immunisations as specified above. Addition of R(b1) resulted both in significantly higher antibody production and lymphocyte proliferation in response to PWM, Con A and *S. aureus* antigens than in the control group. Addition of GS induced a significantly higher lymphocyte proliferation in response to PWM and Con A than the control, but had no additional effect on the antibody production. In conclusion,
both GS and R(b1) were safe adjuvants, and R(b1) had the strongest adjuvant effects, when used for immunisation against S. aureus in dairy cattle. Field trials are warranted to test the ability of GS and R(b1) to enhance the efficacy of mastitis vaccines in protection against intramammary infections.

Dutch workers (Groothuis and Grootenhuis, 1981) conducted a study on the effect of intramammary vaccination in experimental staphylococcal infection in cows. A commercially available vaccine was infused into all 4 or only 2 quarters of 12 cows during the dry period. Another 8 were not vaccinated. Fourteen weeks later, when the cows were lactating; diluted culture of one of three strains of alpha, and/or beta haemolytic S. aureus was infused into the udders. Most of the cows responded with severe mastitis, but vaccinated cows did not develop fever. The alpha-haemolysin titre of blood serum was higher in vaccinated than in unvaccinated cows. Although, vaccination did not prevent acute mastitis, milk yield of vaccinated cows suffered less than that of unvaccinated cows.

A study on the efficacy of a bovine S. aureus vaccine using interleukin-2 as an adjuvant was conducted in USA by Derosa and Sordill (1997). The purpose of this study was to examine the efficacy of a vaccination protocol using recombinant bovine interleukin-2 (rBoIL-2) as an adjuvant with a S. aureus vaccine. Holstein dairy cows were immunized with a S. aureus vaccine in conjunction with either saline solution (n = 3), Freund's incomplete adjuvant (FIA; n = 3) or rBoIL-2 (n = 3). Whey and serum were analyzed for antibody titre to specific S. aureus antigens. Isolated blood mononuclear cells (BMC) were examined for their ability to proliferate and to produce interleukin-2 (IL-2) and interferon (IFN) after either mitogenic or antigenic stimulation in vitro. Efficacy of the vaccination protocol was assessed by challenging experimental animals intramammarily with 100 colony forming units of S. aureus. Regardless of treatment, all cows exhibited similar serum antibody titres to S. aureus pseudocapsule. Cows treated with saline exhibited a significant increase in serum alpha-toxin antibody titre when compared to levels observed in FIA and rBoIL-2-treated cows. However, cows receiving rBoIL-2 treatment exhibited significantly higher lacteal pseudocapsule antibody titre compared to the other adjuvant groups. Administration of rBoIL-2 did not enhance BMC proliferative responses to the mitogens concanavalin A (ConA), phytohemagglutinin
(PHA), pokeweed mitogen (PWM) or interleukin-2 (IL-2) when compared to FIA or saline treated cows. Although, cows receiving rBoIL-2 treatment exhibited enhanced cytokine production upon antigenic stimulation, efficacy of the vaccination protocol was inferior compared to the protection offered by saline treatment.

Zacconi et al. (1999) studied the efficacy of a biological response modifier (obtained from Parapos ovis that was attenuated over 200 tissue culture passages and commercialized in European countries) for preventing S. aureus intramammary infections after calving. A change in the epidemiology of mastitis in recent years emphasized the role of the udder immune system in the pathogenesis of S. aureus. Therefore, if the bovine or udder immune capability could be enhanced, susceptibility to S. aureus could be reduced and antibiotic efficacy could be increased. Immune system defense mechanism could be enhanced by vaccination and by biological response modifiers. This study reported the results of a field trial on the efficacy of this biological response of this biological response modifier in reducing S. aureus intramammary infection (IMI) after calving in pluriparous cows. The trial included 106 cows sampled six times (55 cows from herd A and 51 from herd B) for a total of 2544 quarter milk samples. The analysis of IMI prevalence showed that 25.09% of samples were bacteriologically positive in the placebo group and 23.17% of the positive samples were observed in the biological response modifier group. S. aureus IMI had a frequency of 11.44% in the placebo group and 6.00% in the biological response modifier groups. The dynamic of the hazards showed significantly lower rates in the biological response modifier group than in the placebo group (risk ratio = 0.47). Treatment with the parapox-containing biological response modifier showed significant reduction of S. aureus IMI around calving and this reduction was attributed to an increase in immune defense (Zecconi et al., 1999).

The efficiency of vaccination with S. aureus bacterin in the control of bovine mastitis was evaluated by Brazilian workers (Alberton et al., 2001) in 45 lactating Jersey cows. The bacterin was made from strains of coagulase-positive S. aureus isolated from the same herd tested. The bacterial isolates were inactivated with formalin and 2.5% aluminum hydroxide was added as adjuvanted. The herd was divided into three groups of fifteen animals: Group I (control), without any treatment; Group II (weekly group), where
the animals received weekly injections of 3.0 ml of the vaccine subcutaneously in the region of the mammary lymph node for sixteen weeks; the Group III (bicweekly group), which received the bacterin at two-weekly intervals for sixteen weeks. Samples of milk were collected weekly evaluated with the California Mastitis Test (CMT) and Somatic Cell Counts (SCC). Results demonstrated that the percentage of samples with SCC inferior to 3 x 105 cells/ml and 5 x 105 cells/ml was larger in the weekly group (P>0.05) than other groups. The percentage of positively samples to CMT was 59.60% smaller (P>0.05) in the weekly group than in the control (9.16 and 22.70%, respectively). The percentages of moderate to serve infections according to CMT were smaller (P>0.05) in the weekly group (35.98%) than in the control (63.33%) and biweekly group (57.43%). During the experiment, in all the groups, cases of sub-clinical mastitis were more frequent than other forms of the disease, reaching 81.46% of the total cases, and S. aureus was the most frequent bacterial isolated. The percentage of S. aureus sub-clinical mastitis cases was smaller in the weekly group (25.00%) than in the control (32.25%) and biweekly groups (43.75%). It was concluded that bacterin made from S. aureus isolated from the same herd and containing aluminum hydroxide as adjuvant, when subcutaneously injected repeatedly at one-week intervals in the mammary lymph-node region during the course of lactation was capable of decreasing both the prevalence and severity of mastitis in lactation cows.

Based on a study conducted in rabbits, Korean researchers (Han and Park, 2000) proposed the use of more than one adjuvant. This study was performed to isolate a vaccine strain of S. aureus from clinical or sub-clinical mastitis and to choose the most optimal adjuvant for immune response of alpha toxin and capsular polysaccharide (CPS) of field strain. Of thirty strains of S. aureus isolated from milk of clinical or sub-clinical mastitis, V112 strain isolated from milk of gangrenous mastitis was used in this vaccine. Twenty one of rabbits were allocated into 5 groups based on adjuvants and immunized twice every 2 weeks for 8 weeks. This vaccine was composed of alpha toxin (10 hemolytic units) and formalinized whole cells (1 x 10^{11} cells/ml. Five rabbits received PBS solution as a control group. The highest antibody titres against alpha toxin and CPS were observed in dextran sulfate- and aluminium hydroxide-adjuvant group at 8 weeks after immunization, respectively. Results of the study showed that one adjuvant could not
induce strong and long-term immune response of alpha toxin and CPS antigens. Therefore, the use of combined adjuvants in subunit vaccine may be useful and feasible.

Australian Workers (Watson and Davies, 1993) while examining the influence of different adjuvants (alone and in combination) on the immune response of sheep to a novel *S. aureus* vaccine concluded that dextran sulphate and Freund’s incomplete adjuvant combination resulted in a significantly higher as well as longer sustained anti-pseudocapsule response as compared to dextran sulphate and calcium phosphate combination.

2.10. Toxoids

Indian workers (Pal and Pathak, 1977) conducted immunological studies on staphylococcal mastitis in cows and buffaloes, which were injected with alum-cell-toxoid and aluminium hydroxide gel cell-toxoid of *S. aureus* vaccines. Antitoxin titres increased significantly after vaccination, and *S. aureus* disappeared from the milk. Animals with sub-clinical mastitis recovered. As far as could be ascertained this is the only mastitis vaccination study conducted thus far in which buffaloes were also included.

Adlam *et al.* (1977) investigated the effect of immunization with highly purified alpha- and beta-toxins on staphylococcal mastitis in rabbits. Immunization with alpha- or beta-toxins produced no change in the clinical picture of the disease produced by CN.6708, a strain of *Staphylococcus* responsible for a natural outbreak of abscess-type rabbit mastitis. From these experiments it was found that alpha-toxin is a key antigen in the blue-breast form of rabbit mastitis. Since the abscess form of the disease was not prevented by immunization with either alpha- or beta-toxin. These investigators concluded that other virulence factors must be acting to produce this more localized disease.

*S. aureus* toxoid is commercially available in Australia. It is injected in a 5 mL doses intramuscularly and is repeated in a month to provide protection for 6 months period (Hungerford, 1990). Park et al (1999) conducted a study on immunogenicity of alpha-toxin, capsular polysaccharide (CPS) and recombinant fibronectin-binding protein (r-FnBP) of *S. aureus* in rabbit. This study was conducted to evaluate the antibody levels of alpha-toxin, capsular polysaccharides (CPS) and fibronectin-binding protein (FnBP) in
rabbits immunized with an experimental vaccine against *S. aureus* and to develop the bovine mastitis subunit vaccine in the future. Of three antigen used in vaccine, immunogenicity of CPS was relatively lower, compared with those of alpha toxin and fibronectin-binding protein. Numbers of *S. aureus* in blood of immunized groups were lower than those of control group after bacterial challenge. But the bacterial numbers among immunized groups were not significantly different. *S. aureus* counts in excised organs were significantly lower in all immunized rabbits than in PBS-control group (p<0.05). This study showed that alpha-toxin, capsular polysaccharide and fibronectin binding protein included in a subunit vaccine were protective. Similarly, a study made by Han and Park (2000) on the effects of adjuvants (Dextran sulphate, Aluminium hydroxide, Freund’s complete adjuvant and immunostimulating complexes (ISCOM) on the immune response of response of *S. aureus* alpha toxin and capsular polysaccharide (CPS) in rabbits. Twenty one rabbits were allocated into 5 groups based on adjuvants and immunized twice every 2 weeks for 8 weeks. This vaccine was composed of alpha toxin (10 hemolytic units) and formalized whole cells (1 x 10^{11} cells/ mL). Five rabbits received PBS solution as a control group. The highest antibody titres against alpha toxin and CPS were observed in dextran sulphate and aluminium hydroxide adjuvanted groups at 8 weeks after immunization, respectively. As a corollary, it was found that one adjuvant could not induce strong and long term immune response of alpha toxin and CPS antigens. Therefore, the use of combined adjuvants in subunit vaccines may be useful and feasible (Park *et al.*, 1999).

A trial was made by Ikramm (2004) for production, partial purification of *S. aureus* alpha toxin along with its antigenic response in rabbits. A total of 44 rabbits were divided into three groups i.e A, B and C. Group-A was further divided into 5 subgroups of 4 rabbits each (A1 thru A5). In group-A five subgroups were administered with different amounts of partially purified *S. aureus* alpha toxoid alone i.e. 1:10, 1:20,1:40,1:80,1:160 haemolytic units(HU) respectively. Similarly group-B was further divided into 5 subgroups (B1 thru B5).Group-B was administered with different doses of Dextran sulphate adjuvanted alpha toxoid of *S. aureus*. The group-C was kept as control. The analysis of purified alpha toxin was done by haemolytic activity and dermonecrotic activity in rabbits. The different levels of alpha toxin were determined by haemolytic
units. Blood sampling was done at weekly intervals to collect serum from all subgroups for a period of three weeks in order to measure the antigenic response using the toxin neutralization test (Alpha haemolytic titre). There was production of alpha-haemolytic antibodies in both cases i.e simple toxoid and adjuvanted toxoid. Alpha hemolytic antibodies titre was directly proportional to the concentration of toxoid dilution. As 1:10 HU showed highest alpha-hemolytic antibodies titre as compared to the remaining i.e 1:20, 1:40, 1:80, and 1:160 HU. Dextran sulphate adjuvanted toxoid showed overall better result than simple toxoid at all concentration of toxoid in rabbits. Subsequently, similar type of study was also made by lhsanullah (2004) about the beta toxin of *S. aureus* in rabbits and similar trend of results was there. Both workers considered these toxins (alpha and beta) as the good candidates for subunit vaccine of *S. aureus* for the control of mastitis in buffaloes.

2.11. Efficacy of *S. aureus* Vaccines

It is generally accepted that commercially available *S. aureus* vaccines have limited ability to prevent new infections (Nickerson, 1999; Yancy, 1993). A 3-lactation trial failed to demonstrate a reduction in the number of new *S. aureus* infections in cows vaccinated with a commercial vaccine (Pankey et al., 1985). This study did document an increase in the spontaneous cure rate of cows that received the vaccine. Similar results were found in a separate study conducted in 3 commercial dairy herds in New Zealand (Pankey, 1983). There are several other studies that support the ability of commercially available *S. aureus* vaccines to enhance spontaneous cure rates. Literature published by representatives of the manufacturer suggests that the best use of the vaccine is the reduction of chronic infections rather than the prevention of new infections (Widal, 1994). The ability of commercial *S. aureus* vaccines to reduce the development of chronic infections may be useful in some herds that are involved in *S. aureus* control programs, but for most herds the successful control of *S. aureus* mastitis will result from the prevention of new infections. The failure to prevent new infections is probably the reason that vaccines are used on a limited basis in mastitis control programs.

There have been several approaches to the development of experimental vaccines directed toward the control of *S. aureus* mastitis. Researchers have attempted to develop
vaccines directed toward specific virulence factors responsible for the development of mastitis. Vaccines were formulated based on bacterial cell wall components (protein A), adhesion factors (bacterial factors that allow \textit{S. aureus} to attach to mammary epithelial cells) and \textit{S. aureus} pseudocapsules (a slime layer that surrounds the bacteria and reduce the ability of WBC to destroy the bacteria). The outcomes of these studies were inconsistent and confusing to interpret.

Australian workers have published papers describing results of vaccine trials using an inactivated vaccine produced from \textit{S. aureus} strains that produced pseudocapsules (Watson, 1992; Watson \textit{et al.}, 1996). An experimental challenge study documented that this vaccine can successfully stimulated the development of anti-pseudocapsule antibody and reduced the development of clinical symptoms (Watson, 1992). The vaccine did not significantly reduce SCC or increase milk yields of infected cows. This particular vaccine was further evaluated in a 7-herd field study ((Watson \textit{et al}, 1996). The results of this study were interesting because there was no significant effect of vaccination on SCC or clinical mastitis when data from all 7-herds were included in the analysis. However, this study did not demonstrate that differences were seen between herds. When analysis was restricted to a single herd that had a prevalence of \textit{S. aureus} mastitis, the vaccinated animals had a reduction in signs of clinical mastitis and reduced development of new sub-clinical mastitis.

A Norwegian researchers (Nordhaug \textit{et al.}, 1994a) enrolled 108 heifers from 16 farms in a study of a vaccine that included the pseudocapsule and toxoids (Nordhaug \textit{et al.}, 1994). Almost 20\% of the cows in the enrolled herds were infected with \textit{S. aureus} mastitis. Vaccination did not significantly affect the rate of clinical mastitis or the SCC of enrolled cows. Vaccination did seem to lessen the development of clinical mastitis from sub-clinically infected cows. A vaccine consisting of inactivated highly encapsulated \textit{S. aureus}, unencapsulated \textit{S. aureus} and exopolysaccharides was developed and tested in Argentina (Calzolari \textit{et al.}, 1997; Giraudo \textit{et al.}, 1997). The field trial portion of the studies was conducted in dairy herds with poor milk quality and a moderate prevalence of existing \textit{S. aureus} infections (Calzolari \textit{et al.}, 1997). The experimental unit was quarter and the researchers excluded quarters that were infected prior to beginning the study. Under these conditions, the vaccine successfully reduced new intramammary infections.
with *S. aureus* but did not significantly affect the SCC. In general, there seems to be progress in the development of an effective *S. aureus* vaccine but the efficacy of these vaccines seems to vary by herd. The greatest effect of *S. aureus* vaccines appeared to be a decrease in the development of clinical symptoms and preventive programs were needed to effectively reduce the new infection rate.

### 2.12. Effect of *S. aureus* Vaccination on the Level, Duration and Severity of Intramammary Infection

Bovine mastitis is one of the most problematic diseases and continues to have major economic impact on the dairy industry throughout the world (Dobbins, 1977; Dodd, 1983). Numerous agents can cause mastitis problems in dairy cows or buffaloes but *S. aureus* is the most important etiological agent of bovine mastitis (Bramley and Dodd, 1984; Daniel *et al.*, 1982; Schukken *et al.*, 1993). Therefore, although various management methods for decreasing the prevalence of *S. aureus* mastitis have been improved, many dairies still have some level of infection with *S. aureus* (Fox and Hancock, 1989; Leslie and Schukken, 1993; Schukken *et al.*, 1993). To cope with this problem, vaccination to prevent *S. aureus* mastitis has been the subject of concern of many researchers (Colditz and Watson, 1985; Foster, 1991; Mallard and Barum, 1993). These vaccines were employed as live (Watson, 1984) or killed *S. aureus* (Brock *et al.*, 1975) or combined preparations of killed cells and toxoids (Niekerson *et al.*, 1993; Nordhaug *et al.*, 1994a,b; Watson and Schurkoff, 1990). These vaccines were reported to increase specific antibody to *S. aureus* antigens both in serum and milk, but only partially prevent intramammary infections.

Most *S. aureus* vaccines were directed at reducing new IMI in heifers by the use of farm derived bacterins (autogenous bacterins) or by selecting vaccines that give the good cross protection. Both adjuvants and toxoids were added to enhance the immune response and reduce the severity of the infection. Most of these vaccines were designed to increase spontaneous cure (often referred to as reducing new infections) or reduce clinical symptoms.

Oil based adjuvants are routinely used to elicit a strong immune response as seen with complete and incomplete Freund’s adjuvant. However, other adjuvants can produce
similar responses without the adverse tissue reaction observed with oil adjuvants. Dextran Sulphate has been reported (Watson et al., 1996) to increase the levels of IgG2 which is an antibody class that is highly effective in opsonizing bacteria (antibody coating) which allows phagocytosis (engulfing by neutrophils). There are several components of S. aureus that are not only antigenic but act as mitogens (stimulate cell division) towards B-cells and T-cells. Protein A is a good B-cell mitogen and Teichoic acid, a cell wall fraction of S. aureus is both a B-cell and T-cell mitogen (Sears, 1980). Thus, S. aureus used in combination with most adjuvants stimulates good antibody production.

Protein A is a cell wall component of S. aureus that binds IgG at the Fc region and blocks its attachment to neutrophils, thus inhibiting phagocytosis and bacterial killing. Vaccines directed towards protein A to block this bacterial defence was successful in inducing antibody production, but did not significantly protect against new infection. However it was effective in reducing the clinical symptoms similar to that seen when vaccines included toxoids (Pankey et al., 1985)

The first step in infection by S. aureus is the adherence to the gland tissue which is facilitated by surface proteins called adhesions. The most important of these is clumping factor A (ClfA). ClfA has provided partial protection in a mouse model to protect against S. aureus. Brouillette et al. (2002) reported the effectiveness of a DNA vaccine in inhibiting adherence to fibronectin and promoting phagocytosis of S. aureus. Many virulent S. aureus produce a pseudocapsule that surrounds the bacteria and makes it inaccessible to neutrophils and inhabits phagocytosis. There were two capsular types; C5 and C8 that were the most common serotypes found in S. aureus associated with bovines (Opdebeek and Norcross, 1983; Poutrel et al., 1988). Capsular vaccines were used to protect heifers in challenge trials. In a vaccination trial using capsular vaccine (Sears et al., 1990), heifers were vaccinated 4 and 2 weeks before calving. After calving, quarters were challenged with a heterologous strain of S. aureus. Vaccinates demonstrated a 52% reduction in new IMI and non-vaccinated controls had 64% chronic infections as compared to 12% in vaccinates. The quarters of the non-vaccinated controls were 6 times more likely to become infected. Others (Nordhaug et al., 1994; Watson, 1996) also used these two capsular strains to develop vaccines for use in field studies. In
a Nordic study (Nordhaug et al., 1994a), heifers vaccinated 8 and 2 weeks before calving had a 46% reduction in new IMI during the first lactation.

Sears (2002) described the autogenous vaccines that contained only the *S. aureus* strains isolated from the herd were compared to vaccines containing the modified autogenous + Smith strains in 10 herds, 153 cows with 284 *S. aureus* infected quarters. Of the cows that were vaccinated with autogenous-only vaccine, only 26% cleared the infection as compared to 80% of the cows that were vaccinated with the autogenous + Smith Strain. Although both groups had a better cure rate than the antibiotic-only treatment (3%), autogenous vaccines did not work with this protocol.

An oil-based adjuvant was compared with an aluminum hydroxide (AlOH) adjuvant in vaccination / treatment studies (Sears, 1998). Cure rates for the oil-based vaccine was far lower with a 25% quarter cure as compared to 62% for AlOH based vaccine. Although, AlOH did not produce a prolonged antibody response or protect as well against new IMI, it produced a more rapid antibody response that makes it a better choice when incorporated into treatment protocols.

Sears (2000) commented that the use of autogenous vaccines was not practical because of the difficulty of developing a unique vaccine for each herd. Guidry (1998) reported that 41% of the 273 *S. aureus* isolates from USA dairy herds cross-serotyped with either capsular type 5 or 8. However, 59% cross reacted with a new strain 336 (Nabi, Rockville, MD). These three strains accounted for all of the tested mastitis isolates. Samples of *S. aureus* collected from herds in Europe and Korea could also be cross-typed by these three strains. Therefore, a trivalent vaccine with these three serotypes (Provided by Nabi, Rockville, MD) was tested against the autogenous + Smith strain. This vaccine was used in four herds having 61 cows infected 31 quarters, 42 cows received the trivalent vaccine and 37 cows were vaccinated with autogenous + Smith Strain. It was concluded that there was no significant difference between the two vaccines with 76% quarter cures for the trivalent vaccine and 67% cures for the autogenous + Smith Strain. In cows treated with antibiotic only, *S. aureus* was eliminated from 22% of the quarters. Similarly, use of *S. aureus* vaccine in conjunction with dry cow treatment also enhanced a lot of cure rate (Sears, 2002) which was 91% in vaccinated group and
52% in prevaccination dry cow treatment group of cows. The author concluded that all herds would not be expected to respond as well, but this strategy warranted further investigation.

An investigation was made to evaluate the clinical effects of autogenous toxoid-bacterin treatment for *S. aureus* sub-clinical mastitis in lactating cows. It was concluded that autogenous toxoid-bacterin treatment against *S. aureus* sub-clinical mastitis in lactating cows increased the cure rate of infections, reduced the severity of the infections and also prevented occurrence of the new infections (Hwang et al., 2000).

Another trial was conducted by Guidry et al. (1997) about the prevalence of capsular serotypes among 273 *S. aureus* isolates from cows with mastitis in the United States. This study characterized *S. aureus* serotypes isolated from cases of bovine mastitis obtained from veterinary diagnostic laboratories in Wisconsin, Michigan, New York, Texas and California, servicing 44% of the dairy cattle in the USA. Sub-samples of mastitic milk that contained *S. aureus* were frozen and sent to the laboratory for strain serotyping. Serotype 5 accounted for 18% of the 273 isolates and serotype 8 for 23%. It was suggested that development of a *S. aureus* vaccine for bovine mastitis should take into account regional variation in *S. aureus* serotypes.

### 2.13. Need for a Polyvalent Mastitis Vaccine

Mastitis is caused by a wide variety of the microorganisms including bacteria, fungi, yeast and mycoplasma. However, bacteria are by far the most frequent pathogens associated with mastitis. Among bacteria, mastitis is caused mainly by *S. aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Corynebacterium pyogenes*, *Str. dysgalactiae* and *Str. Uberis* (Radositiis et al., 2000). The four most common bacteria – *S. aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* – generally cause 90 to 95% of all infections in an average herd (Philpot, 1984). More recently, there is evidence that *E. coli* is becoming very prevalent in some herds of cattle (Smith et al., 1985) in New York and buffaloes (Ahmad, 2001; Iqbal et al., 2004) in Pakistan. Obviously it is unlikely that a single vaccine could protect against all forms of mastitis (Derbyshire, 1962). Likewise Koiranen (1977) pointed out that vaccination cannot solve the mastitis problem but it alleviates it and gives time for other, slow-acting
treatments to take effect. He gave the range of vaccines including a polyvalent mastitis vaccine to tackle the problem effectively. Preventing the establishment of an infection and development of an inflammatory response to get rid of the infection are two of the ideal attributes of a mastitis vaccine. These are often unattainable with the present day vaccines. However, because of and colossal economic losses associated with mastitis, even the lesser achievement of reducing the severity of the disease and obtaining a more rapid clearance of established infections with vaccines would be of great value (Nordhaug et al., 1994a).

Several studies in cows or ewes have reported the efficacy of monovalent mastitis vaccines like those of *S. aureus* (Voigt and Bergmann, 1979; Watson, 1982; Miles et al., 1992; Watson, 1998; Watson and Franklin, 1988; Watson and Watson, 1989; Watson and Schwartzkoff, 1990; Nickerson et al., 1993; Nordhaug et al., 1994a; Nordhaug et al., 1994b; Watson et al., 1996), *Str. agalactiae* (Opdebeeck and Norcross, 1985), *Str. Uberis Hill*, 1988; Finch et al., 1994; Leigh, 1999; Leigh et al., 1999). and *E. coli* (Gonzales et al., 1989; Cullor, 1991; Hogan et al., 1999; Smith et al., 1999; Tomita et al., 2000). However, in view of the association of a wide variety of microorganisms with mastitis, a polyvalent vaccine would seem conceivably more pragmatic (Hill, 1990; Calzolari et al., 1997; Giraudo et al., 1997; Yancey, 1999).

Some very encouraging results have been obtained with bacterins prepared from the J5 mutant strain of *E. coli*. This is an epimerase-negative strain in which a terminal sugar is absent from the lipopolysaccharide moiety of the cell wall, thus exposing the lipid A determinant. The J5 vaccine has been shown to reduce clinical coliform mastitis by up to 80% in trials in California and, importantly, it appears to have efficacy against a range of Gram-negative udder pathogens (Gonzalez et al., 1990).

A considerable degree of effort seems to have been made to investigate the role of polyvalent vaccines in the control of bovine mastitis (Savov, 1977; Savov and Suchvarova, 1977; Petrick et al., 1978; Ungureanu et al., 1978. Lincan, 1982; Bozhilov et al., 1985; Bozhkova et al., 1985; Bozhilov et al., 1986; Stolle and Beck, 1993; Calzolari et al., 1997; Giraudo et al., 1997). As for as could be ascertained, the role of vaccination in the control of mastitis in dairy buffaloes, the mainstay of dairy industry of
Pakistan, India, Egypt, Nepal, etc. (Moioii, 1996) has not so far caught the imagination of researchers anywhere in the world. In Pakistan, where extremely small herd size is hallmark of dairying and dairy industry is fragmented in nature and hand-milking is in vogue, contagious pathogens including S. aureus and Str. agalactiae have been found the most prevalent mastitogens followed by the environmental pathogens, especially the E. coli. These have accounted for about 80% of the total mastitogens prevalent in Pakistan. To blunt the colossal economic losses associated with mastitis in dairy buffaloes, development, evaluation and mass use of effective vaccines against these pathogens seems a national animal health imperative.

Keeping this scenario of predominance of the three topmost three mastitogens (S. aureus, Str. agalactiae and E. coli), in view, a doctoral degree study was conducted on the preparation and evaluation of inactivated polyvalent vaccines for the control of mastitis in 100 mastitis free Nili-Ravi dairy buffaloes. It was found that formaline-inactivated plain vaccine showed immediate but a short term protection against the S. aureus, Str. agalactiae and E. coli. Dextran sulphate adjuvanted bactrin provided higher antibody titres than the plain vaccine, which persisted at higher level for the entire study period comprising 6 months. Al (OH)₃ – adjuvanted vaccine provided comparatively better protection to mastitogens than dextran sulphate adjuvanted vaccine. Combined adjuvanted vaccine (dextran sulphate and aluminium hydrochloride) resulted in the highest and sustained protection against common mastitogens in buffaloes (Athar, 2005). Similar study was conducted by Butt (2005) in lactating cows and buffaloes using different oil adjuvanted polyvalent mastitis vaccines (coconut oil, sunflower oil and mineral oil) comprising three mastitogens (S. aureus, Str. agalactiae and E. coli). In conclusion canola oil adjuvanted polyvalent vaccine gave the highest and sustained antibodies levels throughout the span of study consisting of six months.
present study was conducted as first ever effort for the development of mastitis 
vaccine for buffaloes in Pakistan. In view of the functional and technical considerations, 
the study was divided into six phases detailed below:

**PHASE-I:**

3.1. **Isolation and Biocharacterization of Field Isolates**

Foremilk samples were collected aseptically from 100 clinically mastitic quarters 
of buffaloes. These samples were streaked onto Blood agar plates containing 5 \% sheep 
eythrocytes and Staph-110 medium. Colony morphology and haemolytic patterns of the 
isolates were noted. Gram positive, catalase positive, coagulase-positive (at 4 hours) 
isolates were identified as *Staphylococcus aureus* (National Mastitis Council, Inc., 1990).

A typical alpha, beta haemolytic *S. aureus* field isolate was selected and biotyped 
using a commercially available kit (API-Staph Identification Codebook; 1986; API® 
Analytab Products, Division of Sherwood Medical 200 Express Street Plainview, New 
York 11803).

3.2. **Pathogenicity of Selected Vaccinal Isolate of *S. aureus***

An activated growth (6-hours of incubation) of *S. aureus* from Nutrient Broth was 
adjusted to $10^6$ cells per ml in Phosphate buffered saline. A single dose of 0.25 mL was 
administered subcutaneously to 4 healthy adult rabbits. All the animals were kept under 
observation for 24 hours (Marchant and Packer, 1983).

3.3. **Optimisation of Cultural Conditions**

Optimal cultural conditions were standardized in order to get the encapsulated *S. 
aureus*. Pseudocapsule is an extracellular glycocalyx comprised largely of hydrated 
polysaccharides responsible for enhancing virulence of the organism by impairing
complement- and antibody-mediated opsonization and inhibiting phagocytosis and expression of which usually ceases when organism is grow under in vitro conditions in conventional bacteriological media. For this purpose, the selected field isolate was grown on blood agar (BA) plate, nutrient broth (NB), modified NB, brain heart infusion broth (BHI:B), and modified BHIB. Modifications of NB and BHIB was done by incorporating bovine sterile whey @ 10% v/v. Extent of the expression of the pseudocapsule in different cultural conditions was confirmed and compared by using India ink method (Buxton and Fraser, 1980; Appendix-VI), Hiss’s method (Marchant and Packer, 1983; Appendix-V), Anthony’s method (Cruick Shank, 1975; Appendix-IV) and autoagglutination method (Watson and Watson, 1989).

3.3.1. Detection of Expression of Pseudocapsule (Watson and Watson, 1989)

Cultures of S. aureus were centrifuged (1200 x g, 10 min, 4°C) and the cells were washed once with sterile phosphate buffered saline (PBS), pH 7.2, then resuspended in sterile PBS and adjusted spectrophotometrically to a concentration of 5 x 10⁹ cells/mL. A 0.1 ml aliquot of the suspension was added to each well of a U-bottomed microtitre tray. Then to each well was added 0.1 ml of sodium hydroxide solution prepared at various molarities ranging between 0.001 M and 0.1 M. These solutions were made by diluting 1.0 M sodium hydroxide with sterile PBS. A PBS control (without sodium hydroxide) was included, the tray covered, allowed to stand for 16 hours at ambient temperature (approximately 20°C), then the autoagglutination titre was recorded.

3.3.2 Preparation of Sterile Milk Whey (Watson and Watson, 1989)

One litre milk whey was prepared by rennin precipitation from 1.5 litre whole milk collected commercially. A 0.5 mg of rennin was dissolved in 27 ml of saline and the suspension was added to 1.5 litre of delipidized milk. After 30 minutes at 37°C, the samples were centrifuged at 10,000 x g for 20 minutes. The whey samples were then sterilized by filtration gradually from 0.45 and 0.22 micrometer (µm) membrane filter. Filtered milk whey was stored at —20°C for future use.
PHASE-II:

3.4. Evaluation of Antigenicity in Rabbits

For this purpose, two experiments were conducted. In first experiment, antigenic property of above field isolate was checked in rabbits, whereas in the second experiment, dose-dependent immune response was monitored in rabbits.

3.4.1. Preparation of Antigen

The culture suspension was checked for purity through Grams' staining method and Formaline (0.2%) was added into the broth for 12 hours at room temperature. Sterility of the suspension was assessed through cultural examination on Blood agar plate in the next 24 hours. Total bacterial count was calculated using spectrophotometric. Later the suspension was centrifuged (at 200 x g) and resuspended the pellet in Phosphate Buffer Saline (pH.7.2) to maintain $10^6$ cells/mL and stored under refrigeration temperature for future use (Awan and Rahman, 2002).

3.4.2. Evaluation of Antigenicity in Rabbits

Fifteen rabbits were divided into 3 groups. R1-group of rabbits were inoculated with 0.2 ml of inactivated antigen through subcutaneous route as single dose. The rabbits of group R2 were inoculated with 0.2ml of inactivated antigen through subcutaneous route and booster with 7 days apart with the same dose rate. R3-group of rabbits was kept as unvaccinated control. The rabbits of groups were maintained separately in the cages and given fodder and water ad-libitum (Table 3.1).

Serum samples were collected from each group randomly from 3 rabbits at an interval of a week till 21 days postinoculation. Antibody titres were measured using standard technique of indirect haemagglutination (IHA) test (Rahman et al., 2005) as detailed in Appendix-XII.
3.4.3. Preparation of Hyperimmune Sera in Rabbits

Whole culture formalin inactivated antigen (15ml) of *S. aureus* containing 1.2. X 109 cells/ml was prepared (Watson and Davies, 1993; Giraudo *et al.*, 1997). Ten healthy rabbits were selected to raise the hyperimmune sera (Malik, 1996). Progressively increasing doses of antigen were given-intravenously on day 1 (0.1ml), 3 (0.2ml), 5 (0.3ml), 7 (0.4), and 9 (0.5ml). Fourteen days after the last injection, the rabbits were bled to collect the sera. Five buffalo-calves (4-6 months of age) were inoculated with 2 ml of formalin-inactivated antigen as detailed in table 3.2. Second injection (4ml) was repeated after 7 days and blood was collected after 14 days of last injection. Serum was separated, heat inactivated to 56°C for 30 minutes in water bath and stored under −20°C.

3.4.4. Indirect Haemagglutination Test

Indirect haemagglutination test (IHA) method already standardized by Rahman *et al.* (2005) was adopted for the evaluation of immune response in rabbits, buffaloes vaccinated with live and killed mastitis vaccines. Sonicated *S. aureus* antigen was used to sensitized sheep RBCs and glutaraldehyde was used as coupling agent. One percent sensitized sheep RBCs were finally used for conducting IHA test as detailed in Appendix-XII.

3.4.5. Evaluation of Immune Response in Rabbits to Different Concentrations of Local Isolate

This experiment was conducted in another set of 15 adult rabbits (weighing approx. 2.5 kg) divided randomly into 3 groups (R-1, R-2, R-3) comprising 5 rabbits each. For this experiment, antigen was prepared in the same manner as given above.
Table 3.2: Protocol for the preparation of hyperimmune serum in rabbits and buffalo-calves

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mL) and route (I/V)</th>
<th>Intermittent administration of antigen at days</th>
<th>Serum collection time from the last injection (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R2</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R3</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R4</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R5</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R6</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R7</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R8</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R9</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>R10</td>
<td>0.1, 0.2, 0.3, 0.4, 0.5</td>
<td>1, 3, 5, 7, 9</td>
<td>14</td>
</tr>
<tr>
<td>B1</td>
<td>2, 4</td>
<td>1, 7</td>
<td>14</td>
</tr>
<tr>
<td>B2</td>
<td>2, 4</td>
<td>1, 7</td>
<td>14</td>
</tr>
<tr>
<td>B3</td>
<td>2, 4</td>
<td>1, 7</td>
<td>14</td>
</tr>
<tr>
<td>B4</td>
<td>2, 4</td>
<td>1, 7</td>
<td>14</td>
</tr>
<tr>
<td>B5</td>
<td>2, 4</td>
<td>1, 7</td>
<td>14</td>
</tr>
</tbody>
</table>

R = rabbits
B = buffalo-calf

The rabbits of group R-1 were immunized with the antigen preparation containing $10^8$ cells/mL of *S. aureus* inactivated antigen. To the rabbits of group R-2, preparation containing $10^{10}$ cells/mL. Group R3 was administered with $10^{12}$ cells per mL serum samples were collected at weekly intervals for 3 consecutive weeks. Antibody response to the different antigen concentrations was also evaluated through indirect haemagglutination (IHA) test (Watson and Davies, 1993).
PHASE-III:

3.5. Formulation of Plain and Adjuvanted S. aureus Vaccines

This phase of the study was accomplished by three experiments. The optimum cultural conditions were laid down and cultivation of S. aureus was conducted and formulation of vaccines was undertaken.

3.5.1. Preparation of Plain S. aureus Vaccine (PSAV) (Watson and Davies, 1993)

The selected field isolate of S. aureus was grown independently in modified nutrient broth at 37°C for 48 hours on an orbital shaker set at 60 rpm under aerobic conditions. After confirming the purity and pseudocapsule elaboration of the culture the suspension was inactivated with formalin (0.4% v/v), centrifuged (at 6,000 × g for 1 hour at 4°C), and resuspended in PBS at pH 7.2. The suspension was preserved at 4°C until required. Bacterial concentration was adjusted to 10^10 cells/mL spectrophotometrically. Sodium azide, thimerosal, and formalin were added as preservatives at a final concentrations of 0.001% (w/v), 0.001% (w/v) and 0.4% (v/v), respectively (Plate 1).

3.5.2. Incorporation of crude toxin extract of S. aureus (Giraudo et al., 1997)

For incorporation of crude extract, supernatant fluid was collected from 48-hour broth culture of S. aureus. The supernatant was separately autoclaved at 121°C for 20 minutes. This preparation was then centrifuged at 6000 x g for 30 minutes at 4°C and the supernatant was added to the vaccine preparation at a concentration of approximately 5 mg of dry weight per dose. Finally the plain S. aureus vaccines was prepared which contained the availability of individual component as detailed in appendix-XI.

3.6 Preparation of Dextran Sulphate-Adjuvanted S. aureus Vaccine (DSAV) (Watson and Davies, 1993)

Dextran sulphate (DXS, M.W. 500,000; sigma-Aldrich Co., St. Louis, USA) was added as an adjuvant in the vaccine at a final concentration of 50 mg DXS^3ml for 5 ml dose as detailed in Appendix-IV (Plate 1).
3.7 Preparation of Oil-Adjuvanted *S. aureus* Vaccine (OSAV)

(Nordhaug et al., 1994a & b; Shauket et al., 1998)

It was prepared as under:

a. Liquid paraffin was used as an adjuvant whereas Tween-80 and Span-80 were used as emulsifiers.

b. Emulsification of oil and Span-80 was done at low speed (12000 rpm) with the help of ultra homogenizer.

c. Tween-80 was added drop-by-drop and homogenized with antigen at 18000 rpm until complete homogenization.

d. Antigen suspension was slowly added and continuously stirred oil phase at 18000 rpm in the ultrahomogenizer under 4°C.

e. Final oil emulsion vaccine (Appendix-V) was dispersed in sterilized glass vials with automatic cap seal system and stored under 4°C future use (Plate 1).

3.8 Preparation of Live Attenuated Vaccine

(Watson and Lee, 1978; Watson, 1989)

An α-β haemolytic selected isolate of *S. aureus* was repeatedly passaged through cultured on 5% sheep blood agar until it lost its haemolytic activity and then maintained in trypsince soy broth. This live attenuated isolate was grown for 24 hrs. in nutrient broth (NB). Organisms were deposited by centrifugation (3000 × g; 15 min.), washed (×2) with phosphate buffered saline (PBS; pH 7.2) and resuspended in sterile PBS. The concentration of bacteria was finally adjusted to 10^{10} cells^{-1} using spectrophotometric method. The composition of live attenuated vaccine is detailed in Appendix-VII; Plate 1).

3.9 Concentration Adjustment of *S. aureus* in vaccines

Concentration of the vaccinal isolate for live and killed vaccines (plain bacterin, dextran sulphate adjuvanted bacterin and oil adjuvanted bacterin) was adjusted spectrophotometrically (U-2001 Spectrophotometer, Japan) at 640 nm.
Plate 1: Sealed packings of 4 different *S. aureus* mastitis vaccines (from L to R: LSAV, PSAV, DASAV & OSAV).
3.10. Purity, Sterility, Safety and Stability Studies

3.10.1. Purity of live attenuated *S. aureus* was checked by streaking a loopful on the vaccine on blood agar to observe the cultural and morphological characteristics.

3.10.2. Sterility of a vaccine was checked by streaking a loopful of the vaccine onto blood agar plate and incubating for 48 hours for the presence of any growth.

3.10.3. Safety evaluation of the vaccines was checked by subcutaneous inoculation of 0.2 ml of the vaccines into 20 rabbits (5 rabbits per vaccines). A second injection was given after 14 days. Seven days later, the vaccinated rabbits were challenged (0.2 ml subcutaneously) with a live inoculum containing $10^{10}$ cells per ml of the vaccinal *S. aureus* (Tizard, 1996).

The study of side effects, if any was made in 16 adult healthy rabbits divided randomly into 4 groups (R1 thru R4). Half of the rabbits of each group were injected with 0.2 ml of a vaccine subcutaneously, while the remaining animals were given 10 times more dose (2 ml) intramuscularly. Any local or systemic reaction was recorded for 7 days.

3.10.3. Stability Studies of Inactivated Vaccines

To conduct this study, the vials of plain bacterin, dextran sulphate adjuvanted *S. aureus* vaccine and oil adjuvanted *S. aureus* vaccine were kept at room temperature (25, 37°C) for a period of 9 months. Each vaccine was injected @ of 0.2 ml per animal subcutaneously to 2 new rabbits every month. Blood samples were collected before and after a week to measure the mean antibody titers by IHA from the serum. This succession of monthly checking of antibody titre was kept continuous up to 9 months' period.

Physical properties of these vaccines were also monitored during the span of 7 months time by placing at 25°C. There was no change in colour up to 7 months, while viscosity remained normal for a period of 6 months and texture was also found normal until 6 months.

3.10.4. Viability of Live *S. aureus* Vaccine

In this study, *S. aureus* was stored into three separate media to assess the length of
period of viability i.e. peptone water, Phosphate buffered saline and Nutrient broth. The standard plate count method was done daily (Awan and Rahman, 2002).

PHASE-IV:

3.11. Immunogenicity Trials of Live Attenuated and Inactivated *S. aureus* Vaccines in Rabbits

For this experiment, 25 adult healthy rabbits divided randomly into 5 groups (R-I thru R-5) were selected. The rabbits of group R-I were vaccinated with live attenuated *S. aureus* vaccine. The Rabbits of group R-2 received plain bacterin. Rabbits of group R-3 were given Dextran sulphate adjuvanted vaccine. Rabbits of group R-4 received Oil-adjuvanted vaccine, whereas the rabbits of group R-5 were kept as unvaccinated control (Table 3.3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Type of vaccines</th>
<th>Dose of Vaccines (s/c)</th>
<th>Duration (months)</th>
<th>Serum sampling time (Days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>5</td>
<td>LSAV</td>
<td>0.2ml</td>
<td>2</td>
<td>0,15,30,45,60</td>
</tr>
<tr>
<td>R2</td>
<td>5</td>
<td>PSAV</td>
<td>0.2ml</td>
<td>2</td>
<td>0,15,30,45,60</td>
</tr>
<tr>
<td>R3</td>
<td>5</td>
<td>DSAV</td>
<td>0.2ml</td>
<td>2</td>
<td>0,15,30,45,60</td>
</tr>
<tr>
<td>R4</td>
<td>5</td>
<td>OSAV</td>
<td>0.2ml</td>
<td>2</td>
<td>0,15,30,45,60</td>
</tr>
<tr>
<td>R5</td>
<td>5</td>
<td>UC</td>
<td>0.2ml (placebo)</td>
<td>2</td>
<td>0,15,30,45,60</td>
</tr>
</tbody>
</table>

SAV = Live attenuated *S. aureus* Vaccine
PSAV = Plain Bacterin *S. aureus* Vaccine
DSAV = Dextran Sulphate-Adjuvanted *S. aureus* Vaccine
OSAV = Oil Adjuvanted *S. aureus* Vaccine
UC = Unvaccinated Control
PHASE-V:

3.12. Evaluation of *S. aureus* Vaccines under Experimental Conditions in Pregnant Buffaloes

A total of 25 pregnant non-mastitic buffaloes in their last trimester were randomly assigned to 5 groups (B1 thru B5). Animals of each group were respectively vaccinated with live attenuated *S. aureus* (LSAV), plain bacterin *S. aureus* vaccine (PSAV), dextran sulphate-adjuvanted *S. aureus* vaccine (DSAV) and oil adjuvanted *S. aureus* vaccine (OSAV) whereas buffaloes in B5 were kept as unvaccinated control (UC). In groups B1 thru B4 ml of the respective vaccine was administered IM 8 and 4 weeks prepartum (Watson, 1988) (Table 3.4).

**Table 3.4. Distribution of Buffaloes inoculated with Different mastitis vaccines**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No of Buffaloes</th>
<th>Type of vaccines</th>
<th>Dose of vaccines followed by boosting at a month apart</th>
<th>Duration 6 months</th>
<th>Serum sampling time (Months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>5</td>
<td>LSAV</td>
<td>5ml i/m</td>
<td>2 month prepartum and 4 month postpartum</td>
<td>0,1,2,3,4,5,6</td>
</tr>
<tr>
<td>B2</td>
<td>5</td>
<td>PSAV</td>
<td>5ml i/m</td>
<td>2 month prepartum and 4 month postpartum</td>
<td>0,1,2,3,4,5,6</td>
</tr>
<tr>
<td>B3</td>
<td>5</td>
<td>DSAV</td>
<td>5ml i/m</td>
<td>2 month prepartum and 4 month postpartum</td>
<td>0,1,2,3,4,5,6</td>
</tr>
<tr>
<td>B4</td>
<td>5</td>
<td>OSAV</td>
<td>5ml i/m</td>
<td>2 month prepartum and 4 month postpartum</td>
<td>0,1,2,3,4,5,6</td>
</tr>
<tr>
<td>B5</td>
<td>5</td>
<td>UC</td>
<td>5ml i/m (Placebo)</td>
<td>2 month prepartum and 4 month postpartum</td>
<td>0,1,2,3,4,5,6</td>
</tr>
</tbody>
</table>

**Legend:**

- LSAV = Live attenuated *S. aureus* Vaccine
- PSAV = Plain Bacterin *S. aureus* Vaccine
- DSAV = Dextran Sulphate-Adjuvanted *S. aureus* Vaccine
- OSAV = Oil Adjuvanted *S. aureus* Vaccine
- UC = Unvaccinated Control
- Im = Intramuscular
3.13. Challenge/Protection Test

At approximately 5 weeks post-partum, the teats of all groups were dipped in a live inoculum of *S. aureus* in sterile buffer containing $10^{10}$ cfu ml-1 (Calvinho et al., 1989). Evaluation was made on the basis of the presence of mastitis by CMT and SFMT, change in SCC, for 14 days (Plate 2).

**PHASE-VI**

**FIELD EVALUATION OF LIVE ATTENUATED AND DEXTRAN SULPHATE ADJUVANTED *S. aureus* VACCINES IN LACTATING BUFFALOES**

3.14. Vaccines and experimental buffaloes

Based on the results of evaluation of vaccines in pregnant, non-lactating, non-mastitic buffaloes, live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV) were ranked better than the other two vaccines (plain bacterin and oil adjuvanted bacterin). As a final phase of the study, these two vaccines were evaluated in 100 lactating buffaloes under field conditions (Table 3.5).

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of buffaloes</th>
<th>Types of vaccine</th>
<th>Dose and route of administration*</th>
<th>Duration of study (days)</th>
<th>Sampling time points (days post vaccination)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>50</td>
<td>LSAV</td>
<td>5 mL i.m.</td>
<td>180</td>
<td>0,30,60,90,120,150,180</td>
</tr>
<tr>
<td>B7</td>
<td>50</td>
<td>DSAV</td>
<td>5 mL i.m.</td>
<td>180</td>
<td>0,30,60,90,120,150,180</td>
</tr>
<tr>
<td>B8</td>
<td>50</td>
<td>UC</td>
<td>5 mL (normal saline) i.m.</td>
<td>180</td>
<td>0,30,60,90,120,150,180</td>
</tr>
</tbody>
</table>

LSAV = live attenuated *S. aureus* vaccine  
DSAV = Dextran sulphate adjuvanted *S. aureus* vaccine  
SC = Unvaccinated control (placebo control)  
m. = Intramuscular  
Booster inoculation 1 month later
Plate 2: Post milking teat dip challenge with live inoculum of selected *S. aureus*. 
A total of 150 Nili-Ravi buffaloes that had calved 1.5 to 2.5 months earlier were selected for the field trial. The buffaloes were selected from those maintained at Government (Livestock Production Research Institute, Bahadarnagar Okara; Agronomy-Livestock Section, Ayub Agriculture Research Institute, Faisalabad; Fodder Research Institute, Sargodha; Livestock Experimental Station, University of Agriculture, Faisalabad) and six private dairy farms. Buffaloes with blind quarters, congenital abnormalities and clinical mastitis were not included in the trial. All the buffaloes were managed in ‘tie-stall’ cum loose housing system during the experimental period. The buffaloes received a diet of concentrate mixture and green fodder. Field trial started in March and culminated in August. In a cut-and-carry feeding system, chopped green fodder plus chaffed wheat strew was fed ad lib. Berseem (Trifolium alexandrinum) and maize (Zea mays) were the green fodder fed. The concentrate mixture was fed at the time of milking and its quantity roughly corresponded to milk production level of the individual animal. Fresh tap water was made available 3–5 times per day and animals were hosed once or twice daily depending upon the weather. The buffaloes were hand-milked twice a day between 3 – 5 am and 3 –5 pm. No mastitis control practices (e.g., post milking antiseptic teat dipping, dry period antibiotic therapy) were in place. The screening of the buffaloes for mastitis was based on the results of California mastitis test, Sure’field mastitis test as well as culturing of the quarter fore milk samples. Experimental buffaloes were randomly divided into 3 groups (B6, B7 & B8) of 50 animals each. The protocol of vaccination and sampling schedule has been depicted in Table 3.5 (Plate 3).

**Evaluation of *S. aureus* Vaccines in Buffaloes**

Evaluation of the vaccines was conducted by comparing the following parameters in the pre and post-vaccinal periods at monthly intervals.

**Parameters under Study:**

1. Serum and milk whey antibody titres against *S. aureus* were measured using standard modified indirect Haemagglutination (IHA) test (Rahman et al., 2004).
Plate 3: Collection of blood samples from vaccinated buffaloes.
### 14.2. Dinitrochlorobenzene test (DNCB Test) (Reddi et al., 1981)

DNCB test (a field test for evaluation of cell-mediated immunity) after two weeks of booster immunization. The DNCB (2, 4-dinitrochlorobenzene) was applied at the neck region 15 cm anterior to the shoulder blade. In this region, two areas of 3 cm diameter were marked with the help of a metallic ring and these areas were clipped close to the skin with scissors. The metallic ring was placed over the prepared area and the 2% solution of DNCB in acetone was dropped slowly, 2 to 3 drops at a time using a 1 ml tuberculin syringe with a hypodermic needle. The solution was allowed to dry immediately by blowing so as to prevent it from running down the neck region. The minimum dose level and number of applications was found to be 0.5 ml of 2% DNCB in acetone applied for three consecutive days for eliciting a minimum response. All the animals that were sensitized by the above method were challenged at two sites on the 14th day on the opposite side. The challenge dose was 0.5 ml of 2% DNCB in acetone. The skin thickness was measured before and 24 and 48 hours following challenge. The same experimental procedure was repeated with 0.5 ml of acetone without DNCB. This served as control. This test was conducted on randomly selected 15 vaccinated buffaloes from 5 groups (B1, B2, B3, B4 and B5) and each group had 5 five buffaloes. So three animals belonged to each group in Table 3.6 (Plate 4 and 5).

**Table 3.6: Protocol of DNCB test in pregnant buffaloes vaccinated with different *S. aureus* mastitis vaccines**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Vaccines</th>
<th>No. of Animals</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Site 1</td>
</tr>
<tr>
<td>B1</td>
<td>LSAV</td>
<td>3</td>
<td>2% DNCB 0.5 ml</td>
</tr>
<tr>
<td>B2</td>
<td>PSAV</td>
<td>3</td>
<td>-do-</td>
</tr>
<tr>
<td>B3</td>
<td>DSAV</td>
<td>3</td>
<td>-do-</td>
</tr>
<tr>
<td>B4</td>
<td>OSAV</td>
<td>3</td>
<td>-do-</td>
</tr>
<tr>
<td>B5</td>
<td>UC</td>
<td>3</td>
<td>-do-</td>
</tr>
</tbody>
</table>

**Legend:**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSAV</td>
<td>Live attenuated <em>S. aureus</em> Vaccine</td>
</tr>
<tr>
<td>PSAV</td>
<td>Plain Bacterin <em>S. aureus</em> Vaccine</td>
</tr>
<tr>
<td>DSAV</td>
<td>Dextran Sulphate-Adjuvanted <em>S. aureus</em> Vaccine</td>
</tr>
<tr>
<td>OSAV</td>
<td>Oil Adjuvanted <em>S. aureus</em> Vaccine</td>
</tr>
<tr>
<td>UC</td>
<td>Unvaccinated Control</td>
</tr>
</tbody>
</table>
Plate 4: DNB test kit showing its basis essentials.
Plate 5: Areas marked on the neck of buffaloes to perform DNCB test.
3.14.3. Modified Somatic Cell Count Technique

Effect on milk quality in terms of somatic cell count was determined. Somatic cell count was performed with a little modification to the original method of Schalm et al., (1971). A 10 µl of fresh milk was spread over a glass slide having a marked area of 10 mm \times 10 mm using a micropipette. The fine milk smear so prepared was dried in an oven at temperature of 30-40°C the slides were then dipped in xylene for 1 to 2 minutes to remove the fat globules and dried subsequently. The slides were then stained using Newman-Lampert’s stain for a period of 15 minutes, and were dried at room temperature. The excess of stain was removed from the smears with tap water and the slides were again dried at room temperature. The poorly stained smears were further stained with blue (basis) aliquot of Dip-Quik stain (J-332-A3, blue portion, Jorgensen Labs. Inc. Loveland, Colorado, 80538, USA) for 10-15 seconds followed by tap water rinsing and drying. This significantly enhanced the differentiation among cells and the substrate (Appendix-II). The somatic cell counts were measured under microscope with a magnification of 15 x 40 in 50 fields and were multiplied by the microscopic factor to get the cells per ml of milk (Plate 6).

3.14.4. Butter Fat Concentration

Gerber’s fat test as described by Aggarwala and Shamma (1961) was followed for determination of fat percentage in milk. Fresh milk measuring 11 ml from the representative milk samples and 1 ml of amyl alcohol was gently added to 10 ml of sulphuric acid already poured in each Gerber’s acidobutyrometer and was carefully mixed after properly closing the butyrometers with rubber stoppers. The butyrometers were then centrifuged for 3-4 minutes and the percentage of fat was recorded.

3.14.5. Milk Protein Concentration

Milk protein was determined by formal titration method (Davide, 1977). Ten milliliters of well-mixed milk were poured into Erlenmeyer flask. One milliliters of phenolphthalein indicator followed by 0.4 ml of neutral saturated Potassium oxalate solution were added and mixed. These were set aside for two minutes.
Plate 6: A glimpse of milk somatic cell count (SCC) after being treated with a modified Newman-Lempert's Staining Technique.
Titration with standard 0.1 N Sodium hydroxide solution was carried out to a faint pink colour. Then 2 ml of 40 per cent formalin solution were added and mixed. The contents of the flask were then titrated with 0.1 N sodium hydroxide solution to the same pink shade and the volume of alkali used was recorded. The percentage of protein in buffalo milk was obtained by multiplying the volume of 0.1 N Sodium hydroxide used (already corrected for slight acidity of formalin as determined by titrating 2 ml added to 10 ml of distilled water) by the formal factor 1.91.

3.14.6. Effect on the milk quantity

Most of the animals belonged to an institutional herds where daily milk yield of animals was recorded regularly.

3.14.7. Prevalence of mastitis: Determination of prevalence of mastitis (Thrusfield, 1995) was based on the results of microbiological examination of quarter foremilk samples for S. aureus (National Mastitis Council, Inc., 1990), Surf field mastitis test (Muhammad et al., 1995) and California mastitis test (Schalm et al., 1971). For microbiological examination of milk, one hundredth of a millilitre (0.01mL) of milk was cultured on blood agar plates. Guidelines for significance of colony numbers of S. aureus were based on those recommended by National Mastitis Council, Inc. (1980). Sampling time points were day 0, 30, 60, 90, 120, 150, and 180. The udder quarters were the units of concern.

3.14.8. Cumulative incidence: Determination of cumulative incidence (Thrusfield, 1995) of S. aureus mastitis was based on the results of microbiological examination of quarter foremilk samples. Incidence was determined for the periods intervening between day 0 and day 30, day 31 and day 60, day 61 and day 90, day 91 and day 120, day 121 and day 150 and day 151 and day 180. Cumulative incidences for the period between day 0 and day 120 and day 121 and day 180 were calculated separately.

3.14.9. Severity of clinical episodes: This was derived by clinical severity score modified from (Faull & Hughes, 1996; Appendix-III).

3.14.10. Number of colonies of S. aureus in the milk of vaccinates and control (National Mastitis Council, Inc., 1990). Pre-vaccination (day 0) colony numbers in the respective
group were compared with those recorded at day 180 post vaccination to determine the effect of vaccination on colony forming units of *S. aureus*.

### 3.14.11. Vaccine efficacy

Preventative efficacies of live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSA V) at day 120 and day 180 were calculated. Vaccine efficacies at these time points were defined as the proportion of *S. aureus* intramammary infections prevented by the respective vaccine in vaccinated buffaloes (Martin et al., 1987). To this end, an estimate of the vaccine efficacy was obtained from the odds ratio (OR) using the following formula for estimating an attributable fraction:

$$\text{Estimated Attributable Fraction} = \text{OR} - 1 / \text{OR}$$

Odds ratios for the two vaccines at day 120 and day 180 were calculated by the following formula:

$$\text{Odds Ratio (OR)} = \frac{a d}{b c}$$

Where:

- **a** = No. of quarters with *S. aureus* intramammary infection out of a total of 200 quarters of unvaccinated control group at the sampling time point in question.
- **b** = No. of quarters uninfected with *S. aureus* in unvaccinated control group at the sampling time point in question.
- **c** = No. of quarters infected with *S. aureus* in vaccinated buffaloes at the sampling time point in question.
- **d** = No. of quarters uninfected with *S. aureus* in the vaccinated group at the sampling time point in question.

### 3.14.12. Cost-benefit analysis of mastitis control through vaccination

(Martin, *et al.*, 1987; Thrusfield, 1995)

The mean milk yield of each group of buffalos at each sampling time point starting from booster vaccination was multiplied by 30 to calculate the total milk production over
one month period. The monthly milk yields of each group were aggregated to obtain total milk produced in a period between booster vaccination and final sampling time point (day 180). The differentials of milk yield between vaccinates (LSAV and DSAV) and control group were calculated by subtracting the milk yield of unvaccinated control group (B8) from total milk production of the respective vaccinal group (B6 & B7). These differentials were than multiplied by 15 (the prevailing price of milk per liter at the farm gate) to calculate the economic benefit of using each vaccine. Two doses of live attenuated S. aureus vaccine (LSAV) for one vaccinate were estimated to cost Rs. 225/= (inclusive of cost of maintaining a cold chain), whereas two doses of dextran sulphate adjuvanted vaccine (DSAV) were estimated to cost Rs. 500/= (the adjuvant dextran sulphate is very expensive).

3.15. Data Analysis

The data thus generated was subjected to statistical analysis using ANOVA and other appropriate designs on SAS-2000 computer programme. For prevalence, incidence and somatic cell count, the unit of concern was the mammary quarter, whereas for antibody titres, milk fat and protein concentration, and DNBC test, buffalo was the unit of concern. Significance was declared at P< 0.05.
RESULTS

PHASE-I:

4.1. Isolation and Biocharacterization of Isolates from Mastitis Cases

Sixty four *S. aureus* isolates were collected from 100 clinical cases of mastitis in buffaloes following the procedure described by National Mastitis Council, Inc. USA (1990). Gram-positive, catalase-positive, coagulase-positive and α/β-haemolysis producing cocci were regarded as the virulent isolates of *S. aureus*. Thirty were found to be alpha haemolytic; four alpha beta haemolytic and thirty beta haemolytic. Out of these a typical alpha – beta *S. aureus* isolate was selected as a candidate for vaccines (Plate 7). This isolate gave the following 7 digit API-Staph biochemical profile:

636153; Good identification (97.5%) (API-Staph Identification Codebook; 1986; API® Analytab Products, Division of Sherwood Medical 200 Express Street, Plainview, New York 11803) (Plate 8).

4.2. Pathogenicity of Selected Local Isolate of *S. aureus*

All the rabbits died within 18 hours. Post-mortem examination indicated septicaemia, petechiation on the intestinal serosal surface, straw colored fluid in abdomen and swollen kidneys. Stained blood and straw colored smear indicated the *S. aureus*.

4.3 Selection of the Best Culture Media

Modified nutrient broth proved as the best pseudcapsule developing medium in which autoagglutination was observed in molar concentration solution of NaOH from 0.04 to 0.001, while partial agglutination was found in modified brain heart infusion broth in molar concentration solution of NaOH between 0.04 and 0.03 (Table 4.1).
Plate 7: A typical α-β *S. aureus* selected isolate on 5% blood agar plate.
Plate 8: Seven digit API Staph. biochemical profile of selected *S. aureus* isolate.
India ink and Anthony's method failed to demonstrate the development of capsule formation around the cells in any of the culture media whereas autoagglutination method demonstrated successfully the presence of capsule in modified nutrient broth. All other culture media did not exhibit the presence of any pseudocapsule.

Table 4.1: Comparative *S. aureus* capsule production recorded in various culture media at 37°C for 48 hours using auto-agglutination test.

<table>
<thead>
<tr>
<th>Molarities of NaOH Solution</th>
<th>Plate</th>
<th>NB</th>
<th>MNB</th>
<th>BHIB</th>
<th>MBHIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.75</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.09</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.07</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.04</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>0.03</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>0.02</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.005</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.001</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.000</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Plate = Blood agar plate  
NB = Nutrient broth  
MNB = Modified Nutrient broth (10% sterile whey added)  
BHIB = Brain heart infusion broth  
MBHIB = Modified Brain heart infusion broth (10% sterile whey added)
PHASE-II:  

4.4.a. Results of Immune Response of *S. aureus* in Rabbits  

There was a clear-cut primary immune response in group R-1 which was 25 at day 14 followed by slight decrease at day 21 (Fig. 4.1). In group R-2, there was a distinct primary and secondary immune response that was 8 (GMT) at day 7 and was maximum (26 GMT) at day 21. There was nothing such response in unvaccinated group of rabbits. In a nutshell, it may be said that this trial proved the antigenicity of the selected *S. aureus* isolate.

4.4.b. Sterility of Toxoid  

Streaking of toxoid on 5% sheep blood agar did not show any growth even after 72 hours of incubation at 37°C.

4.5. Result of Immune Response to Different Concentrations of *S. aureus*  

The trend of antibody production was about the same in three groups. It was maximum (32) at day 14 in group R-2 along with consistency unto day 21. It was less (30) in group R-1 than that of group R-3 in which antibody titre was 30 at day 14 and 27 (GMT) at day 21.

The immunogenic response was the highest in that group which was administered the dose $10^{10}$/mL concentration, while it was the lowest in case of $10^8$ cells/mL concentration. The immune response elicited by $10^{12}$ cells/mL concentration was in between the response shown by the other two concentrations i.e. $10^8$ and $10^{12}$ cells/mL. It was less than one concentration ($10^{10}$ cells/mL) and higher than that of other ($10^8$ cell/mL).

This study indicated that $10^{10}$ cells/ml concentration proved to be able to stimulate the immune response at the maximum. This trial gave us the clue that this very concentration be used for vaccine preparation (Fig. 4.2; Appendix-XIII).
FIG. 4.1: GEOMETRIC MEAN IHA ANTIBODY TITRES (GMT) AGAINST S. aureus AT DIFFERENT POST PRIMARY

- Primary inoculation (R1)  - Booster inoculation (R2)  - Control (R3)

Secondary Immune Response

Primary Immune Response

GMT against the vaccine S. aureus

Days Post Primary and Secondary Inoculation
FIG. 4.2: GEOMETRIC MEAN IHA ANTIBODY TITRES (GMT) AGAINST THREE DIFFERENT CONCENTRATIONS OF S. aureus AT DIFFERENT DAYS POST VACCINATION

$R_1 \times 10^8 = 10^1$
$R_2 \times 10^{10} = 10^{10}$
$R_3 \times 10^{12} = 10^{12}$
4.6. Purity of the Live Attenuated *S. aureus* Vaccine (LSAV)

This was checked by Gram’s staining by making smear from the vaccine. No other organism was found with the exception of Gram positive, cocci indicating the purity of the live attenuated *S. aureus* vaccine.

4.6.a. Safety Evaluation of Vaccines in Rabbits

One rabbit of group R1 and 4 of group R5 were died within 24 hours. Safety percentage of plain bacterin *S. aureus* vaccine (PSAV). Dextran sulphate adjuvanted *S. aureus* vaccine (DSA) and oil adjuvanted *S. aureus* vaccine (OSAV) was 100% while it was 80% in case of live attenuated *S. aureus* vaccine (Table 4.2).

The dose of 0.2 ml of each vaccine produced very mild type of swelling at the site of injection in all groups. When this dose was increased to 10 times then it produced mild swelling in the animals of group R1, R3 and R4 and it subsided within 3-4 days. The body temperature remained within normal limits in rabbits belonging to all groups till the end of observation period (seven days) (Table 4.3).
## Table 4.2: Evaluation of Safety of *S. aureus* Vaccines in Rabbits.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name of Vaccines</th>
<th>Dose &amp; Route</th>
<th>Total No. of Rabbits</th>
<th>No. of Rabbits survived</th>
<th>No. of Rabbits killed</th>
<th>Safety percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>LSAV</td>
<td>0.2 mL SC</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>80%</td>
</tr>
<tr>
<td>R2</td>
<td>PSAV</td>
<td>0.2 mL SC</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>R3</td>
<td>DSAV</td>
<td>0.2 mL SC</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>R4</td>
<td>Osav</td>
<td>0.2 mL SC</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100%</td>
</tr>
</tbody>
</table>

LSAV = Live-attenuated *S. aureus* vaccine  
PSAV = Plain bacterin *S. aureus* vaccine  
DSAV = Dextran sulphate-adjuvanted *S. aureus* vaccine  
OSAV = Oil-adjuvanted *S. aureus* vaccine
### Table 4.3: Evaluation of Side Effects of *S. aureus* Vaccines in Rabbits.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Name of Vaccines</th>
<th>No. of Rabbits</th>
<th>Dose/Route</th>
<th>Mortality</th>
<th>Morbidity</th>
<th>Swelling pattern at injection site</th>
<th>Body Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>LSAV</td>
<td>4</td>
<td>0.2 ml SC</td>
<td>0</td>
<td>1</td>
<td>Very Mild</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 ml IM</td>
<td>0</td>
<td>2</td>
<td>Mild</td>
<td>Normal</td>
</tr>
<tr>
<td>R2</td>
<td>PSAV</td>
<td>4</td>
<td>0.2 ml SC</td>
<td>0</td>
<td>0</td>
<td>Very Mild</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 ml IM</td>
<td>0</td>
<td>2</td>
<td>Very Mild</td>
<td>Normal</td>
</tr>
<tr>
<td>R3</td>
<td>DSAV</td>
<td>4</td>
<td>0.2 ml SC</td>
<td>0</td>
<td>0</td>
<td>Very Mild</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 ml IM</td>
<td>0</td>
<td>2</td>
<td>Mild</td>
<td>Normal</td>
</tr>
<tr>
<td>R4</td>
<td>OSAV</td>
<td>4</td>
<td>0.2 ml SC</td>
<td>0</td>
<td>0</td>
<td>Very Mild</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2 ml IM</td>
<td>0</td>
<td>2</td>
<td>Mild</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Very Mild = Swelling at the injection site subsided within 48 hrs.
Mild = Swelling at the injection site subsided within 72-96 hrs.

LSAV = Live-attenuated *S. aureus* vaccine
PSAV = Plain bacterin *S. aureus* vaccine
DSAV = Dextran sulphate-adjuvanted *S. aureus* vaccine
OSAV = Oil-adjuvanted *S. aureus* vaccine
4.6.b Stability of Vaccines

4.6.b.i. Stability of Plain and Adjuvanted Vaccines

To conduct this study, the vials of plain bacterin, dextran sulphate adjuvanted S. aureus vaccine and oil adjuvanted S. aureus vaccine were kept at room temperature (25, 37°C) for a period of 9 months. Each vaccine was injected at 0.2 ml per animal subcutaneously to 2 new rabbits every month. Blood samples were collected before and after a week to measure the mean antibody titres by IHA from the serum. This succession of monthly checking of antibody titre was kept continuous until 9 months’ period. Immunogenic quality of plain bacterin, Dextran sulphate adjuvanted vaccine and oil adjuvanted S. aureus vaccine remained stable up to 7 months while it waned afterwards (Table 4.4, 4.5). Physical properties of these vaccines were also monitored during the span of 7 months time by placing at 25°C. There was no change in color up to 7 months while viscosity remained normal for a period of 6 months and texture was also found normal until 6 months. These vaccines remained stable up to 7 month period followed by deterioration.

4.6-b.ii Viability of Live S. aureus Vaccine

In this study, S. aureus was stored into three separate media to assess the length of period of viability i.e. peptone water, Phosphate buffered saline and Nutrient broth. The standard plate count method was done daily. It was found that S. aureus can remain viable for a period of 3 days in PBS up to the required level while in Nutrient broth, it was for 11 days. The viability remained up to the required quality till 5 days in peptone water (Table 4.6).
Table 4.4: Results of IHA test recorded to elaborate antigenic Stability of monovalent mastitis vaccine stored at 25.0±2°C for nine months period.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Months 1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSAV</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>DSAV</td>
<td>1:32</td>
<td>1:32</td>
<td>1:32</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:8</td>
<td>1:4</td>
</tr>
<tr>
<td>OSAV</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:8</td>
<td>1:4</td>
</tr>
</tbody>
</table>

Declared stability ≥ 6 months

P SA V = Plain bacterin *S. aureus* vaccine

D SA V = Dextran sulphate-adjuvanted *S. aureus* vaccine

O SA V = Oil-adjuvanted *S. aureus* vaccine
Table 4.5: Results of IHA antibodies against *S. aureus* recorded after 7 days post inoculation in rabbits to mastitis vaccine stored at 37°C for nine months period.

<table>
<thead>
<tr>
<th>Vaccines</th>
<th>Months</th>
<th>Months</th>
<th>Months</th>
<th>Months</th>
<th>Months</th>
<th>Months</th>
<th>Months</th>
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<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>PSAV</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:4</td>
<td>1:4</td>
<td>1:4</td>
<td>1:4</td>
<td>1:4</td>
</tr>
<tr>
<td>OSAV</td>
<td>1:32</td>
<td>1:32</td>
<td>1:32</td>
<td>1:16</td>
<td>1:16</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:4</td>
</tr>
<tr>
<td>OSAV</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:16</td>
<td>1:8</td>
<td>1:8</td>
<td>1:8</td>
<td>1:4</td>
</tr>
</tbody>
</table>

Declared stability ≥ 4 months

SAV = Plain bacterin *S. aureus* vaccine
OSAV = Dextran sulphate-adjuvanted *S. aureus* vaccine
OSAV = Oil-adjuvanted *S. aureus* vaccine
Table 4.6: Viability studies of *S. aureus* maintained in different media at temperature below 10°C.

<table>
<thead>
<tr>
<th>Days</th>
<th>Peptone Water (CFU/ML)</th>
<th>Phosphate Buffer Saline (CFU/ML)</th>
<th>Nutrient Broth (7.5% NaCl) (CFU/ML)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{12}$</td>
<td>$10^{12}$</td>
<td>$10^{12}$</td>
</tr>
<tr>
<td>3</td>
<td>$10^{12}$</td>
<td>$10^{10}$</td>
<td>$10^{12}$</td>
</tr>
<tr>
<td>5</td>
<td>$10^{10}$</td>
<td>$10^{5}$</td>
<td>$10^{12}$</td>
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<td>7</td>
<td>$10^{6}$</td>
<td>$10^{2}$</td>
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<td>$10^{10}$</td>
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<td>$10^{7}$</td>
</tr>
<tr>
<td>15</td>
<td>$10^{1}$</td>
<td>-</td>
<td>$10^{6}$</td>
</tr>
</tbody>
</table>
PHASE-IV:

4.7. Immune Response of Live and Inactivated Plain and Adjuvanted S. aureus Vaccines in Rabbits

Antibody titres (GMT) were 25 at day 15 and 82 at day 30 with constant increase until day 45 followed by slight decrease (110) at day 60. For group R-3, mean antibody titre (GMT) was 25 at day 15 followed by sharp increase (125) at day 30 with consistency till day 45. There was slight decrease in antibody titre at day 60 when it was 125. For group R-4 vaccinated with oil adjuvanted S. aureus vaccine, the mean antibody titre (GMT) was 18 at day 15 followed by an upward trend reaching 62 at day 30 and had this consistency until the 60th day post primary vaccination.

In case of group R-2 vaccinated with plain bacterin S. aureus vaccine, mean antibody titres was 8 at day 15 with a progressive trend to 25 at day 30 and it persisted till day 45 followed by a downward trend reaching 20 at day 60. There was absolutely no such trend in case of R-5, which was unvaccinated control. On the basis of results emerged from this trial, it is crystal clear that dextran sulphate adjuvanted S. aureus vaccine attained the higher peak antibody titre than that of live attenuated S. aureus vaccine having the similar antibody titre during the last 15 days of trial as that of live attenuated S. aureus vaccine. Live attenuated S. aureus vaccine acquired the highest antibody titre at day 45. A constant sharp upward trend of antibody titre was observed from day 15 to 45 (Fig. 4.3).

4.8. Immune Response of Four Different S. aureus Vaccines in Pregnant Buffaloes

Serum IHA antibody titres against vaccinal isolate of S. aureus have been depicted in Table 4.7 and stylised in Fig. 4.4. For group B1 and B4 the highest IHA titre was achieved at day 30 post-partum (PP). A parturition (day 0), which decreased to almost baseline titre at day 120. For group B3, serum IHA titres were the highest at day 0 parturition) followed by a steady decrease till day 120 PP. In group B5 (Placebo control, the titers remained at the baseline level throughout the study period as can be seen in Fig. 4.5 and the B4 and B2 respectively. On the basis of GMT, group B1 had significantly.
Fig. 4.3: GEOMETRIC MEAN IHA SERUM ANTIBODY TITRES IN RABBITS VACCINATED WITH *S. aureus* VACCINES

![Graph showing geometric mean IHA serum antibody titres](image)

LSAV = Live attenuated *S. aureus* vaccine  
PSAV = Plain bacterin *S. aureus* vaccine  
DSAV = Dextran sulphate adjuvanted *S. aureus* vaccine  
OSAV = Oil adjuvanted *S. aureus* vaccine  
UC = Unvaccinated control
Table 4.7. Serum IHA antibody titres (GMT) against vaccinal *S. aureus* in 4 groups of buffaloes vaccinated twice during dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>IHA Antibody Titres (GMT) at Different Pre- and Post-partum Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-partum</td>
</tr>
<tr>
<td></td>
<td>-60</td>
</tr>
<tr>
<td>B1</td>
<td>3.0</td>
</tr>
<tr>
<td>B2</td>
<td>4.6</td>
</tr>
<tr>
<td>B3</td>
<td>3.5</td>
</tr>
<tr>
<td>B4</td>
<td>5.3</td>
</tr>
<tr>
<td>B5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA V) group  
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group  
5 = Unvaccinated control (UC) group
FIG. 4.4: COMPARATIVE SERUM IHA ANTIBODY TITRES AGAINST S. aureus IN DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH 4 DIFFERENT VACCINES

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
higher titres as compared to those of group B3 and B4 the later two did not differ significantly. However, the cumulative titres of these three groups were significantly higher (P<0.05) than those attained in groups B1 and B5 (Fig. 4.5).

6.9. Milk Whey IHA Antibody Titres of 4 Different S. aureus Vaccines

Milk whey IHA antibody titres were highest in all vaccinal groups (B1, B2, B3 and B4) at the time of parturition (Fig. 4.6 and Table 4.8). The titres registered a steep decline at day 30 postpartum in all group but B2 (plain bacterin group). In this group as well although a marked a decay in antibody titre was registered over the ensuing 2 months period (from parturition to day 60 PP), antibody levels were higher than those in her 3 vaccinal groups. A gradual waning in IHA antibody titer was seen in all vaccinal group till the end of the study period (day 120).

6.10. Cellular Immune Response of Vaccinated Groups of Pregnant Buffaloes

This test was performed to assess the cellular mediated immune (CMI) response of the pregnant buffaloes vaccinated with 4 different S. aureus mastitis vaccines (LSAV, SAV, DSAV & OSAV). This was conducted on randomly selected 15 vaccinated buffaloes from five groups and each group had 5 buffaloes. So 3 animals belonged to each group. The five groups were designated as B1 through B5.

In group B1 (LSAV), the mean skin thickness of site 1 and 2 before sensitization as 9.067+0.24 and 9.700+0.862 millimetres respectively. It increased significantly to 9.300+1.834 and 11.467+0.521 millimetres respectively after 24 hours being sensitized with DNCB and acetone. The mean skin thickness of first site further increased to 1.267±2.293, while a slight increased was observed at site 2 (11.533±0.581). In group B2 (SSAV) and B3 (DSA) a similar trend as mentioned above was found starting from hour to 48 hours post sensitization. For group B4 (OSAV), a significant increase with relevant to site 1 was observed from zero hrs to 48 hrs post sensitization.
VACCINATED TWICE DURING THE DRY PERIOD

BB1 = Live-attenuated *S. aureus* vaccine (LSAV) group
B2 = Plain bacterin *S. aureus* vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group
B4 = Oil-adjuvanted *S. aureus* vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
Fig. 4.6: COMPARATIVE WHEY IHA ANTIBODY TITRES (GMT) OF 4 DIFFERENT S. aureus VACCINES IN BUFFALOES VACCINATED TWICE DuRING DRY PERIOD

<table>
<thead>
<tr>
<th>TIME (MONTHS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

Geometric Mean Titer

- **LASV (B1)**
- **PSAV (B2)**
- **DSAV (B3)**
- **OSAV (B4)**

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
Table 4.8. Whey IHA antibody titres (GMT) against *S. aureus* in four different groups of buffaloes vaccinated twice during dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>55.7</td>
</tr>
<tr>
<td>32</td>
<td>48.5</td>
</tr>
<tr>
<td>33</td>
<td>48.5</td>
</tr>
<tr>
<td>34</td>
<td>42.2</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group  
4 = OiI-adjuvanted *S. aureus* vaccine (OSAV) group
A slight decrease was found from 12.133±0.371 at 24 hours to 11.967±0.233 millimetres at 48 hours post sensitization with regard to site 2. For group B5 (unvaccinated control), a statistically non-significant difference between site 1 and 2 was recorded in the thickness of skin (Table 4.9 and Fig. 4.7, 4.8, 4.9, 4.10 and 4.11).

Statistical analysis using ANOVA indicated a highly significant (P<0.01) difference between the two sites at different times in skin thickness. There was also a highly significant difference (P<0.01) among the all groups. While the interaction between site and time was highly significant while all other interaction i.e. site x group, time x group, site times x groups were non-significant.

11. Somatic Cell Count (SCC)

Highest values of somatic cell count were recorded at parturition in all groups (7.55 x 10^5 to 7.94 x 10^5 mL^-1). A sharp decrease in SCC was registered at day 30 PP in all groups (Table 4.10 and Fig. 4.12). At this sampling time point SCC was the highest (5.365±0.592) in placebo control animals (group B5) followed by B2, B1, B3 and B4 in the given order. The difference among the four vaccinal groups was non-significant (P<0.05). At day 60 PP, a further decreased in SCC was registered in all vaccinal groups.

On the other hand, in placebo control group B5, at this sampling the count almost quadrupled to that recorded at previous sampling point (day 30 PP). The difference among the vaccinal groups were non-significant (P<0.05). From day 60 PP onward till the end of the study (day 120), SCC remained almost steady in all vaccinal groups, which differed non-significantly from each other. From day 60 onward difference in SCC between vaccinal groups and placebo control were highly significant (P<0.01).
Table 4.9: Mean skin thickness (mm±SE) at various time intervals in response to DNBC test in group B1 vaccinated with live attenuated *S. aureus* mastitis vaccine.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Before Sensitization</th>
<th>After Sensitization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNBC (Site 1)</td>
<td>Acetone (Site 2)</td>
</tr>
<tr>
<td>SAV 31</td>
<td>9.697 ±0.240</td>
<td>9.700 ±0.862</td>
</tr>
<tr>
<td>SAV 32</td>
<td>9.267 ±0.612</td>
<td>9.200 ±0.577</td>
</tr>
<tr>
<td>SAV 33</td>
<td>9.000 ±0.115</td>
<td>9.100 ±0.058</td>
</tr>
<tr>
<td>SAV 34</td>
<td>8.667 ±0.296</td>
<td>8.700 ±0.306</td>
</tr>
<tr>
<td>C 35</td>
<td>8.600 ±0.115</td>
<td>8.667 ±0.888</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group
2 = Plain bacterin *S. aureus* vaccine (PSAV) group
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group
5 = Unvaccinated control (UC) group
Fig. 4.7: COMPARATIVE MEAN SKIN THICKNESS±SE OF TWO SITES USING DNBC TEST IN BUFFALOES VACCINATED WITH LIVE ATTENUATED *S. aureus* VACCINE (LSAV)

S1 = DNBC treated site
S2 = Acetone treated site
H = hours
Fig. 4.5: COMPARATIVE MEAN SKIN THICKNESS OF TWO SITES USING DNCB TEST IN BUFFALOES VACCINATED WITH SIMPLE BACTERIN S. aureus VACCINE (SSAV)

- **S1**: DNCB treated site
- **S2**: Acetone treated site
- **H**: hours
S1 = DNCB treated site
S2 = Acetone treated site
H = hours
Figure 3: Comparative study of DNCB test in buffaloes vaccinated with oil adjuvanted S. aureus vaccine (OSAV)

S1 = DNCB treated site
S2 = Acetone treated site
H = hours
Fig. 4.11: COMPARATIVE MEAN SKIN THICKNESS ± SE OF TWO SITES USING DNCB TEST IN BUFFALOES OF UNVACCINATED CONTROL GROUP

S1 = DNCB treated site
S2 = Acetone treated site
H = hours
Table 4.10. Somatic cell count (Mean±SD: × 10^5) in quarter milk samples of 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Somatic Cell Count (× 10^5) at Different Post-partum Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>7.773 ±</td>
</tr>
<tr>
<td></td>
<td>0.815</td>
</tr>
<tr>
<td>B2</td>
<td>7.839 ±</td>
</tr>
<tr>
<td></td>
<td>0.835</td>
</tr>
<tr>
<td>B3</td>
<td>7.707 ±</td>
</tr>
<tr>
<td></td>
<td>0.806</td>
</tr>
<tr>
<td>B4</td>
<td>7.943 ±</td>
</tr>
<tr>
<td></td>
<td>0.522</td>
</tr>
<tr>
<td>B5</td>
<td>7.556 ±</td>
</tr>
<tr>
<td></td>
<td>0.712</td>
</tr>
</tbody>
</table>

Values in columns with different superscripts differed highly significantly (P<0.01)

1 = Live-attenuated *S. aureus* vaccine (LSAV) group
2 = Plain bacterin *S. aureus* vaccine (PSAV) group
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group
5 = Unvaccinated control (UC) group
FIG. 4.12: COMPARATIVE MEAN SOMATIC CELL COUNT RECORDED AT MONTHLY INTRAVELS IN DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (OSAV) group
B5 = Unvaccinated control (UC) group
Post Challenge Somatic Cell Count in Buffaloes Vaccinated with Live and Inactivated Mastitis Vaccines

In response to challenge with live inoculum of *S. aureus* by teat dipping, the animals of groups live attenuated *S. aureus* (B1) and dextran sulphate adjuvanted *S. aureus* vaccinated group (B3) got no infection. In case of LSAV (B1) SCC remained within 1.41±0.10 – 2.14±0.16, while it was 1.60±0.14 – 2.39±4.14 in DSAB. A single quarter of one animal contracted *S. aureus* infection among the animals of group B4 (oil adjuvanted *S. aureus* vaccinated). After 6 day post challenge, SCC became 9.10±1.50 × 10³ /ml followed by prompt response advancing towards normalcy (2.54±0.46) at 48 hours after the previous rise (Table 4.11 and Fig. 4.13). In an unvaccinated control group B5, SCC remained high throughout the study period from 3.82±1.07 to 9.18±3.65 × 10³ /ml due to contraction of *S. aureus* infection of several quarters in response to challenging procedure.

Milk Fat Percentage

Milk fat contents were the highest (range 6.48 – 8.18 %) at parturition followed by a decrease (range 6.21 – 7.18 %) for the remaining study period. The differences among various groups at different sampling days were non-significant (Table 4.12 and Fig. 4.14). When compared to the basis of 120 days study period, the group B1 and B2, which did not differ significantly between them. The mean fat percentage also remained significantly higher (P<0.05) in groups B1 and B2 as compared to that of control group.

Milk Protein Concentration

Milk protein contents were the highest in the colostrums (range 15.26 – 17.14 %) followed by a sharp decrease (range 3.44 – 4.30 %) and then remained plateau throughout the remaining part of the study period (Table 4.13 and Fig. 4.15 & 4.16). When compared for 4 months, mean protein concentration of group B3 was significantly (P<0.05) higher compared to those of group B1 and B2, which differed non-significantly between them. The difference in protein concentration was highly significantly (P<0.01) when compared to that of group B4 and B5, which differed non-significantly between them. The mean protein concentration was also statistically higher in group B1 and B2 as compared to those of group B4 and B5.
Table 4.11: Mean post challenge somatic cell count recorded at two days’ intervals among different groups of buffaloes vaccinated twice during dry period with four different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Days</th>
<th>LSAV (B1)</th>
<th>PSAV (B2)</th>
<th>DSAV (B3)</th>
<th>OSAV (B4)</th>
<th>UC (B5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.14 ±0.164</td>
<td>3.89 ±0.919</td>
<td>2.39 ±0.146</td>
<td>2.15 ±0.151</td>
<td>3.82 ±1.078</td>
</tr>
<tr>
<td>2</td>
<td>1.99 ±0.069</td>
<td>3.82 ±1.11</td>
<td>1.69 ±0.146</td>
<td>1.7 ±0.15</td>
<td>4.2 ±1.15</td>
</tr>
<tr>
<td>4</td>
<td>1.69 ±0.109</td>
<td>4.22 ±1.309</td>
<td>1.8 ±0.194</td>
<td>2.16 ±0.173</td>
<td>6.01 ±2.167</td>
</tr>
<tr>
<td>6</td>
<td>1.700 ±0.133</td>
<td>5.02 ±1.845</td>
<td>2 ±0.149</td>
<td>2.31 ±0.685</td>
<td>7.67 ±2.88</td>
</tr>
<tr>
<td>8</td>
<td>1.41 ±0.10</td>
<td>3.62 ±0.996</td>
<td>1.99 ±0.152</td>
<td>8 ±1.5</td>
<td>9.18 ±3.655</td>
</tr>
<tr>
<td>10</td>
<td>1.85 ±0.108</td>
<td>3.43 ±1.049</td>
<td>1.6 ±0.145</td>
<td>2.54 ±0.462</td>
<td>4.9 ±1.333</td>
</tr>
<tr>
<td>12</td>
<td>2.08 ±0.121</td>
<td>3.13 ±0.695</td>
<td>2.15 ±0.143</td>
<td>2.57 ±0.498</td>
<td>4.6 ±1.251</td>
</tr>
<tr>
<td>14</td>
<td>1.94 ±0.111</td>
<td>3.11 ±0.791</td>
<td>1.53 ±0.118</td>
<td>2.45 ±0.358</td>
<td>4.59 ±1.156</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSAV) group  
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group  
5 = Unvaccinated control (UC) group
FIG. 4.13: COMPARATIVE MEAN POST CHALLENGE SOMATIC CELL COUNT RECORDED AT TWO DAYS' INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (OSAV) group
B5 = Unvaccinated control (UC) group
Table 4.12. Milk Fat (Mean±SD; %) in 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Milk Fat (%) at Different Post-Partum Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>8.18 ± 0.319</td>
</tr>
<tr>
<td>B2</td>
<td>7.52 ± 0.311</td>
</tr>
<tr>
<td>B3</td>
<td>7.94 ± 0.114</td>
</tr>
<tr>
<td>B4</td>
<td>7.94 ± 0.207</td>
</tr>
<tr>
<td>B5</td>
<td>6.48 ± 0.311</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA V) group  
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group  
5 = Unvaccinated control (UC) group
FIG. 4.14: COMPARATIVE FAT (%) RECORDED AT MONTHLY INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES

<table>
<thead>
<tr>
<th>B1</th>
<th>B2</th>
<th>B3</th>
<th>B4</th>
<th>B5</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSAV</td>
<td>PSAV</td>
<td>DSAV</td>
<td>OSAV</td>
<td>UC</td>
</tr>
</tbody>
</table>

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
Table 4.13. Milk protein (Mean±SD; %) in 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean Milk Protein (g/dL) at Different Post-partum Days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>16.34 ±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B2</td>
<td>16.36 ±0.47&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B3</td>
<td>17.14 ±0.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>B4</td>
<td>15.26 ±0.76&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>B5</td>
<td>15.36 ±0.46&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in columns with different superscripts differ significantly higher (P<0.01)

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group  
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group  
5 = Unvaccinated control (UC) group
FIG 4.15: COMPARATIVE PROTEINS (%) RECORDED AT MONTHLY INTERVALS AMONG DIFFERENT GROUPS OF BUFFALOES VACCINATED TWICE DURING DRY PERIOD WITH FOUR DIFFERENT \( S. \textit{aureus} \) VACCINES

- LSAV
- PSAV
- DSAV
- OSAV
- UC

B1 = Live-attenuated \( S. \textit{aureus} \) vaccine (LSAV) group
B2 = Plain bacterin \( S. \textit{aureus} \) vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted \( S. \textit{aureus} \) vaccine (DSA) group
B4 = Oil-adjuvanted \( S. \textit{aureus} \) vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
Fig. 4.10: GROUP MEAN MILK PROTEIN CONCENTRATION (%) DURING 120 DAYS POST CALVING PERIOD IN 4 GROUPS OF BUFFALOES VACCINATED TWICE WITH 4 DIFFERENT S. aureus VACCINES

<table>
<thead>
<tr>
<th>Groups</th>
<th>Proteins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>6.33</td>
</tr>
<tr>
<td>B2</td>
<td>6.32</td>
</tr>
<tr>
<td>B3</td>
<td>6.66</td>
</tr>
<tr>
<td>B4</td>
<td>5.95</td>
</tr>
<tr>
<td>B5</td>
<td>5.88</td>
</tr>
</tbody>
</table>

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
15. Milk Production Recorded in Vaccinated and Unvaccinated Groups of Buffaloes

The effect of vaccination on milk yield (L/24 hr) of the buffaloes vaccinated with different S. aureus vaccines is given in Table 4.14 and presented graphically in Fig. 4.17 and 4.18. A decrease in the milk yield vis-à-vis that at parturition was recorded in all groups, which different non-significantly (P<0.05) till day 30 PP. The difference was significant (P<0.05) between vaccinated and control animals at day 60 and day 120 PP. When compared on the basis of overall milk yield for 4 months, a non-significant difference was found among vaccinated group, which differed significantly (P<0.05) with the control group.

16. Quarter-based Point Prevalence of S. aureus Mastitis among Vaccinated and Unvaccinated Buffaloes

None of quarters of animals from group B1, B3 and B4 tested positive for S. aureus mastitis during 120 days postpartum (PP). In group B2, point prevalence was 0%, 10% 5% and 5% on day 30, 60, 90 and 120, respectively. Quarter-based point prevalence value of S. aureus mastitis in group B5 at the corresponding time point were 5%, 20%, 20% and 20% respectively (Table 4.15 and Fig. 4.19).

17. Quarter-based Incidence Rate of S. aureus Mastitis among Vaccinated and Unvaccinated Buffaloes

None of the quarters of animals from group B1, B2 and B4 tested positive for S. aureus mastitis during 120 days PP. In group B2 incidence rate was 10%, 0% and 0% in period intervening between parturition and day 30, day 31 and day 60, day 61 and day 90, day 91 and day 120, respectively. Quarter-based incidence rate of S. aureus mastitis in group B5 (control) in the corresponding period was 15%, 5%, 0% and 0% respectively (Table 4.16 and Fig. 4.20).
Table 4.14. Milk yield (Mean±SD; L/24 hrs.) of 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Milk yield (L/24) of vaccinated and control buffaloes at different postpartum days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>B1</td>
<td>9.9 ± 0.4</td>
</tr>
<tr>
<td>B2</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>B3</td>
<td>9.9 ± 0.3</td>
</tr>
<tr>
<td>B4</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>B5</td>
<td>9.7 ± 0.1</td>
</tr>
</tbody>
</table>

Columns values with different superscripts differ significantly (P<0.05)

1 = Live-attenuated *S. aureus* vaccine (LSAV) group
2 = Plain bacterin *S. aureus* vaccine (PSAV) group
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA) group
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group
5 = Unvaccinated control (UC) group
FIG. 4.17: COMPARATIVE MILK YIELD (L/24 hrs.) OF 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT S. aureus VACCINES

- LSAV
- PSAV
- DSAV
- OSAV
- UC

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSA V) group
B4 = Oil-adjuvanted S. aureus vaccine (PSAV) group
B5 = Unvaccinated control (UC) group
Table 4.15. Quarter-based prevalence (%) of *S. aureus* mastitis in 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prevalence of <em>S. aureus</em> mastitis at different post-partum days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>Parturition</td>
</tr>
<tr>
<td>B1</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td></td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group
2 = Plain bacterin *S. aureus* vaccine (PSAV) group
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA V) group
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group
5 = Unvaccinated control (UC) group
FIG. 4.19: QUARTER-BASED PREVALENCE (%) OF S. aureus IN 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT VACCINES

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (OSAV) group
B5 = Unvaccinated control (UC) group
Table 4.16. Quarter-based incidence rate (%) of *S. aureus* mastitis in 4 groups of buffaloes vaccinated twice during the dry period with 4 different *S. aureus* vaccines.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Incidence rate (%) of <em>S. aureus</em> mastitis at different post-partum period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Parturition</td>
</tr>
<tr>
<td>B1</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>-</td>
</tr>
<tr>
<td>B4</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>-</td>
</tr>
</tbody>
</table>

1 = Live-attenuated *S. aureus* vaccine (LSAV) group  
2 = Plain bacterin *S. aureus* vaccine (PSAV) group  
3 = Dextran sulphate-adjuvanted *S. aureus* vaccine (DSA V) group  
4 = Oil-adjuvanted *S. aureus* vaccine (OSAV) group  
5 = Unvaccinated control (UC) group  
P = post-partum
FIG. 4.20: QUARTER-BASED INCIDENCE RATE (%) OF S. aureus MASTITIS IN 4 GROUPS OF BUFFALOES VACCINATED TWICE DURING THE DRY PERIOD WITH FOUR DIFFERENT VACCINES

VACCINES (GROUPS)

B1 = Live-attenuated S. aureus vaccine (LSAV) group
B2 = Plain bacterin S. aureus vaccine (PSAV) group
B3 = Dextran sulphate-adjuvanted S. aureus vaccine (DSAV) group
B4 = Oil-adjuvanted S. aureus vaccine (OSAV) group
B5 = Unvaccinated control (UC) group
.18. Serum IHA Antibody Titres Against S. aureus in Lactating Buffaloes

Dextran sulphate adjuvanted S. aureus vaccine (DSAV) elicited higher antibody titres than live attenuated S. aureus vaccine (LSAV) (Table 4.17). In buffaloes vaccinated with LSAV (group, B6), IHA – antibody titres (GMT) against the vaccinal S. aureus at day 0, 30, 60, 90, 120, 150, and 180 post partum were 6.24, 128, 291, 261, 261,142, and 8, respectively. The corresponding values in the group vaccinated with dextran sulphate adjuvanted S. aureus vaccine group (B7) were 4, 135, 363, 302, 258, 193 and 90 respectively (Fig. 4.21). For unvaccinated control (B8), IHA antibody titres (GMT) was 1.3 at day 0, which remained almost same till the termination of trial. The comparison between vaccinates and control indicated highly significant difference (P< 0.01).

.19. Milk whey IHA antibody titres against vaccinal S. aureus in Lactating Buffaloes

Milk whey IHA antibody titres against vaccinal S. aureus were by and large similar in animals vaccinated either with LSAV or DSAV. In group B6, whey IHA antibody titres at day 30, 60, 90, 120, 150 and 180 post vaccination were 4.3, 4, 3.5, 2.8, 3 and 2.3, respectively. The corresponding values at these time points in animals vaccinated with dextran sulphate adjuvanted S. aureus vaccine (B7) were 3.5, 4, 3.2, 2.6, 3 and 2.0, respectively (Table 4.18). It is apparent that serum IHA antibody titres were not correlated with the milk whey IHA antibody titres. In unvaccinated control (B8) IHA antibody titre was 3.2 at day 0 and it remained almost at the same level during the span of 80 days’ study. The comparison of GMT antibody titres among three groups (B6, B7& B8) indicated a non-significant (P>0.05) during the whole study period (Fig. 4.22).

.20. Milk Somatic Cell Count in Lactating Buffaloes

Table 4.19 depicts the somatic cell count (SCC) in two groups of buffaloes vaccinated twice during the post-partum period with live attenuated S. aureus vaccine LSAV, group B6) or dextran sulphate adjuvanted S. aureus vaccine (DSAV; group B7). In buffaloes vaccinated with LSAV, SCC was highest at day 0 (12.72±4.15×10⁵/mL of
Table 4.17. Geometric mean serum IHA antibody titres in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Geometric Mean Serum IHA Antibody Titers</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
<td>DSAV (Group B7)</td>
<td>Unvaccinated control (Group B8)</td>
</tr>
<tr>
<td>0</td>
<td>4.3</td>
<td>4.0</td>
<td>5.3</td>
</tr>
<tr>
<td>30</td>
<td>128</td>
<td>135</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>291</td>
<td>363</td>
<td>4.3</td>
</tr>
<tr>
<td>90</td>
<td>261</td>
<td>302</td>
<td>4.9</td>
</tr>
<tr>
<td>120</td>
<td>261</td>
<td>258</td>
<td>2.3</td>
</tr>
<tr>
<td>150</td>
<td>142</td>
<td>193</td>
<td>3.0</td>
</tr>
<tr>
<td>180</td>
<td>58</td>
<td>90</td>
<td>4.6</td>
</tr>
</tbody>
</table>

6 = Live attenuated *S. aureus* Vaccine (LSAV)
7 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)
8 = Unvaccinated Control (UC)
GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED *S. aureus* VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVATED *S. aureus* VACCINE (DSAV)

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

**Graph Details:**
- GMT on the y-axis
- Days on the x-axis
- B6 = Live attenuated *S. aureus* Vaccine (LSAV) group
- B7 = Dextran sulphate adjuvant *S. aureus* Vaccine (DSAV) group
- B8 = Unvaccinated Control (UC) group
Table 4.18. Geometric mean milk whey IHA antibody titres in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvated *S. aureus* vaccine (DSA).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Geometric Mean Serum IHA Antibody Titers</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
<td>DSAV (Group B7)</td>
<td>Unvaccinated control (Group B8)</td>
</tr>
<tr>
<td>0</td>
<td>2.0</td>
<td>2.2</td>
<td>2.8</td>
</tr>
<tr>
<td>30</td>
<td>4.3</td>
<td>3.5</td>
<td>3.2</td>
</tr>
<tr>
<td>60</td>
<td>4.0</td>
<td>4.0</td>
<td>2.5</td>
</tr>
<tr>
<td>90</td>
<td>3.5</td>
<td>3.2</td>
<td>2.3</td>
</tr>
<tr>
<td>120</td>
<td>2.8</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>2.3</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>180</td>
<td>2.3</td>
<td>2.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

66 = Live attenuated *S. aureus* Vaccine (LSAV)
67 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSA)
68 = Unvaccinated Control (UC)
Fig. 4.22: COMPARATIVE GEOMETRIC MEAN MILK WHEY HYALURONIDASE TITERS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED *S. aureus* VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVATED *S. aureus* VACCINE (DSA V).

- **LSAV (Group B6)**
- **DSA (Group B7)**
- **Unvaccinated Control (Group B8)**

B6 = Live attenuated *S. aureus* Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant *S. aureus* Vaccine (DSA V) group
B8 = Unvaccinated Control (UC) group
Table 4.19. Milk somatic cell count (Mean ±SD; 10^5/mL of milk) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvated *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Somatic cell count (×10^5/mL of milk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
</tr>
<tr>
<td>0</td>
<td>12.72±4.15</td>
</tr>
<tr>
<td>30</td>
<td>4.58±2.43</td>
</tr>
<tr>
<td>60</td>
<td>3.03±1.82</td>
</tr>
<tr>
<td>90</td>
<td>2.46±2.03</td>
</tr>
<tr>
<td>120</td>
<td>2.36±1.17</td>
</tr>
<tr>
<td>150</td>
<td>3.09±1.30</td>
</tr>
<tr>
<td>180</td>
<td>4.04±0.70</td>
</tr>
</tbody>
</table>

36 = Live attenuated *S. aureus* Vaccine (LSAV)
37 = Dextran sulphated adjuvant *S. aureus* Vaccine (DSAV)
38 = Unvaccinated Control (UC)
(milk) followed by decline to 4.58±2.43, 3.03±1.82, 2.46±2.03 and 2.36±1.17 × 10^5/mL at day 30, 60, 90 and 120, respectively. From this sampling point onwards with advancing lactation, the mean SCC registered a steady increase; the values at day 150 and day 180 being 3.01±0.18 and 4.04±0.70×10^5/mL of milk, respectively. The same biphasic trend decrease in SCC till sampling day 120 followed by a steady increase till the termination of study at day 180 was recorded in buffaloes vaccinated with dextran sulphate adjuvanted _S. aureus_ vaccine. In buffalo of unvaccinated control group (B8) mean somatic cell count was 13.52±4.15×10^5/mL milk at the onset of trial and kept on declining till day 60 followed by a slight rise to 10.26±3.70 at day 90. It waned to 6.15±1.92×10^5/mL at day 150 followed by a moderate rise to 9.00±3.02 at the last sampling time (day 180). The comparative somatic cell count between the two groups (B6 & B7) was statistically significant (P<0.05). In contrast to this, it was highly significant (P<0.01) between vaccinates and unvaccinated control (B8) (Fig. 4.23).

### 21. Milk Fat Percentage in Lactating Buffaloes

In buffaloes vaccinated with live attenuated _S. aureus_ vaccine (group B6), the average milk fat percentage was 6.10±0.58 at the initiation of the field trial (day 0) which increased slightly to 6.22±0.49 at day 30 and to 6.24±0.48 at day 60 post vaccination (Table 4.20). After registering a slight decrease to 6.10±0.54 at day 90, a progressive increment in the milk fat contents was recorded till the termination of the trial; the values at day 120, 150 and 180, respectively being 6.18±0.55, 6.43±0.52 and 6.77±0.43. In buffaloes vaccinated with dextran sulphate adjuvanted _S. aureus_ (group B7), by and large same trend in the changes of milk fat contents was noted. The differences in the milk fat contents between the two groups were statistically significant (P<0.05).

In animals kept as unvaccinated control, mean milk fat percentage was 6.00±0.38 and kept on decreasing till day 90 when it was 5.2±0.38. It slightly dropped 5.0±0.20 at day 120 and remained same till day 150, followed by it rose to 5.5±0.45 at day 180. Comparison between two vaccinates (B6 and B7) indicated a significant difference (P>0.05) while it differed significantly (P<0.05) between the vaccinates (B6, B7) and unvaccinated control (B8) (Fig. 4.24).
Fig. 4.23: COMPARATIVE MILK SOMATIC CELL COUNT (MEAN ±SD; 10⁷/ML OF MILK) IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVATED S. aureus VACCINE (DSAV).

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

B6 = Live attenuated S. aureus Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group
Table 4.20. Milk fat percentage (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period either with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvated *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Milk Fat Percentage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
<td>DSAV (Group B7)</td>
</tr>
<tr>
<td>0</td>
<td>6.10±0.58</td>
<td>6.01±0.85</td>
</tr>
<tr>
<td>30</td>
<td>6.22±0.49</td>
<td>6.22±0.80</td>
</tr>
<tr>
<td>60</td>
<td>6.24±0.48</td>
<td>6.11±0.57</td>
</tr>
<tr>
<td>90</td>
<td>6.16±0.54</td>
<td>6.03±0.55</td>
</tr>
<tr>
<td>120</td>
<td>6.18±0.55</td>
<td>6.03±0.57</td>
</tr>
<tr>
<td>150</td>
<td>6.43±0.52</td>
<td>6.25±0.55</td>
</tr>
<tr>
<td>180</td>
<td>6.77±0.43</td>
<td>6.70±0.48</td>
</tr>
</tbody>
</table>

6 = Live attenuated *S. aureus* Vaccine (LSAV)
7 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)
8 = Unvaccinated Control (UC)
LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD EITHER WITH LIVE ATTENUATED *S. aureus* VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVATED *S. aureus* VACCINE (DSAV)

- **LSAV (Group B6)**
- **DSAV (Group B7)**
- **Unvaccinated Control (Group B8)**

B6 = Live attenuated *S. aureus* Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant *S. aureus* Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group
22. Milk Protein Concentration in Lactating Buffaloes

In group of buffaloes (n=50) vaccinated with live attenuated *S. aureus* vaccine (LSAV; group B6), protein concentration (percent) was 3.39±0.24 at the start of the trial (day 0) which increased to 3.43±0.19 at day 30 post vaccination. It kept on increasing till day 90 (3.47±0.21) and declined slightly to 3.43±0.17 at day 120 post vaccination. The milk protein level recorded at day 150 was the same as that registered in day 120. A slight increase in milk protein concentration was noted at day 180 post vaccination.

In animals vaccinated with dextran sulphate adjuvanted vaccine (DSAV; group B7), milk protein concentration was 3.34±0.32 at day 0, which remained unchanged till day 30 post vaccination. It rose slightly to 3.36±0.21 at day 60 followed by a slight dip at sampling day 90 and 120. A non-significant increase (P<0.05) in milk protein concentration was recorded at day 150 and 180 post vaccination.

In case of unvaccinated control (group B8) a constant decline in milk protein concentration was recorded from day 0 to day 120 post vaccination. A non-significant difference (P<0.05) increase in this milk constituent was registered at day 150 and 180.

A comparison between vaccinates (B6, B7) and control (B8) indicated a significant differences (p<0.05), while it was also a significant (p<0.05) between G5 and 6 in term of milk protein concentration (Table 4.21 and Fig. 4.25).

23. Milk Yield in Lactating Buffaloes

In the group of 50 buffaloes vaccinated with live attenuated *S. aureus* vaccine, mean milk yield (L/24 hr) was 11.7±2.33 in the beginning followed by a slight increase 1.9±2.03; 12.1±0.13) till 2nd month of post vaccination. It diminished to 10.9±2.10 and 5±0.17 at 3rd and 4th month post vaccination followed by a progressive decrease till the termination of the study (Table 4.22 and Fig. 4.26). In animals vaccinated with dextran sulphate adjuvanted vaccine (group B7), mean milk yield stood at 11.0±1.85 the start of the field trial (day 0). Milk yield peaked at day 30-post vaccination followed by a declining trend towards the end of the study. In the unvaccinated buffaloes (placebo
Table 4.21. Milk protein concentration (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvated *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Milk Protein Concentration (Percent)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
<td>DSAV (Group B7)</td>
</tr>
<tr>
<td>0</td>
<td>3.39±0.24</td>
<td>3.34±0.32</td>
</tr>
<tr>
<td>30</td>
<td>3.43±0.19</td>
<td>3.34±0.25</td>
</tr>
<tr>
<td>60</td>
<td>3.46±0.20</td>
<td>3.36±0.21</td>
</tr>
<tr>
<td>90</td>
<td>3.47±0.21</td>
<td>3.35±0.19</td>
</tr>
<tr>
<td>120</td>
<td>3.43±0.17</td>
<td>3.31±0.18</td>
</tr>
<tr>
<td>150</td>
<td>3.43±0.19</td>
<td>3.3±0.20</td>
</tr>
<tr>
<td>180</td>
<td>3.45±0.15</td>
<td>3.37±0.22</td>
</tr>
</tbody>
</table>

6 = Live attenuated *S. aureus* Vaccine (LSAV)  
7 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)  
8 = Unvaccinated Control (UC)
Fig. 42. COMPARATIVE MILK PROTEIN CONCENTRATION (RAW %) IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVATED S. aureus VACCINE (DSAV).

<table>
<thead>
<tr>
<th></th>
<th>LSAV (Group B6)</th>
<th>DSAV (Group B7)</th>
<th>Unvaccinated Control (Group B8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>Live attenuated S. aureus Vaccine (LSAV) group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>Unvaccinated Control (UC) group</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Days

Protein (%)

0  0.5  1  1.5  2  2.5  3  3.5  4
Table 4.22. Milk yield (Mean ±SD) in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvated *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Milk Yield (L/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (Group B6)</td>
</tr>
<tr>
<td>0</td>
<td>11.7±2.33</td>
</tr>
<tr>
<td>30</td>
<td>11.9±2.03</td>
</tr>
<tr>
<td>60</td>
<td>12.1±1.81</td>
</tr>
<tr>
<td>90</td>
<td>10.9±2.10</td>
</tr>
<tr>
<td>120</td>
<td>8.5±1.38</td>
</tr>
<tr>
<td>150</td>
<td>6.8±1.04</td>
</tr>
<tr>
<td>180</td>
<td>4.9±0.97</td>
</tr>
</tbody>
</table>

6 = Live attenuated *S. aureus* Vaccine (LSAV)

7 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)

8 = Unvaccinated Control (UC)
Fig. 4.26: COMPARATIVE MILK YIELD (L/24 hrs) of Buffaloes Vaccinated Twice During Postpartum Period with Live Attenuated S. aureus Vaccine (LSAV) and Dextran Sulphate Adjuvated S. aureus Vaccine (DSAV).

- □ LSAV (Group B6)
- □ DSAV (Group B7)
- □ Unvaccinated Control (Group B8)

B6 = Live attenuated S. aureus Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group
control, group B8) the mean milk yield at the onset of the trial was 12.0±0.39), which started declining towards the termination of the study. Comparison of vaccinated groups with unvaccinated control indicated a significant difference till day 30-postvaccination. Differences between vaccinated groups and unvaccinated control were significant (P<0.05) at the subsequent sampling time points. Comparison between vaccinated groups (B6 and B7) indicated a significant difference in the mean milk yield throughout the study period.

24 Prevalence of Mastitis in Lactating Buffaloes

24.a. Surf Field Mastitis Test Based Quarter Point Prevalence of Mastitis

At the initiation of field trial (day 0), 48, 49.5 and 48.5 % quarters reacted positive in surf field mastitis test (SFMT) (Table 4.23). Comparison between vaccinates (groups B6 & 7) and unvaccinated control (group B8) in term of reactivity in SFMT indicated that both vaccines (LSAV & DSAV) affected an appreciable reduction in quarter point prevalence till day 120. Percent reduction in quarter point prevalence during this period ranged from 9 – 14 and 13 – 15 with LSAV and DSAV, respectively. In both vaccinates groups SFMT-based quarter point prevalence at the two subsequent sampling points (day 150 & 180) tended to decrease towards those recorded at day 0. In unvaccinated control (placebo, group B8) prevalence of SFMT positive quarters remained almost unchanged throughout the course of the field trial (Fig. 4.27).

24.b. California Mastitis Test Based Quarter Point Prevalence of Mastitis

Screening of quarters foremilk samples with California mastitis test (CMT) at the initiation of field trial (day 0) indicated that 48.5, 49.5 and 48.5 % quarters respectively of B6, B7 and B8 were positive (Table 4.24). Comparison between vaccinates (groups B6 & 7) and unvaccinated control (group B8) in term of reactivity in CMT indicated that both vaccines (LSAV & DSAV) affected an appreciable reduction in quarter point prevalence till day 120. Percent reduction in quarter point prevalence during this period ranged from 9.5 – 4.5 and 13 – 15 with LSAV and DSAV, respectively. In both vaccinates groups CMT-based quarter point prevalence at the two subsequent sampling points (day 150 & 180) tended to decrease towards those recorded at day 0. In unvaccinated control (placebo, group B8) prevalence of CMT positive quarters remained almost unchanged throughout the course of the field trial (Fig. 4.28).
Table 4.23. Surf Field Mastitis Test-based quarter point prevalence rate (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Quarter Point Prevalence (%) of Mastitis Based on Surf Field Mastitis Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
</tr>
<tr>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>60</td>
<td>39</td>
</tr>
<tr>
<td>90</td>
<td>37.5</td>
</tr>
<tr>
<td>120</td>
<td>39</td>
</tr>
<tr>
<td>150</td>
<td>41.5</td>
</tr>
<tr>
<td>180</td>
<td>45</td>
</tr>
</tbody>
</table>

5 = Live attenuated *S. aureus* Vaccine (LSAV)  
7 = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)  
8 = Unvaccinated Control (UC)
RATE (%) OF MASTITIS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV).

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

B6 = Live attenuated S. aureus Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group
Table 4.24. California Mastitis Test-based Quarter point prevalence (%) of mastitis in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Quarter Point Prevalence (%) of Mastitis Based on California Mastitis Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
</tr>
<tr>
<td>0</td>
<td>48.5</td>
</tr>
<tr>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>60</td>
<td>39</td>
</tr>
<tr>
<td>90</td>
<td>37</td>
</tr>
<tr>
<td>120</td>
<td>39</td>
</tr>
<tr>
<td>150</td>
<td>41.5</td>
</tr>
<tr>
<td>180</td>
<td>45</td>
</tr>
</tbody>
</table>

S = Live attenuated *S. aureus* Vaccine (LSAV)

* = Dextran sulphate adjuvanted *S. aureus* Vaccine (DSAV)

UC = Unvaccinated Control (UC)
(% OF MASTITIS IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSAV).

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

B6 = Live attenuated S. aureus Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group
24.c. Quarter Point Prevalence of *S. aureus* Intramammary Infection

Microbiological examination of quarter foremilk samples revealed that at the initiation of the field trial (day 0), 28, 29.5 and 28.5% quarters harbored *S. aureus* (Table 25 and Fig. 4.29). Comparison between vaccinates (groups B6 & B7) and unvaccinated control (group B8) in terms of levels of *S. aureus* infection indicated that both vaccines (LSAV & DSAV) affected an appreciable reduction in infection rates till sampling day 120. Reduction in quarters point prevalence during this period (till day 120) ranged between 9–13 and 14–17 with LSAV and DSAV, respectively. In both vaccinate groups quarter point prevalence on day 150 and 180 rose again towards those recorded at day 0.

In unvaccinated control (group B8) waxes and wanes in prevalence of *S. aureus* infection were recorded throughout the study period. The terminal prevalence value (6.5%) at day 180 was higher than the value (28.5%) registered at the start (day 0) of the field trial (Fig. 4.29).

25. Cumulative Incidence

During first 4 months (day 0–120) post vaccination, cumulative incidences of *S. aureus* intramammary infection in animals vaccinated with LSAV and DSAV, were 0.096 and 0.063, respectively (Table 4.26). Corresponding value (0.11) in unvaccinated control (group B8) during this period was 1.14 and 1.74 folds higher than that of group B6 (LSAV) and B7 (DSAV), respectively.

During the last 2 months of the study (i.e., day 121–180), in vaccinates as well as unvaccinated control, a relatively more frequent occurrence (on per month basis) of new cases of *S. aureus* intramammary infection was recorded; cumulative incidence values being 0.082, 0.14 and 0.139 in group B6, B7 and B8, respectively.

Over the entire six months study period (day 0–180), cumulative incidence of *S. aureus* intramammary infection in unvaccinated control (group B8), group B7 and group B6 was in that order. Thus cumulative incidence of *S. aureus* infection in unvaccinated control for the entire duration of the study was 1.39 and 1.18 magnitudes higher than that recorded in buffaloes vaccinated with LSAV and DSAV, respectively (Fig. 4.30).
Table 4.25: Quarter point prevalence (%) of *S. aureus* intramammary infection in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV).

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>Quarter Point Prevalence (%) of <em>S. aureus</em> Intramammary Infection Based on Microbiological Examination of Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
</tr>
<tr>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td>90</td>
<td>16</td>
</tr>
<tr>
<td>120</td>
<td>19</td>
</tr>
<tr>
<td>150</td>
<td>21.5</td>
</tr>
<tr>
<td>180</td>
<td>25</td>
</tr>
</tbody>
</table>

*LSAV* = Live attenuated *S. aureus* Vaccine

*DSAV* = Dextran sulphate adjuvanted *S. aureus* Vaccine

*UC* = Unvaccinated Control
INFECTION IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED S. aureus VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE (DSA V).

- **LSAV (Group B6)**
- **DSA V (Group B7)**
- **Unvaccinated Control (Group B8)**

**Legend:**

- B6 = Live attenuated S. aureus Vaccine (LSAV) group
- B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSA V) group
- B8 = Unvaccinated Control (UC) group
Table 4.26: Cumulative incidence of \textit{S. aureus} intramammary infection over a six month period in two groups of lactating buffaloes vaccinated twice during post partum period with live attenuated \textit{S. aureus} vaccine (LSAV) and dextran sulphate adjuvanted \textit{S. aureus} vaccine (DSAV).

<table>
<thead>
<tr>
<th>Period (in days) post vaccination</th>
<th>Cumulative incidence of \textit{S. aureus} intramammary infection in vaccinated and unvaccinated groups of buffalo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
</tr>
<tr>
<td>-30</td>
<td>-</td>
</tr>
<tr>
<td>-60</td>
<td>0.055</td>
</tr>
<tr>
<td>-90</td>
<td>-</td>
</tr>
<tr>
<td>-120</td>
<td>0.041</td>
</tr>
<tr>
<td>Total (till day 120)</td>
<td>0.096</td>
</tr>
<tr>
<td>1 – 150</td>
<td>0.034</td>
</tr>
<tr>
<td>1–180</td>
<td>0.048</td>
</tr>
<tr>
<td>Total (from day 1–180)</td>
<td>0.082</td>
</tr>
<tr>
<td>Grand Total (from day 0–180)</td>
<td>0.178</td>
</tr>
</tbody>
</table>

- = Live attenuated \textit{S. aureus} Vaccine (LSAV)
- = Dextran sulphate adjuvanted \textit{S. aureus} Vaccine (DSAV)
- = Unvaccinated Control (UC)
Fig. 4.30: CUMULATIVE INCIDENCE OF *S. aureus* INTRAMAMMARY INFECTION OVER A SIX MONTH PERIOD IN TWO GROUPS OF LACTATING BUFFALOES VACCINATED TWICE DURING POST PARTUM PERIOD WITH LIVE ATTENUATED *S. aureus* VACCINE (LSAV) AND DEXTRAN SULPHATE ADJUVANTED *S. aureus* VACCINE (DSA).

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

B6 = Live attenuated *S. aureus* Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant *S. aureus* Vaccine (DSA) group
B8 = Unvaccinated Control (UC) group
26. Clinical Severity Score

Table 4.27 depicts cumulative severity score of S. aureus clinical mastitis over a six-month period in two groups of lactating buffaloes vaccinated twice during post partum period with live attenuated S. aureus vaccine (LSAV) and dextran sulphate adjuvanted S. aureus vaccine (DSAV). Vaccination with both live attenuated and dextran sulphate adjuvanted S. aureus vaccines tended to decrease the severity of S. aureus clinical mastitis. The mean clinical severity score at the initiation of trial decreased from 2.15 to 1.8 and 2 to 1.84 at the termination of trial in buffaloes vaccinated with LSAV and DSAV, respectively. Contrarily, the clinical severity score in unvaccinated control (B8) increased from 1.75 to 1.84 at the end of the field trial (day 180).

27. Colony Count

Staphylococcus aureus colony count in two groups of buffaloes vaccinated with live attenuated S. aureus vaccine (LSAV) and dextran sulphate adjuvanted S. aureus vaccine (DSAV) have been depicted in Table 4.28. Both vaccines affected a significant (<0.05) reduction in S. aureus colony count over 180 day period of field trial. Contrarily, statistically non-significant differences in the colony count of S. aureus were recorded in unvaccinated control group (Fig. 4.31).

28. Vaccine Efficacy

At day 120 post vaccination, vaccine efficacy (45%) of live attenuated S. aureus vaccine was lower (Table 4.29) than that of dextran sulphate adjuvanted vaccine (57%) (Table 4.30). Contrarily, at day 180, efficacy of the former vaccine (42 %) was higher than that of dextran sulphate adjuvanted S. aureus vaccine (39%) (Table 31).

At the termination of field trial (day 180) efficacy of live attenuated S. aureus vaccine (42%) was higher than that of dextran sulphate adjuvanted vaccine (39%) (Table 31). Between day 120 and 180 a decrease of 3% was registered in the efficacy of live attenuated vaccine where as efficacy of dextran sulphate adjuvanted vaccine decreased by 8% during this period (Table 4.32).
Table 27. Cumulative severity scores of *S. aureus* clinical mastitis over a six-month period in two groups of lactating buffaloes vaccinated twice during post-partum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV)

<table>
<thead>
<tr>
<th>Days Post Vaccination</th>
<th><em>S. aureus</em> Vaccinates</th>
<th></th>
<th>Unvaccinated Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
<td>DSAV (group B7)</td>
<td>(group B8)</td>
</tr>
<tr>
<td></td>
<td>No. of quarters</td>
<td>Severity Scores</td>
<td>No. of quarters</td>
</tr>
<tr>
<td></td>
<td>with <em>S. aureus</em></td>
<td>*</td>
<td>with <em>S. aureus</em></td>
</tr>
<tr>
<td></td>
<td>clinical mastitis</td>
<td>1  2.25  2.5</td>
<td>clinical mastitis</td>
</tr>
<tr>
<td>0</td>
<td>8  1  5 2</td>
<td>17.25 (2.15)</td>
<td>7  2  2 3</td>
</tr>
<tr>
<td>30</td>
<td>16  2  3 1</td>
<td>11.25 (1.87)</td>
<td>4  2  1 1</td>
</tr>
<tr>
<td>60</td>
<td>5  2  2 1</td>
<td>9 (1.8)</td>
<td>3  2  1 0</td>
</tr>
<tr>
<td>90</td>
<td>4  2  1 1</td>
<td>6.75 (1.68)</td>
<td>3  2  1 0</td>
</tr>
<tr>
<td>120</td>
<td>6  4  1 1</td>
<td>8.75 (1.45)</td>
<td>4  4  0 0</td>
</tr>
<tr>
<td>50</td>
<td>2  5  1 1</td>
<td>9.75 (1.39)</td>
<td>5  3  1 1</td>
</tr>
<tr>
<td>180</td>
<td>5  2  2 1</td>
<td>9 (1.8)</td>
<td>4  2  1 1</td>
</tr>
</tbody>
</table>

* Clinical Severity Score modified from Faull and Hughes, (1985)

1 = A quarter with visible changes in milk (usually a few clots in the fore-milk). The quarter, however, felt normal and the buffalo was not ill.

2.25 = A quarter with visible and palpable changes but the buffalo was not ill.

2.5 = A quarter which was hard and lumpy and not painful; the buffalo not being ill.
Table 4.28. Prevaccination (day 0) and post-vaccination (day 180) colony count of *S. aureus* in two groups of buffaloes vaccinated twice during postpartum period with live attenuated *S. aureus* vaccine (LSAV) and dextran sulphate adjuvanted *S. aureus* vaccine (DSA).

<table>
<thead>
<tr>
<th>Sampling time points with respect to vaccination</th>
<th>Colony Count of <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LSAV (group B6)</td>
</tr>
<tr>
<td>Pre vaccination (day 0)</td>
<td>32±27.71^a</td>
</tr>
<tr>
<td>Post vaccination (day 180)</td>
<td>23±21.40^b</td>
</tr>
</tbody>
</table>

Values in columns with unlike superscripts differ significantly (P<0.05)
- Live attenuated *S. aureus* Vaccine (LSAV)
- Dextran sulphate adjuvanted *S. aureus* Vaccine (DSA)
- Unvaccinated Control (UC)
Fig. 1: COMPARATIVE PREVACCINATION AND POSTVACCINATION COLONY COUNT OF S. aureus IN TWO GROUPS OF BUFFALOES VACCINATED TWICE DURING POSTPARTUM PERIOD WITH LIVE ATTENUATED VACCINE AND DEXTRAN SULPHATE ADJUVANTED S. aureus VACCINE

- LSAV (Group B6)
- DSAV (Group B7)
- Unvaccinated Control (Group B8)

B6 = Live attenuated S. aureus Vaccine (LSAV) group
B7 = Dextran sulphate adjuvant S. aureus Vaccine (DSAV) group
B8 = Unvaccinated Control (UC) group

Colony Count

Pre vaccination (day 0)  Post vaccination (day 180)

Time
Table 4.29. Day 120 preventative efficacy of live attenuated *S. aureus* vaccine (LSAV) in a group of 50 lactating buffaloes (200 quarters).

<table>
<thead>
<tr>
<th></th>
<th>No of quarters +ve for <em>S. aureus</em></th>
<th>No of quarters -ve for <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated buffaloes (n=50)</td>
<td>60 (a)</td>
<td>140 (b)</td>
</tr>
<tr>
<td>Vaccinated buffaloes (n=50)</td>
<td>38 (c)</td>
<td>162 (d)</td>
</tr>
<tr>
<td>Odds Ratio (OR)</td>
<td></td>
<td>1.83</td>
</tr>
<tr>
<td>Atributable Fraction (AF)</td>
<td></td>
<td>0.45</td>
</tr>
<tr>
<td>Vaccine Efficacy</td>
<td></td>
<td>45(%)</td>
</tr>
</tbody>
</table>
Table 4.30. Day 120 preventative efficacy of dextran sulphate adjuvanted *S. aureus* vaccine (DSAV) in a group of 50 lactating buffaloes (200 quarters).

<table>
<thead>
<tr>
<th></th>
<th>No. of quarters +ve for <em>S. aureus</em></th>
<th>No of quarters –ve for *S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated buffaloes (n=50)</td>
<td>60</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Non-vaccinated buffaloes (n=50)</td>
<td>31</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>(c)</td>
<td>(d)</td>
</tr>
<tr>
<td>Odds Ratio (OR)</td>
<td></td>
<td>2.34</td>
</tr>
<tr>
<td>d / b c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>attributable Fraction (AF)</td>
<td></td>
<td>0.57</td>
</tr>
<tr>
<td>OR – 1 / OR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine Efficacy</td>
<td></td>
<td>57 (%)</td>
</tr>
</tbody>
</table>
Table 4.31. Day 180 preventative efficacy of live attenuated \textit{S. aureus} vaccine (LSAV) in a group of 50 lactating buffaloes (200 quarters).

<table>
<thead>
<tr>
<th></th>
<th>No. of quarters +ve for \textit{S. aureus}</th>
<th>No of quarters –ve for \textit{S. aureus}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unvaccinated</td>
<td>73</td>
<td>127</td>
</tr>
<tr>
<td>Buffaloes (n=50)</td>
<td>(a)</td>
<td>(b)</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>50</td>
<td>150</td>
</tr>
<tr>
<td>Buffaloes (n=50)</td>
<td>(c)</td>
<td>(d)</td>
</tr>
<tr>
<td>odds Ratio (OR) =</td>
<td></td>
<td>1.72</td>
</tr>
<tr>
<td>d /b c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attributable Fraction (AF) =</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>OR – 1 / OR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vaccine Efficacy</td>
<td></td>
<td>42 (%)</td>
</tr>
</tbody>
</table>
Table 4.32. Day 180 preventative efficacy of dextran sulphate adjuvanted *S. aureus* vaccine (DSAV) in a group of 50 lactating buffaloes (200 quarters).

<table>
<thead>
<tr>
<th></th>
<th>No. of quarters +ve for <em>S. aureus</em></th>
<th>No of quarters –ve for <em>S. aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unvaccinated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffaloes (n=50)</td>
<td>73 (a)</td>
<td>127 (b)</td>
</tr>
<tr>
<td><strong>Vaccinated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>buffaloes (n=50)</td>
<td>52 (c)</td>
<td>148 (d)</td>
</tr>
<tr>
<td>Odds Ratio (OR)</td>
<td></td>
<td>1.64</td>
</tr>
<tr>
<td>d / b c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Attributable Fraction (AF)</td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>(OR - 1 / OR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaccine Efficacy</td>
<td></td>
<td>39 (%)</td>
</tr>
</tbody>
</table>
9. Cost-Benefit Analysis of Mastitis Control Through Vaccination

When compared with an unvaccinated control buffalo, each of the 50 buffaloes vaccinated with LSAV produced 279 liters additional milk during the 180-day (6 month) period of field trial. The corresponding value for a buffalo vaccinated with DSAV was 8 liters. Given a farm gate price of Rs. 15/= per liter, additional milk produced by each buffalo vaccinated with LSAV would be valued at Rs 4185/= and one vaccinated with SAV at Rs. 2970/= The cost-benefit ratio for an immunological control of mastitis with no shots live attenuated *S. aureus* vaccine translated into 1:18.6. The corresponding ratio for the dextran sulphate adjuvanted vaccine stood at 1:5.9 (Table 4.33).
Table 4.33. Cost-benefit ratio in two groups of lactating buffaloes vaccinated twice during postpartum period with live attenuated \textit{S. aureus} vaccine (LSAV) and dextran sulphate adjuvated \textit{S. aureus} vaccine (DSAV) \textit{vis-à-vis} unvaccinated control.

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>LSAV (Group B6)</th>
<th>DSAV (Group B7)</th>
<th>Unvaccinated control (Group B8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.7±0.15</td>
<td>11.0±0.16</td>
<td>12±0.39</td>
</tr>
<tr>
<td>30</td>
<td>11.9±0.18</td>
<td>11.3±0.15</td>
<td>11.2±0.40</td>
</tr>
<tr>
<td>60</td>
<td>12.1±0.13</td>
<td>11.2±0.14</td>
<td>9.5±0.23</td>
</tr>
<tr>
<td>90</td>
<td>10.9±0.15</td>
<td>11.0±0.19</td>
<td>8.6±0.25</td>
</tr>
<tr>
<td>120</td>
<td>8.5±0.17</td>
<td>8.1±0.17</td>
<td>7.5±0.16</td>
</tr>
<tr>
<td>150</td>
<td>6.8±0.15</td>
<td>6.3±0.15</td>
<td>5.5±0.12</td>
</tr>
<tr>
<td>180</td>
<td>4.9±0.16</td>
<td>4.5±0.18</td>
<td>3.5±0.13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Liteer of milk produced/animal per 180 day trial period</th>
<th>1653</th>
<th>1572</th>
<th>1374</th>
</tr>
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</table>

Additional milk (L) produced over a 180 day period \textit{vis-à-vis} unvaccinated control | 279 | 198 |
DISCUSSION

Average daily milk yield (3.47L for cow; 5.23L for buffalo) (Economic survey of Pakistan, 2003-04) in Pakistan leaves a lot to be desired. Increase in per animal productivity would require simultaneous improvement in health management, nutrition, reproduction, breeding and marketing of livestock and their products (Ahmad and Saji, 1997). Mastitis is one of the significant impediments to realize this goal (Ahmad, 1966; humman, 1967; Ahmad 1968; Amin, 1973; Hashmi, 1978; Sahi, 1983; Shireen, 1984; ghal, 1991; Qamar, 1992; Fazal-ur-Rehman, 1995; Razzaq, 1998; Memon et al., 1999). In countries, where dairying is well-developed (e.g. European countries, U.S.A., australi and New Zealand etc.), post milking teat dipping, dry cow therapy, segregation, pulling etc. are practiced quite despotically to control mastitis. Pakistani farmers being neducated, non-progressive and non-commercial are not inclined to these stringent mastitis strategies.

Dairy industry in Pakistan has characteristics similar to those prevailing in developing countries. These include very small herd size, poor genetic potential of animals for milk, low quality feed, high risk of epidemics, improper marketing channels, lack of quality premium program, high environmental stresses, reproductive failure and high udder abnormalities, orthodox management practices, poor extension services and lack of commercial rations (Sarwar et al., 2002). Notwithstanding these problems, dairy animals (buffalo and cattle mainly) are producing 28.62 million tones of milk in Pakistan Pakistan Economic Survey, 2003-04). These above mentioned conditions do not create a milieu for adoption of standard mastitis control practices. Farmers, however, routinely get their animals vaccinated against several diseases like haemorrhagic septicaemia (HS), lack quarter (BQ), foot and mouth disease (FMD). Mastitis control through vaccination could easily be integrated into the already ongoing vaccination program. However, the role of vaccination in the control of mastitis remains to be investigated. The present study as undertaken in this vein. As for as could be ascertained, this study is the first attempt
evaluate mastitis control through vaccination in buffalo herds lacking standard mastitis control practices.

The need to develop an immunological control of *S. aureus* mastitis stems from:

1. finding ways to circumvent the use of antibiotics which otherwise may potentially taint the milk
2. the need to reduce the somatic cell count (SCC) in order to meet the limits prescribed by the industry
3. the extremely poor bacteriological cure rate of *S. aureus* intramammary infection (IMI) using antibiotics (Dodd, 1987; Sandholm *et al.*, 1990; Nickerson and Owens, 1994; Wilson and Sears, 1996; Leitner *et al.*, 2004)
4. very high prevalence of mastitis in developed countries despite adoption of standard mastitis control practices (Watson, 1984)

The present study attempted to evaluate an immunoprophylactic control of mastitis in dairy buffaloes following the criteria recommended by OIE manual (Truszoynski and Blancou, 1996) and some recent studies (Giraudo *et al.*, 1997; Jordhaug *et al.*, 1994a,b and Watson, 1989).

**Isolation and Biocharacterization of Selected Isolate of *S. aureus***

In the present study, isolation and biocharacterization of *S. aureus* isolate was conducted following the standard procedure (National Mastitis Council Inc., 1990). This procedure for *S. aureus* is in line with those of many workers (Gonzalez *et al.*, 1989; Nickerson, 1992; Chaudhry and Azam, 1995; Fazal-ur-Rehman, 1995; Watts and Nickerson, 1986).

**Antigenicity of Vaccinal *S. aureus* Isolate**

Selected isolate of *S. aureus* was found to be antigenic in rabbits and an amanastic response could also be observed with booster dose administered at day 7. This confirmed the antigenicity of *S. aureus*. This was in line with the recommendations of OIE Manual (Truszoynski and Blancou, 1996).
Dose Dependent Immune Response of S. aureus

Antigenic/immune response to different concentrations of the *S. aureus* was evaluated in rabbits so as to find out the optimal concentration of it. A concentration of $10^6$ cells/mL was found to be more immunogenic as compared to other concentrations studied. It was also found that a concentration of $10^{12}$ is rather immunosuppressent initially contrary to the other two concentrations. Therefore, an increase in concentration above $10^6$ cell/mL did not enhance the immune response rather a decrease could be observed. These findings are in line with those of Opdebeeck and Norcross (1985) and Watson and Davies (1993) who reported a concentration above $10^6$ did not elicit a significantly higher immune response in the experimental animals. It is also possible, of course, that doses of cells even lower than $10^9$ may give satisfactory results with different adjuvant formulations but it is clearly impractical to give > $10^{11}$ cells (Watson and Davies, 1993). Therefore, in the vaccines of the present study, a dose of $10^{10}$ cells was selected and used both in laboratory as well as target animals. It is in line with the findings of Opdebeeck and Norcross (1982) who conducted a dose dependent study of three different bacterial antigens (*S. aureus* bacterin, a *Strep. agalactiae* bacterin, and staphylococcal alpha xoid) in cattle and concluded that the antigen dosages previously used in mastitis vaccine studies represented less than the optimum. It is pertinent to mention that this study was made by administering the antigenic preparations in inguinal lymph nodes.

Optimum Culture Conditions for Expression of Pseudocapsule of *S. aureus*

Many organisms when grown inside the udder or *in vivo* produce a large, well-defined pseudocapsule outside the cell wall (Watson and Watson, 1989). Although a few strains of *S. aureus* produce a true capsule, in general the organism is not encapsulated. However, when grown under *in vivo* conditions, in a variety of lesions, *S. aureus* produces an extracellular glycocalyx comprised largely of hydrated polysaccharides. Pseudocapsule is neither a true capsule nor a slime layer. This glycocalyx enhances virulence of the organism by impairing complement- and antibody-mediated opsonization and inhibiting hagocytosis. Expression of the glycocalyx usually ceases when *S. aureus* is cultured under *in vitro* conditions in conventional bacteriological media (Watson and Watson, 1989).
In the present study, the selected field isolate of *S. aureus* was grown in different cultural conditions e.g., Blood agar plates, Nutrient broth, Modified nutrient broth, Brain art infusion broth and Modified brain heart infusion broth. The simple methods of pule demonstration (e.g. India ink, Hiss’s and Anthony’s methods; Marchant and cker, 1983) and procedure of autoagglutination (Watson and Watson, 1989) were used assess the extent of pseudocapsule elaboration by *S. aureus*. India ink, Hiss’s and Anthony’s methods failed to demonstrate any pseudocapsule formation around any of the bacteria because of the absence of true capsule. It is in accordance with the findings of rack and Reiter in 1976 as cited by Watson and Watson (1989) who described that on the bases of India ink test, it can not be a true capsule nor is it simply the extracellular me, which is produced in generous amounts when *S. aureus* is cultured in modified 110 edium. Thus pseudocapsule is both an accurate and convenient description of theycalyx distinguishing it from a true capsule and from slime. This is also in agreement ith the findings of Mero et al. (1991) who enhanced the virulence of *S. aureus* from govine mastitis upon growth in milk whey compare to homologous organisms grown in yptic soy broth (TSB). In the mouse mastitis model, *S. aureus* grown in milk whey caused more severe lesions than homologous strains grown in TSB. *S. aureus* strain 1140 grown in milk whey increased 75% mortality and local necrotic reaction in abutaneously inoculated mice, whereas the homologous strain grown in TSB caused only 5% mortality, extracellular capsule on milk whey grown *S. aureus* they could not be demonstrated. However, diffuse type colony morphology could be correlated with an increased virulence of *S. aureus* towards mice, while Watson and Prideaux (1979) revised a method to enhance the pseudocapsule formation by implanting a dialysis sacaving *S. aureus* suspension in the peritoneal cavity of sheep but in the present study the inexpensive and simple autoagglutination test was carried out.

Anthony’s method also did not demonstrate pseudocapsule/capsule formation because of not having the true capsule by the organism, whereas, autoagglutination method was able to demonstrate pseudocapsule formation. It was found that nutrient roth enriched with sterile whey obtained from fresh defatted bubaline milk elicited a higher concentration of pseudocapsule around bacteria. Organisms grown in the presence of milk whey showed a strong propensity to autoagglutinate and produced pseudocapsule
material, which contained antigens in common with staphylococci grown *in vivo* (Watson and Watson, 1989). Increasing the concentrations of sodium hydroxide progressively inhibit autoagglutination by the bacteria. In the present study, a concentration of more an 0.04 M was found to inhibit the autoagglutination process which is slightly higher an that of Watson and Watson (1989) who reported a concentration of >0.03 M hibited the autoagglutination process and concluded that the autoagglutination assay as a useful preliminary test for the expression of pseudocapsule by *S. aureus* in odified nutrient broth.

**Virulence Factors of Vaccinal *S. aureus* Incorporated in the Vaccines**

*S. aureus* produces a variety of virulence factors. Of these pseudocapsule, alpha and beta toxins are considered to be the most important ones which should be found in a *S. aureus* mastitis vaccines to make those more effective. That is why Nutrient Broth was enriched by adding 10% sterilized bubaline whey v/v for making a simulated *vivo* condition required for the maximum development of pseudocapsule. Crude toxin extract as also incorporated in the vaccines in order to have both types of toxins.

**Safety, Sterility, Safety and Stability of Vaccines**

After preparing the four vaccines to be used in this study were subjected to the different abovementioned criteria following the standards of OIE manual (Truszoynski and Blancou, 1996).

**Immune Response of *S. aureus* Vaccination in Rabbits**

In rabbits vaccinated with either of four vaccines or kept as control, the highest antibody titres against *S. aureus* were recorded in R3 group received the vaccine containing dextran sulphate (DXS) as an adjuvant (DSAV). This is in agreement with the finding of (Han and Park, 2000) while there was also very good response elicited by the 1 group receiving LSAV. It is in accordance with the results of Watson and Prideaux (1979) who concluded that following immunization with a live *S. aureus* vaccine, the organisms multiply in the skin or tissues and an abscess develops. In this situation the animals were exposed to the *in vivo* antigens and produced antibody against them. This is major reason for the lack of protection conferred by traditional (Laboratory-grown)
iled staphylococcal vaccines. It could be their deficiency of protection in vivo surface antigens. Oil adjuvanted S. aureus vaccinated (OSAV) group (R4) gained the peak titre only followed by a persistency and it is in accordance with the results of Watson (1987) and Nordhaug et al. (1994a) who also ascribed it to the mineral oil component of the vaccine, which constituted a depot from which vaccine components were slowly released. The animals of group (R2) receiving plain bacterin showed the less peak titres than the other three (LSAV, DSAV and OSAV) followed by a sharp decline and it is in line with the findings of Watson and Prideaux (1979). In case of staphylococcal mastitis, the literature is replete with papers describing experiments in which systemic immunisation of killed bacterial vaccines and/or toxoids, with or without adjuvants, has induced little immunological protection (Singleton et al., 1967; McDowell and Watson, 1974). It is worth noting that, unlike most other mammals, ruminants have an almost prominent local (IgA) immune system in mammary tissue under normal conditions, but significant local IgA responses can be evoked with appropriate local antigenic stimulation. Furthermore, it has been established that a degree of protection from staphylococcal mastitis can be conferred on the mammary gland by prepartum infusion of sterile staphylococcal cell or cell-toxoid vaccines (Outeridge et al., 1971; McDowell and ascelles, 1971). However as a practical measure, local immunization of the mammary gland has some serious disadvantages; Firstly, the lactational stage at which the infusion is carried out is critical in order to avoid damage to secretory tissue. Secondly, the procedure requires strict asepsis to avoid inadvertently infecting the gland with other pathogens. Thirdly, when the dose of a sterile staphylococcal vaccine is increased to a level at which the specific local immune response is maximized, there is a concomitant reduction in milk production from locally-infused glands. This suggests that a large dose of sterile vaccine infused locally will destroy secretory tissue within the gland, and therefore such a vaccination procedure could not be considered a practical proposition. The highest titres at day-45 and D-60 in the same group is also in agreement with that of Fan and Park (2000). A higher titres at day -30 followed by sharp decline at D-45 and D-90 in rabbits of group R-3 concur with the pattern of antibody titres in sheep. It has been observed that addition of dextran sulphate (DXS) to the vaccine provoked significantly higher antibody response that did sustain for a longer period in sheep (Watson, 1987;
In the present study, dextran sulphate (DXS) was used at 50 g ml\(^{-1}\). Its dose could be enhanced to 250 mg ml\(^{-1}\). According to Watson and Davies (1993), an increasing dose of DXS could neither increase the antibody response nor could it have any benefit in terms of durability of the response. Therefore, a lower dose level (50 g ml\(^{-1}\)) was selected at the least to minimize the local tissue reaction (van der Meer et al., 1977; Watson and Davies, 1993).

**Serum and Milk Whey Antibody Titres**

In order to find out the best vaccine to control mastitis, estimation of serum and milk whey antibody titres for a certain duration is mandatory to establish its effectiveness for that period. Worldwide, antibody titres are being measured through antigen-specific enzyme linked immunosorbant assay (ELISA) by many workers (Watson, 1984; Watson, 1985; Kirlin and Watson, 1987; Watson, 1987; Watson and Franklin, 1988 Nickerson et al., 1993; Nordhaug et al., 1993 a,b; Watson and Davies, 1993). In a country like Pakistan, where facilities to conduct such tests are lacking, other methods needed to be evaluated and standardized. Currently, tests like direct bacterial agglutination, haemagglutination, haemagglutination inhibition and indirect haemagglutination are being used to fulfil the requirement. In the present study, the method of indirect haemagglutination (IHA) was used that was standardized with certain modifications to assess the antibody titres against *S. aureus* isolated from milk of mastitic buffaloes (Rahman et al., 2004).

In pregnant buffaloes, group B1 showed the highest serum IHA antibody titres followed by those in groups B3, B4, B2 and B5, respectively. While in case of lactating buffaloes, highest antibody titre was shown by B6 (DSAV) group followed by B7 (LSAV). These findings are in line with those of Opdebeeck and Norcross (1983), Watson, (1981), Watson (1984), Kirlin and Watson (1986), Kirlin and Watson (1987), Watson (1987), Nickerson et al. (1993) Watson and Davies (1993), Calzolari et al. (1997), Giraudo et al. (1997), Han and Park (2000) and Leitner et al. (2003a, 2003b). A higher IHA antibody titres shown by DSAV in the pregnant buffaloes may be attributed to the addition of an adjuvant (DXS) which has a specific ability for triggering the IgG2 response. (Watson, 1984; Norcross, 1991; Watson,1987; Watson, 1988) who concluded...
Dextran sulphate (DXS) is a useful adjuvant with S. aureus vaccines in promoting both quantity and quality (isotype and affinity) of antibody, but it was recognized that antibody responses were not adequately durable.

Oil adjuvants enhance the immune response of most antigens and prolong the duration of immunity. Field trials of haemorrhagic septicaemia (HS) vaccine using oil adjuvants have proved their superiority over alum hydroxide and saponin for protecting against different diseases (De-Alwis, 1981; Jolles and Paraf, 1973; Mackercher and Raves, 1977). The limiting factors in their widespread use are high viscosity, poor stability and adverse reaction at the site of inoculation (Mackercher, 1986). The oil adjuvanted S. aureus vaccine (OSAV) prepared for the trial of pregnant buffaloes was free of these side effects i.e. it was stable, had low viscosity and did not produce any adverse effect. Oil adjuvant are readily adopted to many livestock disease antigens and are widely used in water in oil emulsions vaccines (Shauket et al., 1998) as was in this study. The oil adjuvants used (mineral oil – liquid paraffin) in mastitis vaccine did not fulfil the quality for instigating the production of IgG₂ antibody but produces IgG₁ antibody which is not required to subjugate the infection.

In the present study, LSAV also produced a higher IHA titre in pregnant buffaloes. It is also consistent with the results of many workers (Watson, 1987; Watson and Colditz, 1983) who concluded that the synthesis of IgG₂, which is cytophilic for neutrophils and which confers enhanced phagocytic capacity to these cells. Live S. aureus multiply in subcutaneous abscess or intramuscular tissue following vaccination express novel pseudcapsular antigens; antibody directed against these in vivo antigens is important for opsonization of staphylococci during subsequent infection of the mammary gland. The high antibody titre incited by LSAV is in accordance with the findings of several research workers (Watson and Lee, 1978; Watson, 1981; Watson, 1984; Colditz and Watson, 1985; Watson, 1987 and Calvinho et al., 1989).

Veterinary profession had a wide range of safe effective vaccines, it is often necessary to choose between modified live and inactivated products. The outcome of this choice, commonly based on economic considerations as well as on efficacy, has resulted in the widespread and deliberate use of modified live vaccines in animals. Unfortunately
odified live vaccines as a class, present considerable hazards, not only to the vaccinated animal, but also to its contacts. These hazards take 2 forms: disease attributable to residual virulence and disease attributable to contamination.

The process of attenuation is designed to develop an organism that can transiently establish itself in a vaccinated animal, while at the same time, not cause disease. The vaccine manufacture, therefore, seeks to achieve minimal virulence while retaining animal immunogenicity. This balance may be readily achievable in clinically normal adult animals, but may be unattainable in animals with even minor defects in immune impetuses (Tizerd, 1990). In the present study none of the animals showed this type of hazard.

Under field trials, higher antibody titre elicited by B7 group of buffaloes may also ascribed to the presence of dextran sulphate as an adjuvant in the vaccine as compared to live attenuated vaccine, which showed a little less response. Similar findings werecorded by Watson (1986), Watson (1988) and Amorena (1994) who used dextran sulphate adjuvanted S. aureus vaccine and found high antibody titre leading to a significant reduction in S. aureus mastitis. The results of present study also consistent with the findings of Watson (1992) who conducted a trial in dairy heifers in Australia and corded a very high level of both IgG1 and IgG2 antibodies in serum of vaccinated animals but it is noteworthy that in this trial double adjuvanted (dextran sulphate & ineral oil) S. aureus vaccine was used that is why a high level of IgG1 antibody was found. It is the characteristic feature of mineral oil to incite this subclass of antibodies. This finding is also in conformity with the conclusion drawn by Nickerson et al. (1993) who used S. aureus bacterin to study its effect on serum antibody and new infection in on-lactating dairy cows. At drying off, cows were vaccinated, either intramuscularly or subcutaneously in the area of supramammary lymphnode are were left as unvaccinated control. Vaccinates received booster injection at 6 weeks. Serum antibody concentrations, bacteriologic status, and SCC of quarter milk samples were determined. Four weeks after vaccination, cows were challenged by intramammary infusion of S. aureus. Mean serum antistaphylococcal antibody titre of vaccinated cows during the trial was 4.7 – fold that of control. Challenge resulted in intramammary infection (IMI) rates of 92, 36, and 0% for control cows, cows vaccinated intramuscularly, and cows vaccinated in the area.
the suprammary lymph node. Oil-based adjuvants are routinely used to elicit a strong immune response as is seen with complete and incomplete Freund's adjuvants. Other adjuvants, however, can produce similar immune response without the adverse tissue action that occur with oil-adjuvants (Sears and McCarthy, 2003). Dextran sulphate has been reported to increase the levels of immunoglobulin i.e. IgG₂ (Watson et al., 1996) which is a class of antibody that is highly effective in opsonizing bacteria (antibody coating) that promotes phagocytosis (engulfing by neurophils).

Oil adjuvanted S. aureus vaccine showed less response as compared to the DSAV d LSAV but after gaining the peak concentration of antibody titres, constancy was observed in response which is a conspicuous characteristic of oil based vaccine due to a slow release of antigen from the site of depot for a longer time. (Nordhaug et al., 1994b; eh and Lascelles, 1985; Lascelles et al.,1989; Schroder and Stahl, 1984). Kennedy and Watson (1982) as cited by Kiirlin and Watson (1988) concluded that mineral oil adjuvants combined with killed S. aureus vaccines preferentially promoted IgG₁ rather IgG₂ synthesis in ruminants serum. Mcdowell and Watson (1974) also concluded after making trial of oil-adjuvanted S. aureus vaccine in sheep in late pregnancy that it was less effective though post challenge mastitis was less severe. Similarly an oil-based adjuvant as compared with an aluminium hydroxide (AlOH) adjuvant in vaccination/treatment studies (Sears, 1998). Cure rate for the oil-based vaccine was for lower with a 25% quarter cure as compared 62% for AlOH based vaccine. Although AlOH did not produce prolonged antibody response or protected as well against new IMI, it produced a more rapid antibody response that made it a better choice when incorporated into treatment protocols (Sears, 2003).

Plain bacterin S. aureus vaccine (PSAV) showed the lowest antibody response for short duration of time. This is in accordance with the findings of many workers Derbyshire, 1961; Watson and Lee, 1978; Watson and Prideaux, 1979; Watson and Kennedy, 1981) who also reported the antibody response of limited period in animals like oats, rabbits and sheep. It was also in line with the findings of Davidson (1987) who concluded that it did not stimulate the production of specific antibodies. This type of lowest antibody response may be attributed to very less production of IgG₂ and high reduction of IgG₁ which is supported by many workers (Anderson, 1978; Mellenburger,
When comparative cumulative serum mean antibodies titres of four *S. aureus* vaccine were calculated for a period of six months (2 months prepartum + 4 month postpartum period). It was found that there was 4.5, 3.70, 3.45 folds increase in B1 (SAV), B3 (DSAV) and B4 (OSAV) groups, respectively as compared to B2 (PSAV) group of pregnant buffaloes. Besides this, a similar comparison was made between vaccinated (B1, B2, B3 and B4) and non-vaccinated groups. It indicated an increase of 2.30, 9.39, 34.67, 32.36 folds in groups B1, B2, B3, B4, respectively as compared to the control groups (B5) in which it was 3.6.

The reason for vaccinating the pregnant buffaloes was to stimulate the antibody production against *S. aureus* in order to prevent the likelihood of being infected with *S. aureus* mastitis at parturition (Targowski, 1983) and during the ensuing months when the animals have a great propensity or susceptibility to be infected with mastitis due to immunosuppression (Burton et al., 2001; Madson et al., 2002; Pyorala, 2002; Burton and Erskine, 2003). Bilal et al. (1999) also concluded that high incidence of mastitis was found in cattle during 1-3 months postpartum.

Vaccination during the dry period increases the potential transfer of antitoxins from blood serum to mammary tissue since cellular permeability in the udder increases as parturition approaches (Derbyshire, 1961). In addition to it, any organism entered during the early dry period into the udder, are opsonized by the antibodies impregnated on the cell wall of the neutrophils followed by their distraction. So the chances of being mastitis during the last phase of dry period and at parturition are minimized.

Watson (1988) described that most of the vaccines which have been used in attempts to control staphylococcal mastitis have been made using methods traditionally employed with bacterial vaccines, that is, *in vitro* culture of *S. aureus*, killing of bacteria, injecting systemically into the animal with or without staphylococcal toxoids (similarly prepared) and with or without immunological adjuvants. Unfortunately these approaches have not worked well, even with autogenous vaccines.
Contrasting with this situation, as early as 1907 Bridre in France showed that ewes, which had been immunized by subcutaneous infection with live \textit{S. aureus}, developed quite a significant degree of protection from staphylococcal mastitis. The potential of live \textit{S. aureus} organisms as mastitis vaccines was confirmed by Derbyshire working with goats in England in the 1960s (Derbyshire, 1961). In studies carried out in ewes (Watson and Lee, 1978) and cows (Watson, 1984) in Australia it has been established that live \textit{S. aureus}, given subcutaneously, provides significant protection from experimental staphylococcal mastitis.

The results of the present study show that DSAV and LSAV elicited the maximum serum antibody titres in pregnant buffaloes. This may be due to an increased potential transfer of antitoxins from blood serum to mammary tissue since cellular permeability in the udder increases as parturition approaches (Derbyshire, 1962). The situation in lactating buffaloes was also the same but it was against the findings of Reiter and Bramley (1975) who indicated that serum antibodies cannot cross the mammary epithelial cell barrier during lactation until an irritation such as clinical or sub-clinical mastitis is present. The oil adjuvanted \textit{S. aureus} vaccine elicited immune response lesser an DSAV and LSAV but higher than PSAV in pregnant buffaloes. Dextran sulphate adjuvanted \textit{S. aureus} vaccine showed elicited a little higher immune response than the live attenuated \textit{S. aureus} vaccine in lactating buffaloes under field conditions.

Ideally an effective mastitis vaccine must raise antibody titres to a protective level in the milk whey. In pregnant buffaloes, whey antibody titre was maximum at the time of parturition which was due to the change in permeability of mammary blood barrier and it is a normal physiological process (Watson and Lee, 1978; Targowski, 1983; Nordhaus et al., 1994b). There was a drastic decrease (5 times) in antibody titres at one month postpartum in B1, B3 and B4 groups and same trend was progressive in the downward direction during the following monthly intervals. This may be due to absence of infection in any quarters of the animals belonging to these groups. There was a significant increase in antibody titres in B2 group as compared to B1, B3 and B4 at various monthly intervals. This increase may be due to the presence of infection in certain quarters of the animals belonging to this group. Presence of infection in the udder attracts the neutrophils impregnated with IgG2 antibodies from the general circulation to the site of infection.
ceter and Bramley, 1975) to intensify the process of phagocytosis in order to eliminate the causative agent. This study shows that there is directly no relation found between the rum and whey antibody titre. Pearson (1959) also stated that there is no constant relationship between blood titres and staphylococcal udder infection but presence of infection in the udder increases the antibody titre in milk whey. Nickerson (1994) also stated that antibody concentration in blood were found to be increased after immunization, but concentrations in milk increased only after the inflammatory response to bacterial invasion had occurred. The increase in milk antibodies may be effective in reducing the severity of mastitis but ineffective in preventing new infections.

There was a significant difference between the antibody titres elicited by group 5 and B7 which was due to almost of the same type of level of periodic infection during the six month period of study. This is in line with the findings of Yoshida et al. (1984) who conducted a trial on Staphylococcal capsular vaccine for preventing mastitis in two herds of cattle in Georgia. In one herd, resistance to infection continued for 6 months, when experiment was terminated. Leitner et al. (2003b) conducted a large scale vaccination field trial of a new S. aureus vaccine “MASTIVAC I” (Patent No. PTC/IL 3/00627) in cows (n = 452; n = 228 vaccinated; n = 224 control) over two consecutive years. Antibody response was detected in all vaccinated animals 4-5 weeks post-primary immunization and it was sustained throughout the experimental period (300-330 days). S. aureus infection could be detected in only 3 out of 228 animals (1.3%) in the vaccinated group and in 6 out of 224 (2.7%) in the control group. SCC was low in vaccinated group while milk yield was higher (0.5kg/day) in vaccinated than unvaccinated group. It was concluded that this vaccine elicited a non-specific health improvement of the udder in addition to specific protection against S. aureus.

The results of present study in case of lactating buffaloes (B6) are not in agreement with the findings of Calvino et al. (1989) who evaluated the efficacy of a live commercial vaccine against S. aureus mastitis in two matched groups of 10 each of Olando – Arentino cows (one vaccinated and one non-vaccinated control group). Valuation was made by clinical observation, the California Mastitis Test and bacteriological culture of milk over a 6 - month period. Although the teats of both groups were dipped in a culture of S. aureus three weeks after vaccination of the first group, to
ne an additional challenge, no significant differences could be found between the vaccinated and non-vaccinated animals.

Cow milk contains immunoglobulins originating from blood and the mammary gland (Brandon et al., 1971; Caffin et al., 1983) and their reactivity with *S. aureus* varians has been demonstrated by various workers over the years (Lasmanis and Spencer, 1954; Spencer et al. 1963; Gudding, 1977; Opdebeeck and Norcross, 1981; Ashison et al., 1984; Opdebeeck and Norcross, 1985; Watson and Davies, 1985; Loeffler and Norcross, 1987). Based on these observations, a clinical diagnostic test for detection of *intramammary S. aureus* mastitis was developed which detects the binding of *S. aureus* specific antibodies in milk of infected cows with a purified antigen preparation (Adams et al. 1988). Enzyme-linked immunosorbent assay (ELISA) was used for detection of specific antibodies to a purified antigen fraction of *S. aureus* (Adams et al. 1988). Its accuracy relative to bacterial culture was 98% for intramammary *S. aureus* infection. This method was used to evaluate the efficacy of an immunosuppressed and chemically preserved milk samples.

It is now known that *S. aureus* produces a capsular or slime layer in milk when it is grown in cows' udder. This capsule interferes with mammary defenses by inhibiting the ability of leukocytes to engulf and destroy bacteria. Australian workers have had some success with a novel killed *S. aureus* vaccine also known as a bacterin (Watson and Schwartzkoff, 1990). Immunization with this vaccine also caused a more rapid immune response to bacteria that entered the udder. This vaccine had successfully immunized dairy cattle against experimental mastitis and was effective against many strains of *S. aureus*. This new bacterin promoted the production of IgG2 antibodies that were directed against the capsule, thereby stimulating phagocytosis. In addition, the vaccine contained staphylococcal toxoids and a novel adjuvant i.e. Dextran sulphate (DXS). In an Australian field trial using five commercial dairies, cows were injected intramuscularly at 8 and 4 weeks prior to calving with either the vaccine or placebo. Results showed that vaccination reduced new sub-clinical *S. aureus* intramammary infection (IMI) by 25% and reduced clinical cases by about 50% (Nickerson, 1992).

Numerous attempts have been made to develop effective vaccine against *S. aureus*. Unfortunately, most of the early vaccine did not reduce the incidence of infection.
though they did aid in reducing the severity of clinical infections. The vaccines produced elevated levels of antibodies in the blood but concentrations in the udder increased only after bacterial invasion and inflammatory response. More recently, newer vaccines have been more effective because they target the pseudocapsule surrounding each bacterial cell more normally, interferes with opsonization, phagocytosis and digestion of bacteria. Such vaccines have been shown to be effective in preventing new infections. No doubt, further progress will be made as scientists develop ways to block binding proteins that permit pathogens to attach to epithelial tissues in the mammary gland, inhibit leucocidin that destroys neutrophils and inhibits phagocytosis, and interfere with the action of protein A that prevents immunoglobulin from opsonizing bacteria.

Live *S. aureus* vaccines stimulate the synthesis of large quantities of IgG2 antibody which is cytophilic for neutrophils and there is direct evidence that this antibody enhances the specific phagocytic capacity of neutrophils for *S. aureus* under *in vitro* conditions (Watson, 1976). Conventionally prepared, killed staphylococcal vaccines, on the other hand, predominantly stimulate production of IgG1 anti-staphylococcal antibody (Kennedy and Watson, 1982). Thus the relatively poor results obtained with the conventional. Killed *S. aureus* vaccines were at the least partially explained by the fact that they did not stimulate synthesis of the subclass of IgG2 which mediates protection through its opsonizing activity. Furthermore, live *S. aureus* vaccines are superior to killed vaccines in promoting an enhanced early inflammatory response in the mammary gland upon subsequent intramammary challenge of ewes with viable Staphylococci (Colditz and Watson, 1982). Thus the immunity resulting from immunization with a live vaccine results in more neutrophils arriving in secretion within a few hours of the infection occurring. Importantly, these newly arrived neutrophils are “armed” with cytophilic IgG2 antibody which enhanced their ability to phagocytose *S. aureus*.

**Somatic Cell Count (SCC)**

Dairy researchers in the developed world have passed through many stages of refining quality of milk. Quality now includes protein alone or in some cases, in combination with fat as the farmers are paid premiums for these constituents. Milking hygiene has also become the norm and milk is required to be clean from pathogens.
yment systems penalize the farmers for having less hygienic milk and restrictions are likely to become criteria used for determining whether milk is acceptable for processing or consumption) is the level of somatic cells in the milk.

Somatic or body cells in the milk are of low types, shoughed epithelial cells from udder and leucocytes from the blood. The epithelial cells are present in normal milk a normal breakdown and repair while leucocytes enter in milk from blood, being reacted by chemical substances released from the injured mammary tissue. Most somatic cells are primarily leucocytes, which include macrophages, lymphocytes and neutrophils. Studies showing the cell types in milk have shown that epithelials cells usually range from 0 to 7% of the cell population. During inflammation, however, major increase in somatic cell count (SCC) is because of the influx of neutrophils into the milk (Filler and Paape, 1985). Because the lactating mammary gland is a very active metabolic organ, it is logical to expect variation in cell counts from day to day or milking.

Testing of milk for somatic cells have gone through many phases of development and with the advent of electronic somatic cell counting, SCC has become one of the most popular and most important management aids to most dairymen. About four million cows are tested in the United States alone. The major factor affecting SCC is the infection of mammary gland. One of the most important pathogens causing greatest increase in SCC S. aureus in addition to others Strept. agalactiae, E. coli (Sheldrake et al., 1983). Situation in Pakistan is quite different from the developed world both in terms of quantity of milk produced on per animal basis as well as the quality in terms of constituents and hygiene (Khan, 1997).

Bacterial antigen elicit a specific cell mediated immunity response in the mammary gland of bovine, resulting in the production of lymphokines, this in turn results in an increase in neutrophils in the gland upon subsequent exposure of the gland to the homologous antigen. This influx of neutrophils could have a significant impact on the resistance of the mammary gland to invading pathogens (Guidry, 1985). Somatic cell counts (SCC) have been reported to be an index of mammary health for detection of subclinical mastitis in cows and buffaloes (Dohoo and Meek, 1982). These cells are secreted
ring the normal course of lactation and influenced by a variety of factors like season (hultz, 1977; Rechordon and Owen, 1982, Dohoo and Meek, 1982); Banos and Shook, 90; Lee et al., 1980), management (Bodoh et al., 1976; Goodhope et al., 1980) stages lactation (Cullen, 1968; Reichmuth, 1975; Schultz, 1977; Eberhart et al., 1979), parity heidrake et al., 1983a,b) and vaccination (Tenhagen et al., 2001; Athar, 2005, Butt, 05). In the present study, the values of SCC at parturition was very high in pregnant buffaloes followed by a drastic decrease. This is in line with the findings of Harmon 993), Targowski (1983), Nickerson et al. (1995) and Khan (1997) who also reported an elevated SCC at parturition followed by a steep decrease in the following months in ttle. This is also in consonant with the findings of Yoshida et al. (1984) who also observed decrease in SCC after vaccination. This count was non-significant among B1, 3 and B4, whereas it was high in case of B2 and B5 group. This rise may be ascribed to the presence of infection in certain quarters of the animals belonging to these groups owing to the inability of defense system of the udder at parturition to prevent mastitis [argowski, 1983] whereas similar trend was found in case of lactating buffaloes. The bufalao is more resistant to udder infections than cattle (Wanasinghe, 1985). Therefore, it possible that the quantity and /or quality of somatic cells which appear in buffalo milk a response to mammary infections may be different to that of cattle (Uppal et al., 994). The reasons for selecting the non-mastitic pregnant buffaloes for vaccination was due to the heightened propensity to be effected with mastitis at drying off and parturition s reported by many workers (Targowski, 1983; Sordillo et al., 1997; Burton et al., 001).

In the present study, mean somatic cell count in B1, B3 and B4 groups was gnificantly lower than that of the group B2 and B5 of pregnant buffaloes. This rise of CC in B2 and B5 groups may be ascribed to the presence of infection in some quarters f the buffaloes of these groups. This is in line with the findings of Eberhart et al. (1979) nd Sheldrake et al. (1983) who also found that a higher elevation in SCC is abnormal nd an indication of inflammation in the udder. The major pathogens cause the greatest CC increase and include S. aureus, Strep. agalactiae, coliforms and other streptococcus pecies other than Strep. agalactiae, whereas minor pathogens (C. bovis and coagulase egative staphylococci) usually cause only a moderate increase in SCC (Harmon and
nglois, 1986). Michael, et al. (1991) also concluded SCC increase in *S. aureus* mastitis. Dohoo and Leslie (1991) also conducted a study on the evaluation of changes in somatic cell counts as indicators of new intramammary infections in cattle and concluded that SCC is a standard tool for monitoring the mastitis. SCC increased in mastitis caused by *S. aureus*. Same conclusion was found in response to a study made by Schukken et al. (1991). Nickerson (1997) was also of this view that cell counts were higher during the first 2 weeks after calving due to the presence of colostrums and the stress associated with the onset of lactation. The present finding is also in line with the results reported by van (1997) that SCC are usually high during the early stages of lactation and then decrease gradually followed by slight rise at the end of lactation.

In foreign countries, dairymen who are more aware of their herds' status relative to mastitis, have herds of lower somatic cell count (Hutton et al., 1990) and have higher production fewer cow with fewer intramammary infections, especially *S. aureus* along with environmental streptococci (Jones et al., 1998). This is also in accordance with the conclusion of Miles et al. (1992) who conductive a trial on the economic implications of bioengineered mastitis control (bacteriocin and capsule enriched *S. aureus* bacterin). It was recommended that for practical application and best response, *S. aureus* vaccination be made at drying of in order to provide protection during the high risk period (last trimester and first few weeks of post parturition).

The application of biotechnology to mastitis treatment is opening up new avenues of prevention and control that may have important implications. Bacteriocins consist of enzymes and other bacteriological proteins. These act as catalysts and are very specific to a single chemical reaction. For mastitis treatment, bacteriocins can be either infused into the udder (in the same way as antibiotics) are used in solutions (such as teat dips). Most research work done so far on enzymatic proteins is to control *S. aureus*. Bacteriocins kill targeted organisms rapidly by lysing the cell wall, and they do not require that organism undergo cell division (Davis et al., 1973). The research results inferred so far indicated that these bacteriocins were comparable with antibiotic treatment and twice as effective as penicillin treatment for first time infection in heifers (Smith et al., 1985). The data on the effectiveness of bacterin indicated quite encouraging results. In one study (Watson and Schwartzkoff, 1990) involving 5 commercial herds. The number of clinical cases
pared with the control group was reduced by 45 to 52%. New sub-clinical infections were reduced by 25% (Watson and Schwartzkoff, 1990). In another field study (Norcross and Kenny, 1986), the number of quarters infected by *S. aureus* decreased by 77% whereas the number of new infections was about one-third lower than the control group. Shedding in this herd was very heavy, however (Norcross and Kenny, 1986). The data also suggested a decline in the bulk tank SCC of about 150,000 [187,000 in the Norcross and Kenny Test (1986) and 130,000 in the Norwegian Trial (Gudding, 1988)]. In New York, using 400,000 SCC average adopted, the level declined to about 250,000. The decline in SCC level from 400,000 to 250,000 corresponded to a reduction in yield loss of about 6.

In case of selected lactating buffaloes under field condition, SCC was very high before the commencement of trial followed by a very drastic decrease at a monthly interval and a progressive downward trend was observed at the following three months study period. This decrease may be due to the progressive decrease in the extent of infection in the quarters of the animals and it was high just at the very outset when the trial was initiated after the screening of animals using California Mastitis Test (CMT) and Surf Field Mastitis Test (SFMT). During the last two months a slight upward trend was observed. This increase may be due to the normal physiological process to be found at the end of lactation (Heald, 1985). He also observed that production drops towards the end of lactation, the frequency of elevated somatic cell count increases. This result is in line with the findings of Yoshida et al. (1984) who conducted a study of *S. aureus* vaccine for preventing mastitis in two herds in Georgia. In one herd, SCC of milk samples decreased remarkably 1 week after the booster injection and significant resistance to infection was maintained for 4 months after vaccination. This is also in line with the findings of Morena et al. (1996) who conducted a study on the evaluation of vaccines against *aphylococcal* mastitis in dairy cattle under commercial farm conditions and concluded that vaccinated animals had a lower somatic cell count for a five months period post vaccination and higher milk production in relation to the control group, suggesting that the economic benefit of the vaccine may be 13 times higher than the cost.

Nickerson (1997) also described that somatic cell counts are generally lowest during the winter and highest during the summer months, which coincides with an
increased incidence of clinical mastitis during the summer. Schultz (1977) reviewed earlier studies on the effect of season on cell counts and reported that, although, cell count was elevated by hot weather, evidence was not convincing for field conditions. Richardson and Owen (1982) found lowest values for spring and highest values for fall and winter months. SCC in the milk samples were lowest in winter and highest in summer in the study made by some workers (Dohoo and Meek 1981 & 1982). The interpretation of SCC records is particularly applicable to herds experiencing infections from contagious such as *S. aureus* and *Strep. agalactiae*. Because infections by these pathogens tend to be of long duration, new infections in the herd may lead to increased prevalence of infection and are reflected in elevated SCC for Bulk tank or herd average ores. Jeffery (1986) also conducted a study on the effective use of Dairy herd improvement somatic cell counts in mastitis control and concluded that SCC in infected cows was high at freshening, lowest from peak to mid lactation and highest at drying off. Leitner et al. (2003b) also reached the conclusion about the significant reduction in SCC among cows vaccinated with a newly introduced *S. aureus* mastitis vaccine “MASTIVAC I” in a large-scale vaccination field trial in Israel.

**Milk Yield**

The mean milk yield for a period of 4 months was the highest in B3 (DSAV) followed by B1 (SLAV), B4 (OSAV), B2 (PSAV) and B5 (UC) respectively. Whereas the difference among the vaccinate was non-significant. While it was significant when comparing the control group (B5) with all the vaccinate groups.

The mean milk yield for the entire study period (6 months) was higher in B6 (LSAV) than B7 (DSAV). Statistically it was a significant difference whereas comparing these two groups (B6 and B7) with the control group. There was a highly significant difference. This is in line with the finding of Amorena (1996) who conducted a study on the evaluation of vaccine against staphylococcal mastitis in dairy cattle under commercial farm condition. It was carried out to evaluate the effect of vaccine on SCC and milk production under natural conditions in commercial farms of cows. In the five months following vaccination, vaccinated animals had a lower SCC (20.6% reduction) and higher milk production (6.1% increment) in relation to the control group, suggesting that the
The economic benefit of the vaccine may be 13 times higher than the cost. Similar findings are reported by Leitner et al. (2003b) in cattle while using a new S. aureus vaccine MASTIVAC 1™ (Patent no. PTC/IL98/00627) in a large-scale vaccination field trial in Israel. A total of 452 Israeli Holstein heifers were included in the study over two consecutive years. Approximately half of the heifers (228) were vaccinated while the others (224) served as a control group. Several parameters (Antibody titre, SCC and milk yield) were kept in view. It was concluded that milk yields were 0.5kg per day higher than the non-vaccinated control cows.

It is worth noting that some part of this trial was carried out in summer season during the months of June, July, and August. The overall decreased milk production may be attributed to the heat stress. Nickerson (1997) described that a 10-20% decline in milk production is not unusual for dairy cattle experiencing heat. Collier (1985) described that environmental temperature extremes influence milk composition. Heat stress reduces udder intake, which results in decline in milk yield as well as percentage of fat. Increase during heat stress is due to direct effects of heat stress during lactation and direct or carry-over effects of heat stress during pregnancy. Direct effects of heat stress on milk yield are due to increased maintenance requirements to dissipate excess heat load, decreased metabolic rate, and decreased feed intake. Heat stress results in a much steeper decrease in milk yield than cold stress.

Rosenthal (1991) described that many physiological and environmental factors influence the yield and composition of milk. Factors that influence to a higher milk yield are increased body weight, advancing age, intensive nutrition, fall and winter calving and cool to moderate environmental temperatures. Factors that tend to decrease the yield of milk are infection (especially of udder), advanced lactation, advanced stage of gestation, short dry period, spring and summer calving, high environmental temperatures and humidities etc.

**Milk Fat and Protein Concentration**

An increase in somatic cell count in milk leads to the release of lipolytic (lipases) and caseolytic (plasmin) enzymes which can degrade the triglycerides of milk fat and casein contents of the milk (Saeman et al., 1988; Barbano, 1989; Roux et al., 2003). Hence
ceding to poor quality milk in the mastitis-affected animals. In the present study, milk fat and protein contents in the vaccinated and control buffaloes (B5) differed significantly. There was a very high protein concentration among the all groups (B1 thru B5) in the lostrum. This is in line with the findings of many workers (Eckles et al., 1951; Davis d Macdonald, 1953; Larson, et al., 1956; Smith, 1959; Treece et al., 1961; Erb and shworth, 1962; Mishrs and Nayak, 1962; Slack et al., 1964; Ikram, 1965; Waite et al., 65; Qasi and Manus, 1966; Khan.1967; Anwar, 1975) who conducted a comparative study of colostrum of cow and buffaloes and found a very high protein concentration followed by a decrease to a minimum and thereafter rise steadily until the end of station. It is worth mentioning that all these studies were conducted in normal animals. At the same time, fat percentage was high at the time of parturition in all the oups followed by a slight decrease at the following monthly intervals. This is also in line with the findings of Anwar (1975). This increase may be ascribed to the normal physiological process that goes on at the time of parturition. Jenness (1985) described at cow's and buffaloes’ milk contained more mineral salts (ash) and protein and less close than milk; fat content is often, but not always, higher than that of milk

In a field trial, milk fat was low just at the very outset (bottom line data) and then began to increase at first monthly interval followed by slight decrease during the ensuing months. Then it began to rise to the end of lactation. This rise is attributed to the normal physiological process in which there is an increase of fat percentage with the decrease in the milk production (Treece, 1961; Ikram, 1965). Whereas Calzolari et al. (1997) conducted a trial of vaccines in cattle and found no observable effect on fat production in milk. There was a significant difference in protein concentration between the two groups (B6 and B7) and it was higher in LSAV than DSAV. This may be due to the ability of SAV to improve the quality of milk protein by lowering the somatic cell count as compared to the DSAV.

The results of the present study are in agreement with the findings of Calzolari et l. (1997) and Giraudo et al. (1997) who recorded a slight but non-significant increase of fat content in response to vaccination in cows and are not in agreement with the findings of Gravert (1987) who concluded that fat and protein percentage decreased in mastitis. The increase in fat and protein concentration in the present study would
present an important economical benefit of the vaccines for dairy producers. Several

workers (Mert, et al., 1992; Urech, 1998; Rawdat and Omaima, 2000; Ullah, 2004) to concluded that protein contents decrease in response to sub-clinical mastitis. On the contrary, Kalorey et al., (2001) indicated that protein percentage elevated due to mastitis. Similar inference was drawn by Urech et al. (1999) while studying the changes in milk protein fraction of cows as affected by sub-clinical mastitis.

Collier (1985) described that milk composition is adversely affected by environmental stress. Fat and protein are generally lowest during hot months and highest during cooler months. Decrease in milk fat concentration during heat stress has related to fermentation in rumen fermentation. Decreased forage consumption leads to an altered state: Propionate ratio in rumen fluid, which is associated with reduced milk fat.

Most Challenge Studies

Most studies (Watson, 1984; Nickerson et al., 1993; Nordhaug et al., 1994a; Eitner et al., 2003a) of mastitis vaccines have attempted to give challenge with vaccinal organisms directly into the teat canal. In the present study, however, vaccinated animals were challenged by immersion of teats into a culture of the vaccinal organisms. The procedure of challenge in the present study approximates more closely with the natural transmission under the field conditions (Boddie and Nickerson, 2002). Calvinho et al. (1989) also conducted a study on the evaluation of a commercial live vaccine against S. aureus mastitis in two matched groups of 10 each of Holando-Argentino cows (one vaccinated group and one non-vaccinated control group). Evaluation was made by clinical observation, the California Mastitis Test and bacteriological culture of milk over 6-month period. Although the teats of both groups were dipped in a culture of S. aureus three weeks after vaccination of the first group, to give an additional challenge no significant differences could be found between the vaccinated and the non-vaccinated animals.

In the present study, all 25 buffaloes (randomly divided into 5 groups i.e., B1, B2, B3, B4 and B5) were challenged just after milking. None of the quarters of B1, B3 contracted the infection. Only one quarter of one animal of B4 (Oil adjuvanted vaccinated) group contracted the infection and SCC became very high (8 x 10^5 cells per
and decreased within 48 hour coming to the normal level. This decrease may be attributed to the presence of antibodies in the circulation and their rapid influx with neutrophils into the udder at the site of infection leading to the immediate initiation of sonification followed by phagocytosis. Nickerson (1997) also described that the major mastitis pathogens cause the greatest SCC increase and include *S. aureus, Strept. galactiae, coliforms* etc. The magnitude of SCC responses to major pathogens varies among cows, and differentiation between the types of pathogens does not seem possible with SCC alone. Days, weeks or longer may be required for SCC to decrease after the mastitis causing bacteria have been eliminated from the infected quarter. SCC remained w in those groups, which were immunized with LSAV and DSAV. This decrease may be attributed to the absence of infection in the quarters of animals hailing to these groups. An increased somatic cell count in the B2 and B5 (Plain and unvaccinated control) may be attributed to the contraction of infection by some quarters of the animals belonging to these groups during the two weeks of post challenge study period. These results are also in line with the findings of Leitner et al. (2003a) who conducted challenge trials in cows vaccinated with mastitis vaccine comprising three field isolates of *S. aureus* derived from astitic cows. The vaccine was administrated to nine uninfected cows while 10 other cows were used as control. All the cows were challenged with a highly virulent *S. aureus* strain administered into two quarter's of each cow. Quarters were tested for clinical signs, secretion of *S. aureus* and somatic cell count. No systemic effect were observed in any of the cows, vaccinated or control. Vaccinated cows had 70% protection from infection compared with fewer than 10% in the controls. Moreover, all quarters challenged in the unvaccinated cows, regardless of whether they were successfully infected or not with *S. aureus*, exhibited very mild inflammatory reaction, identified by their low SCCs (<100,000).

**Dinitrochlorobenzine Test (DNCB Test)**

Cell mediated immunity (CMI) plays a vital role in some of the disease conditions. The assessment of cell mediated reactivity has become increasingly common. To assess the CMI, various intracutaneous tests and contact sensitivity test have been developed in recent years. Among these tests, the contact sensitivity with 2, 4-
nitrochlorobenzine (DNCB) and PHA have gained much importance recently and are
duly used in assessing CMI in human beings but only to a limited extent in animals. It
is well established that direct application of chemically reactive compounds like
NCB to the skin results in systemic sensitisation to various metabolites of the
sensitizing compounds. These chemicals react with the skin components to form hapten
molecules. DNCB is a highly reactive substance, which can form dinitrophenyl
protein complexes with various skin proteins (Provost, 1978). The ability of an individual
develop contact sensitivity is a measure of cellular immunity to a new antigen to which
has been exposed previously (Stites, 1978). Although the DNCB sensitivity test has
been used extensively in humans and laboratory animals in assessing CMI response
Sakagawa et al., 1971; Oka et al., 1986; Freedmann, 1990; Upadhye and Maibach,
1992; Brnaugh et al., 1994; Kolde, 1994), reports on its usage in bovines are few and in
buffaloes absolutely lacking.

Brummerstedt and Basse (1973) in their trials in calves applied DNCB for
primary sensitization for seven consecutive days using 2 ml of alcoholic solution of 2 %
NCB. Jennings (1979) in his experiment on calves applied 0.1 ml of a 2 % DNCB in
acetone to a 2.25 cm² areas anterior to the right scapula for the sensitization. The site
elected at the neck region is also very convenient as the animal can be very easily
controlled and at the same time more subcutaneous tissue and the somewhat loose skin in
that region allow the readings to be measured correctly for the thickness and diameter,
but it is possible that the chemical may run down the neck region. This can be avoided by
very slow application and by allowing sufficient time for the acetone to evaporate before
the metallic ring is removed.

In the present study, a strong skin reaction was observed at the site 1 where 2%
DNCB solution was applied in the animals of all groups (B1-B5). This site was raised
strongly above the surrounding normal skin and the reaction involved and extended over
an area of 2.5cm to 5cm around the site. This site became painful, greatly thickened and
ndurated as compared to other site that was treated with simple acetone and served as
control in all groups. The highest skin thickness at 24 and 48 hours was observed in
roup B1 followed by group B3 which were administered LSAV and DSAV respectively.
A severe peeling of epidermis occurred during 7-10 days and during this period severe exudates oozed from it. Skin reaction began to subside from 10th day onward and finished from day 25 to 30 leaving a circular white spot for a period of 2 months followed by the restoration of normal skin colour. Similarly the extent of response was higher in B4 group than B2 which were administered with OSAV and SSAV but the tent of severity was less as compared to B1 and B3. Moderate peeling of epidermal layer occurred during 7-10 days and the site kept on oozing moderately and became normal till 20-25 days. It retained its white impression for a period of one and a half month.

Almost similar trend was recorded in case of control group but was minimum. There was no peeling of skin. The finding about the thickness of skin of site 2 in all groups is in agreement with the findings of Reddi et al. (1981). The severe skin response peeling may be due to species variation of buffalo having much vascular skin. This finding is not in agreement with Reddi et al. (1981) who tried this test to standardize the technique in non-pregnant six cross-bred jersey cows and observed no peeling of the skin necrosis, while normality of skin restored with 4 days. In the present study, this test as applied at the end of last trimester when the immune system is of the animals is very active so this variation in stage of the buffaloes may be attributed to this hyperresponse of skin.

The skin of site two (that was treated only with acetone) of all groups demonstrated a non-significant increase in thickness indicating a very mild inflammatory condition which may be due to the high skin vascularity of buffalo. Otherwise in cattle this did not reveal any inflammatory reaction when examined at 24 and 48 hours post challenge (Reddi et al., 1981). It is quite possible that much more research is required to get it standardized in buffaloes during its various stages of gestation. When this test was applied in non-pregnant lactating animals (cattle and buffaloes) by Butt (2005) who tried different oil-based polyvalent mastitis vaccines. It was concluded that a non significant difference between two sites. Same type of result was recorded by Athar (2005) in case of lactating buffaloes, while making an investigation about the immune response of this species as consequence of polyvalent mastitis vaccines. On the other hand, extent of increase in skin thickness in a descending order (LSAV, DSAV, OSAV & PSAV) is also
responds to the ranking order of immunization among the four groups of buffaloes. It
drove its worth to be applied in pregnant stage but unwanted skin response may be
sensed by decreasing the number of application or the dose of DNCB to be applied on
the skin of buffaloes.

In the present study, a strong skin reaction was observed in response to this test
but there was a non-significant difference between the thickness of the fold of skin at 24
s. and 48hrs post-challenge in both the groups. The findings of the present study
must be correlated with any of the previous studies as there is no report on buffaloes
for this very simple test to evaluate the CMI response. A stronger reaction in buffaloes
may be attributed to more perfusion of the buffalo skin with blood. According to Lall et
(1969), the incidence of tuberculin reactors in India is also higher in buffaloes than in
steer. Similarly, mammalian tuberculin evokes a stronger reaction in buffaloes on
tradermal test as compared to that in cattle (Lall et al., 1969).

Revalence and Incidence of Mastitis in Pregnant and Lactating
buffaloes

Prevalence and incidence of a disease is imperative for the calculation of the
economic importance of a disease. If a medication or vaccine is successful in bringing
down these parameters, then it would significantly curtail the economic losses and hence
save a lot of revenue. In the present study, live attenuated S. aureus vaccine (LSAv),
extran sulphate adjuvanted S. aureus vaccine (DSAv) and oil adjuvanted S. aureus
accine (OSAv) performed equally in terms of reducing the incidence and prevalence at
different sampling intervals as compared to either plain vaccine or unvaccinated control
pregnant buffaloes. Prevalence of S. aureus mastitis was zero during the span of four
month postpartum in case of LSAV (B1), DSAV (B3) and OSAV (B4), while it was 10%
in first two monthly intervals followed by 5% at the ensuing sampling times. This may be
attributed to the presence of infection in certain quarters at these time intervals.

These results are in line with the findings of an Australian workers (Watson and
chwartzkiff, 1990) who used a patented staphylococcal mastitis vaccine (comprising
ilised S. aureus grown under simulated condition plus toxids plus a composite
munological adjuvant) in 5 commercial dairy herds by deep intramuscular injection

197
the gluteal muscles of pregnant cattle 8 weeks and 4 weeks prepartum. It was included that this vaccine had a high level of efficacy. There was considerable variation in herds in term of prevalence of mastitis but among the 5 herds involved in the trials, clinical mastitis caused by *S. aureus* was reduced by 45 - 52% in vaccinates compared with controls. Levels of sub-clinical mastitis were reduced by 18% in vaccinates compared with controls.

Incidence rate of *S. aureus* mastitis remained 10% at day 30 postpartum in plain *aureus* vaccinated group. It was due to the presence of infection in some quarters consistently during this span of time. It was 15% and 5% at day 30 and 60 postpartum in control group and was due to the persistence of infection in the same quarters during the suing months. It is in contrast to the findings of Targowski (1983) and Nickerson *et al.* 1994 who found the incidence of mastitis high at parturition in cattle. Waage *et al.* 2001) also conducted a case-control study of risk factors for clinical mastitis in parturant dairy heifers and concluded that risk being infected with *S. aureus* is high at parturition. Similarly Lescaurret *et al.* (1995) conduct a study on mastitis occurrence thin a lactation and time of mastitis onset in 1000 cows in France. It was concluded at incidence of mastitis was greater at parturition. Pankey *et al.* (1991) made a study on mastitis prevalence in primigravid heifers at parturition and concluded that prevalence of mastitis was high within 3 days postpartum.

In the lactating buffaloes, comparison between the vaccinates (B6 & B7) and unvaccinates control (B8) in terms of reactivity in California Mastitis Test (CMT) dictated that both vaccines (LSAV & DSAV) affected an appreciable reduction in quarter point prevalence till day 120. Percent reduction during this period ranged from 5-14.5 and 13-15 with live attenuated *S. aureus* vaccine and dextran sulphate sjuvant vaccinated vaccine respectively. After words quarter point prevalence increased towards the end of study. In unvaccinated control (B8), Prevalence of CMT positive quarters remained almost unchanged throughout the course of the field trial. Almost similar finding was found in response to Surf-Field Mastitis test-based quarter point prevalence test.

These results are in line with the findings of Hwang *et al.* (2000) who conducted a
to evaluate clinical effects of autogenous toxoid—bacterin treatment for *S. aureus* b-clinical mastitis in lactating cows. Twenty two cows which had at least one *S. aureus* infected quarter, were selected from among cows at a *S. aureus* prevalent dairy farm. Even cows were injected with their own autogenous toxoid—bacterin and the others were maintained as non-injected control. In the toxoid—bacterin injected group, 27% of infected quarters were cured during the 12 week trial, compared to 5% in the control group. He concluded that autogenous toxoid—bacterin treatment against *S. aureus* sub-clinical mastitis in lactating cows may increase the cure rate of the infection, reduce the severity of the infections and also prevent occurrence of the new infections. Similarly, Law (1998) made a comparison of an oil-based adjuvant with an aluminium hydroxide (AIOH) adjuvant in vaccination/treatment studies. Cure rate for the oil-based vaccine was lower with a 25% quarter cure as compared to 62% for AIOH based vaccine. Although, AIOH did not produce a prolonged antibody response, or protect as well against new intramammary infections, it produced a more rapid antibody response. That makes it a better choice when incorporated into treatment protocols.

Quarter point prevalence (%) of *S. aureus* intramammary infection based on microbiological examination of milk was almost same in both live attenuated *S. aureus* vaccine (LSAV; B6) and dextran sulphate adjuvanted *S. aureus* vaccine (DSAV; B7) in which it reduced from 28, 29.5 to 25 and 26 respectively, while in unvaccinated control group waxes and wanes were recorded throughout the study period. The terminal prevalence value (36.5%) at day 180 was higher than the value (28.5%) registered at the start (day 0) of the field trial. These findings are in line with the results of several workers Watson, 1986; Watson, 1988; Nickerson, 1991; Watson, 1992; Amorena et al., 1994) who concluded while using DXS as an adjuvant in their vaccines in cows and sheep that incidence and prevalence of *S. aureus* mastitis was reduced significantly. Similar findings were found by Davidson (1987), Watson (1984) who used live attenuated *S. aureus* vaccines in cattle and sheep. This may be ascribed to the presence of high antibodies titre in the general circulation. Since the exposure to micro-organisms was alike to all animals, it is quite possible that even the buffaloes might have contracted the infection at were cleared off over the month long period between the two samplings.
ROSS Reactivity Studies

This study was made as an M.Sc. thesis research work in the department of veterinary Clinical Medicine and Surgery, Faculty of Veterinary Science, to assess the application of the S. aureus vaccines in different areas of Punjab, Pakistan. For this purpose, S. aureus isolates from mastitic buffaloes of 4 different districts (Faisalabad, Nang, Tobataksing and Sargodha) of Punjab Province of Pakistan were collected. Antisera were raised in rabbits against these isolates following the standard method. Then the cross reactivity of the selected vaccinal S. aureus against the different isolates collected from different districts was checked using direct slide agglutination test. There was 100 percent cross reactivity indicating effectiveness of the vaccines in these areas (Aaeem, 2002).

Clinical Severity Score

Vaccination with both live attenuated and dextran sulphate adjuvanted S. aureus vaccines decreased the severity of S. aureus mastitis. The mean clinical severity score at initiation of the trial decreased from 2.15 to 1.8 and 2 to 1.68 at the termination of trial in buffaloes vaccinated with LSAV and DSAV, respectively. In contrast to it, it increased from 1.75 to 1.84 at the end of field trial in unvaccinated control group (B8). This is in line with the findings of Watson (1984) who conducted a trial of LSAV in lactating heifers and found a significant decrease in severity of S. aureus mastitis. It is so consistent with the findings of Watson (1988), Amorena et al. (1994), Watson (1992), Nickerson (1991) and Philpot (2003) who concluded a decline in the severity of S. aureus mastitis. While using the DSAV in sheep and cows. Hawang et al., (2000) also recorded the similar finding using an autogenous toxoid bacterin in cows. Nordhaug et al. (1994a) also concluded while making a trial of vaccine (pseudocapsule + toxoids) in 108 heifers from 16 farms. It was concluded that vaccination did seem to lessen the development of clinical mastitis from sub-clinically infected cows. In USA a commercially available S. aureus vaccine also reduced the frequency and severity of clinical episodes in cattle (Tyler, 1993).
Colony Count

To properly evaluate a herd mastitis problem, it is necessary to determine the type and extent of bacterial infections. Bacteriological examination of quarter milk sample from all cows in the herd will provide the most accurate evaluation. However, in large herds this is a time consuming and somewhat costly procedure (Farnsworth et al., 1977). The use of composite milk samples was devised to minimize the cost. However, Californian workers indicated an extremely poor correlation between the bulk tank counts of Strep. agalactiae and S. aureus and the percentage of infected cows in the herd (Farnsworth et al., 1977). In addition to it, culturing quarter milk sample is a method to assess the effect of vaccine.

This is also in line with the findings of Giraudo et al. (1997) who conducted a field trials for the evaluation of a vaccine against bovine mastitis in heifers. A vaccine was developed comprising inactivated, highly encapsulated S. aureus cells, a crude extract of S. aureus exopolysaccharides, inactivated unencapsulated S. aureus and staph. p. cells. The vaccine was evaluated in 164 cows from two commercial dairies during their months period. Two doses of vaccine were administered subcutaneously in the pericelial cephalicus muscle of the neck within a 4 weeks interval. It reduced the mammary infection significantly and the colony counts for S. aureus in milk from infected quarter of vaccinated cows were significantly lower than those in milk from infected quarter’s of control cows.

In the present study, both vaccines (LSAV and DSAV) registered a significant reduction in S. aureus colony count over a period of 6 months trial while there was no change in the control group. This is in line with the findings of Hawang et al., (2000) who conducted a study on the effects of autogenous bacterin in lactating cows and found a significant decrease in mean S. aureus cfu per ml of milk.

Vaccine Efficacy

Research on mastitis vaccine has been conducted for at least 30 years and at present several mastitis vaccine are commercially available in different countries for immunization against mastitis caused by S. aureus and E. coli. There are two S. aureus

201
barrier marketed to U.S. dairy producers but they are simply separate licensures of the
same product. The vaccines are marketed as somato-staph® and lysiginate® and are labeled
somatic antigen containing phage type I, II, III, IV and miscellaneous groups of *S.
reus*. An other new *S. aureus* mastitis vaccine “MASTIVAC” (Patent no. IL 122829,
ILC/IL 98/00627, AU 746285, USA 09/582692) is available in USA and Israel (Leitnew
et al., 2004). There are three coliform mastitis vaccines marketed but two of the products
are identical. The two identical coliform bacterin are marketed as J-5 bacterin and
astiguard™. A separate bacterin-toxoid (J-vac®) is also available. A 4th Gram negative
masitis vaccine (Endovac-Bovi®) contains re-17 mutant salmonella typhimurium
toxin toxoid (Ruegg, 2001).

The definition of a successful mastitis vaccine may vary depending upon the herd
circumstance. Farmer may except mastitis to reduce the severity and frequency of mastitis,
event new infections and eliminate existing infections (Yancey, 1993). While these
expectations seem reasonable, it is unlikely that any one vaccine will be able to achieve
these outcomes. Furthermore, the evaluation of mastitis vaccine is complicate by the
underlying biology of the various mastitis pathogens. One of the most frustrating mastitis
pathogens is *S. aureus*. This organism is a highly successful mastitis pathogen in that it
has evolved to produce infections of long duration with limited clinical mature. Most
infections with this pathogen are sub-clinical in nature and are detected by the production
of poor quality milk. While clinical mastitis may occur sporadically, affected animals
can become seriously ill and the major economic effects of this disease is reduced milk
yield and quality premiums received by the producers. Animals are at risk for this
organism throughout the lactation and often becoming infected after prolonged periods of
exposure. Unless vaccine can prevent new infections throughout the lactation and
ramatically reduce the SCC of affected animals, it may be difficult for a producer to
cognize the benefit of using a *S. aureus* vaccine.

In the present study, vaccine efficacy (45%) of LSAV was lower than that of
SAV (57%) at day 120. Contrarily, at day 180 efficacy of the former (42%) was higher
than that of DSAV (39%). Between day 120 and 180, a decrease of 3% was registered in
efficacy of live attenuated *S. aureus* vaccine (LSAV) whereas efficacy of dextran
sulphate adjuvanted *S. aureus* vaccine (DSAV) decreased by 18% during this period. It is
generally accepted that commercially available *S. aureus* vaccines have limited ability to prevent new infections (Nickerson 1999; Yancey, 1993). A 3-lactation trial failed to demonstrate a reduction in the number of new *S. aureus* infections in cows vaccinated with a commercial vaccine (Pankey *et al.*, 1985). This study did document an increase in the spontaneous cure rate of cows that received the vaccine. Similar results were found in separate study conducted in 3 commercial dairy herds in New Zealand (Pankey *et al.*, 1983). There are several studies that support the ability of commercially available *S. aureus* vaccines to enhance spontaneous cure rates and reduce the chronic infections other than prevention of new infections (Widal, 1994).

The ability of commercial *S. aureus* vaccines to reduce the development of chronic infections may be useful in some herds that are involved in *S. aureus* control program but for most herds the successful control of *S. aureus* mastitis will result from prevention of new infections. The failure to prevent new infections is probably the reason that this vaccine is used on a limited basis in mastitis control programs. There have been several approaches to the development of experimental vaccine directed toward the control of *S. aureus* vaccine. Researchers have attempted to develop vaccine directed toward specific virulence factors responsible for the development of mastitis. Vaccines have been formulated based on bacterial cell wall components (protein A), adhesion factors (bacterial factors that allow *S. aureus* to attach to mammary epithelial cells) and *S. aureus* pseudocapsules (a slime layer that surround the bacteria and reduce the ability of WBC to destroy the bacteria). The outcomes of these studies have been inconsistent and confusing to interpret. Australian researchers have published several papers describing results of vaccine trials using an inactivated vaccine produced from *S. aureus* strains that produce pseudocapsule (Watson, 1992; Watson *et al.*, 1996). An experimental challenge study documented that this vaccine can successfully stimulate the development of anti-pseudocapsule antibody and reduce the development of clinical symptoms (Watson, 1992). The vaccine did not significantly reduce SCC or increase milk yields of infected cows. This particular vaccine was further evaluated in a 7-herd field study (Watson *et al.*, 1996). The results of this study were interesting because there was no significant effect of vaccination on SCC or clinical mastitis when data from all 7-herds were included in the analysis. However, this study did demonstrate that differences were
in between herds. When analysis was restricted to a single herd that had a high valence of \textit{S. aureus} mastitis the vaccinated animals had a reduction in signs of clinical mastitis and reduced development of new sub-clinical mastitis infections. A Swedish researcher enrolled 108 heifers from 16 farms in a study of a vaccine that included pseudocapsule and toxoids (Nordhage et al., 1994a). Almost 20\% of the cows in enrolled herds were infected with \textit{S. aureus} mastitis. Vaccination did not significantly affect the rate of clinical mastitis are the SCC of enrolled cows. Vaccination did seem to sen the development of clinical mastitis from sub-clinical infected cows. A vaccine consisting of inactivated, highly encapsulated \textit{S. aureus}, unencapsulated \textit{S. aureus} and opolysaccharides has been developed and tested in Argentina (Calzolari et al., 1997; Maudo et al., 1997). The field trial portion of the studies was conducted in dairy herds th poor milk quality and a moderate prevalence of existing \textit{S. aureus} infections (Calzolari et al., 1997). The experimental unit was quarters and the researchers excluded quarters that were infected prior to the beginning of study. Under these conditions, the caccine successfully reduced new intramammary infections with \textit{S. aureus} but did not significantly highly affect the SCC. In general, there seems to be progress in the development of an affected \textit{S. aureus} vaccine but the efficacy of these vaccines seems to vary by herd. The greatest effect of \textit{S. aureus} vaccines appears to be a decrease in the development of clinical symptoms and preventive management programs are needed to ffectively reduce the new infection rate.

In most herds the most effective control strategy prevention of new infections by e use of good management practices. The use of \textit{S. aureus} vaccines is not universally commended but may be useful in some herds as an adjunct to prevention oriented control programs. It is also important to emphasize that vaccine must be handled propery, used before the expiration date and given to healthy immune competent cattle in the manner recommended by the manufacturer.

\textbf{Cost Benefit Ratio}

Increasingly, veterinarian are interested in the economic impacts of animals diseases and disease control programs. As the more dramatic and devastating diseases ave been eradicated or controlled, the profession has turned its attention to control of
onic diseases which affect production. Mastitis is one such disease. Because, mastitis often insidious, economic justification for implementing mastitis control programs must be made in order to achieve producer interest in and compliance with a mastitis control program (Millar, 1986).

In a view of the economic of mastitis, Jansen (1970) found losses in total milk production which ranged from 5.0 to 25.5%. In addition, decreases in the amount of fat, lds-not fats, lactose and total solids have been documented. Annual loss estimate from mastitis ranged from $23 to $154 per cow prior to implementing control and $28 to $ after initiating control programs. The benefit-cost ratio from mastitis research has been imated at 9.6 to 1 (Blosser, 1979).

Changes in milk production would result in increased revenues realized because increased production resulting from decreased discarded milk and decreased sub-clinical mastitis. The amount of milk which is decreased during treatment of clinical mastitis is only a small part of the production losses associated with mastitis. Sub-clinical mastitis results in decreased production even though the milk is of acceptable quality. Sub-clinical mastitis has been shown to be the major economic component of decreased revenues from loss of milk production (Willett et al., 1982).

The cost benefit ratio for an immunological control of mastitis with two shots live tenuated *S. aureus* vaccine translated into 1:18.6. The corresponding ratio for the extran sulphate adjuvanted vaccine stood at 1:5.9. The LSAV proved itself as an expensive and cheep vaccine because its two doses cost about Rs. 225/- (inclusive of cost of maintaining a cold chain), Whereas DSAV proved to be an expensive because its two doses cost about Rs. 500/- owing to the soaring price of dextran sulphate.

The results of the present study are in line with the findings of Amorena et al. (1996) who conducted a study on the evaluation of vaccines against staphylococcal mastitis in dairy cattle under commercial farm conditions and concluded that the vaccinated animals had a lower SCC and higher milk production in relation to the control group, suggesting that the economic benefit of the vaccine may be 13 times higher than the cost.
If the cost benefit ratio be adjudged on the touch stone of law of production (which, 1992) which describes that when the cost of fixed and variable resources is less than return is called as increasing return to scale. When the cost of fixed variable is equal to return is called as constant return to scale and if the cost of fixed plus variable resources are greater than return is called as diminishing return to marginal scale.

The efficacy of S. aureus vaccines in both pregnant and lactating buffaloes in the present study seems to be quite promising in Pakistan where no control measure (teat tip, immediate treatment, culling, dry cow therapy etc.) for mastitis are in place. That is why the results of this study fall within the preview of increasing return to scale, while it will fall within the horizon of constant/diminishing return to scale wherever there is a strict compliance of mastitis control measures that seems to be impossible ideally.

It is pertinent to mention about the special feature of this study that it was conducted where no mastitis control program was in place.
SUMMARY

Buffalo is the principal dairy animal in Pakistan and mastitis is one of the major production limiting diseases causing colossal economic losses to the dairy industry. *S. aureus* is the most important etiologic agent of mastitis as per results of studies undertaken as far as Pakistan. In over 50% of the cases *S. aureus* is the sole bacterial isolate. Owing to extremely poor cure rate in *S. aureus* intramammary infections, vaccination against this organism in cattle has been a subject of intensive investigation in foreign countries but this search frontier is almost unexplored as far as dairy buffalo is concerned. Keeping in view the importance of *S. aureus* mastitis, 4 vaccines viz live attenuated *S. aureus* vaccine (SAV), plain bacterin *S. aureus* vaccine (PSAV), dextran sulphate adjuvanted *S. aureus* vaccine (DSAV) and oil adjuvanted *S. aureus* vaccine (OSAV) were prepared and evaluated in laboratory animals and in the target species i.e. buffalo.

Sixty four *S. aureus* isolates were collected from 100 clinical cases of mastitis in buffaloes. These were tested for the haemolytic activity on 5% sheep blood agar. Thirty were found to be alpha haemolytic, 4 alpha-beta haemolytic and 30 only beta haemolytic.

A typical *S. aureus* alpha-beta haemolytic was selected and biotyped using API kits. Biotyping of vaccinal *S. aureus*, revealed 7 digit biochemical profiles of 6736153. The presence of pseudocapsule of vaccinal isolate of *S. aureus* was demonstrated by stioagglutination method, while India ink, Hiss’s and Anthony’s methods failed to demonstrate pseudocapsule. For production of inactivated vaccines (PSAV, DSAV and SAV) sterile bubaline whey was added @ 10% to the nutrient broth. To produce live attenuated *S. aureus* vaccine (LSAV), vaccinal isolate was serially passaged on blood agar, till it lost its haemolytic activity (the last passaged no. was 42). For all vaccines the concentration of *S. aureus* was adjusted spectrophotometrically at 10^10 cells per mL.

These vaccines were tested on rabbits for antigenicity, safety and immunogenicity. Dextran adjuvanted and live attenuated vaccines gave better mean geometric antibody titres 8.95, 99.85, respectively for two month’s study and all vaccines were protective.
Experimental trials were carried out in dry pregnant non-mastitis buffaloes in 25 mals divided into 5 equal groups. Two vaccines inoculations were done at -60 and -30 cs of parturition. After 5 weeks of parturition test dip challenge (10^{10} cells/mL) was carried using a virulent S. aureus isolate. None of the quarters contracted infection among AV, DSAV with the exception of OSAV, PSAV and unvaccinated control group of mals. Cumulative mean Serum antibody titres were better in live attenuated (152.3), xtran sulphate adjuvanted (124.8) and oil adjuvanted (116.5) vaccines. Serum antibody es were high but whey titres were low. Somatic cell count (SCC) was significantly lower vaccinated animals. SCC remained the lowest (1.19±0.758×10^5) in LSAV followed by SAV (1.4±0.89×10^5) and OSAV (1.4±1.150×10^5) at the end of study while it was 39±2.450×10^5) in PSAV followed by the highest (11.32±1.13×10^5) in unvaccinated ntrol. Milk yield, fat and protein concentrations were better in the vaccinated animals. Thisference was significant (P<0.05) as compared to control group. Intramammary infections (MI) were not seen till 4 month post partum in LSAV (B1), DSAV (B3) and OSAV (B4) oups but found in PSAV (B2) and unvaccinated control (B5). Dinitrochlorobenine test (NCB test) showed the maximum response in LSAV and DSAV followed by OSAV and SAV groups of buffaloes. As a whole, dextran sulphate adjuvanted S. aureus vaccine (SAV) and live attenuated S. aureus vaccine (SAV) and live attenuated S. aureus vaccine had better efficacy than the others.

In case of lactating buffaloes, two shots were given 30 days apart. Evaluation parameters in 150 lactating buffaloes irrespective of mastitis status for comparative evaluation of two better vaccines (i.e. LSAV & DSAV) included IHA titres in serum and ey milk somatic cell count, incidence and prevalence of S. aureus, milk quality and quantity in terms of fat and protein contents. LSAV got peak antibody titre (291 GMT) at 2 onth post vaccination followed by a progressive decrease and became 60 at 6 month. Whereas, DSAV attained the climax (363 GMT) at 2 month post vaccination followed by a ownward trend at the ensuing monthly intervals and was 90 GMT at the end of 6th month hile, the commulative geometric mean antibody titre was higher (192.14) in DSAV than SAV (163.61) and was 4.05 in unvaccinated control.

Both vaccines reduced the somatic cell count significantly in milk. The cumulative CC in DSAV was lower (4.24×10^5) than LSAV (4.61×10^5) whereas, it was 9.97×10^5mL^{-1} in unvaccinated control. Milk yield, fat and protein concentrations were better in LSAV than
AV significantly (P<0.05) while this difference was highly significant (P<0.01) as compared to control. Point prevalence of sub-clinical mastitis was lower in vaccinated animals. Severity of mastitis was less in clinical cases in vaccinated animals. Both LSAV and DSAV affected a significant (P<0.05), reduction in S. aureus colony count over 180 day period of field trials. Contrarily, statistically non-significant differences in the colony count of S. aureus were recorded in unvaccinated group. Cost benefit ratio was 1:18.6 for the attenuated vaccine and 1:5.9 with dextran sulphate adjuvanted vaccine.

The tentative conclusion of the present study is that both vaccines (LASV & DSAV) may be used both prophylactically and therapeutically in bubaline mastitis. LSAV is found cheaper than DSAV. External validity of this preliminary study remains to be established before recommending integration of vaccine into a mastitis control package customized to the local bubaline farming in Pakistan. It is pertinent to mention that this study was conducted in such field conditions where no mastitis control practices were in place.
FUTURE RECOMMENDATIONS

Latest available adjuvants may be incorporated into the mastitis vaccines to enhance the protective immunity up to one year (liposomal etc.).

Further immunopotentiators like interleukines etc. should be incorporated to enhance the efficacy of present mastitis vaccines.

Various stabilizers and methods like (ultrafreezers/ liquid Nitrogen etc.) may be utilized to enhance the shelf life of attenuated live *S. aureus* vaccine.

More sensitive serological tests (ELISA) may be devised to monitor humoral and cellular immunity of mastitis vaccines in buffaloes.

Further work may be recommended to compare the cost of treatment and vaccines separately and in conjunction.

Work may be extended towards the development of subunit (capsular) molecular *S. aureus* vaccines (DNA) in buffaloes.

Efforts should be made to monitor the effective and best candidates of *S. aureus* (strains) to develop multivalent vaccines against mastitis.

Linear somatic cell count scoring method should be standardized in buffaloes.

National Mastitis council programme should be formulated including the use of vaccine as an integral part of management practices.

Cross reactivity among the prevalent strains of *S. aureus* in different provinces should be made to widen the horizon of effectiveness of vaccines which is to be made available in the country.
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Appendix-I: Geometric mean serum antibody titre in rabbits vaccinated with 4 different *S. aureus* vaccine for a period of 2 months

<table>
<thead>
<tr>
<th>Vaccine / Groups</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live attenuated. <em>S. aureus</em> vaccine</td>
<td>0</td>
<td>32</td>
<td>84.4</td>
<td>128</td>
<td>111.4</td>
</tr>
<tr>
<td>Bacin Bacterin <em>S. aureus</em> vaccine</td>
<td>0</td>
<td>16</td>
<td>32</td>
<td>32</td>
<td>21.1</td>
</tr>
<tr>
<td>Extrax Sulphate adjuvanted <em>S. aureus</em> vaccine</td>
<td>0</td>
<td>32</td>
<td>128</td>
<td>128</td>
<td>111.4</td>
</tr>
<tr>
<td>I adjuvanted <em>S. aureus</em> vaccine</td>
<td>0</td>
<td>16</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

Appendix-II: Newman-Lempert's Staining Technique for Somatic Cell Count

Spread 10 µL of fresh milk sample over a clean glass slide having a marked area of 10 mm × 10 mm using a micropipette.

Dry the fine milk smear so prepared in an oven at a temperature of 30-40°C.

Dip the dried smear in Xylene for 1-2 minutes to remove the fat globules and dry subsequently.

Stain the slides with Newman-Lempert’s stain for a period of 15 minutes.

Dry at room temperature.

Remove the excess stain from smears with tap water.

Dry again at room temperature.

Stain the poorly stained smears with blue (Basis) aliquot of Dip Quick stain (J-332, blue portion, Jorgensen Labs. Inc. Loveland, Colorado, 805 38, USA) for 10-15 seconds.

Rinse by tap water and dry.

0. Count the somatic cells under microscope with a magnification of 15 × 14 in 50 fields.

1. Calculate by multiplying with the microscopic factor to get the cell/mL of milk.

**Interpretation:**

- <500,000 cells/mL ................................................. Normal Milk
- >500,000 cells/mL .................................................. Mastitic Milk
Appendix-III: Guidelines for significance of colony number of specific organisms isolated pure with other colony type (based on 0.01 ml quarter sample streaked on blood agar).

<table>
<thead>
<tr>
<th>Total Number Colonies</th>
<th>One</th>
<th>Several</th>
<th>More than 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pure</td>
<td>Pure</td>
<td>Mixed Two Types</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>4*</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>Streptococci</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>Streptococcal Species</em></td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Staphylococcus</em></td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>Eubacterium</em></td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Molds &amp; Other Fungi</em></td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><em>Pyococcus</em></td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Pyococci</em></td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>Pyococcus</em></td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>Pyococcus</em></td>
<td>2</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

(National Mastitis Council, Inc., 1990)

Degree of confidence in diagnosing infection:
- not significant
- questionable significance
- probable significant
- highly significant
Appendix-IV: India Ink Method of Capsule Staining

Emulsify a small amount of bacteria culture in a loopful of glucose solution at one end of the slide.
Add a loopful of India ink and mix thoroughly.
Spread the mixture over the slide as for a blood film.
Dry in air.
Fix the film by covering with Methanol for 15 seconds.
Drain and dry.
Stain with Methyl violet for 2 minutes.
Wash in water, blot, dry and examine under oil-immersion.

Interpretation: Capsule appears as clear spaces surrounding the violet stained bacteria ion a dark grey background of India ink.

Appendix-V: Hiss's Method of Capsule Staining

Prepare the smear and dry it in air.
Place a few drops of saturated alcoholic solution of Basic fuchsin or Gentian violet (1 part dissolved in 19 parts of distilled water).
Heat to steaming and leave the slide for 30 seconds.
Wash off with 20% aqueous solution of CuSO₄.
Dry in air and examine under oil-immersion.

Interpretation: Capsule appears as faint blue halo around dark purple cell.

Appendix-VI: Anthony's Method of Capsule Staining

Prepare the smear and dry it in air.
Stain for 2 minutes with 1% solution of Crystal violet.
Wash off with 2% aqueous solution of CuSO₄.
Dry in air and examine under oil-immersion.

Interpretation: Capsule appears as faint/colourless halo around deep purple bacteria.
Appendix-VII:

Attenuation of S. aureus isolate

Staph. aureus (API – Staph 6736153)

↓

Serially passaging (B.A)

↓

42 passages

α/β- haemolytic activity lost

↓

Mass cultivation in NB (orbital shaker-60 rpm / min, 37°C for 24 hrs.)

↓

Harvesting & washing twice with PBS

↓

Resuspension in PBS & Conc. Adjustment- 1x10^10/ml → stored 4°C
Appendix-VIII: Composition of live attenuated *S. aureus* vaccine

<table>
<thead>
<tr>
<th>Each 5 ml dose contained (Prepared fresh)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staph. Aureus</em></td>
<td>$5 \times 10^{10}$ cells /ml</td>
</tr>
<tr>
<td>PBS</td>
<td>QS to make 5ml</td>
</tr>
</tbody>
</table>

Appendix-IX: Composition of oil- adjuvanted *S. aureus* vaccine (OSAV)

<table>
<thead>
<tr>
<th>Each 5 ml dose contained</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phylococcus aureus 6736153</em></td>
<td>$5 \times 10^{10}$ cells</td>
</tr>
<tr>
<td>Fluid paraffin</td>
<td>2.3 ml</td>
</tr>
<tr>
<td>Green -80</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>lan -80</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>formalin</td>
<td>&lt; 0.02 ml</td>
</tr>
<tr>
<td>merosal</td>
<td>0.0005 g</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.0005 g</td>
</tr>
</tbody>
</table>

Appendix-X: Composition of dextran sulphate-adjuvanted *S. aureus* vaccine (DSAVal)

<table>
<thead>
<tr>
<th>Each 5 ml dose contained</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phylococcus aureus 6736153</em></td>
<td>$5 \times 10^{10}$ cells</td>
</tr>
<tr>
<td>Crude Toxin Extract</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Dextran sulphate (M.W. 500,000)</td>
<td>250 mg</td>
</tr>
<tr>
<td>formalin</td>
<td>&lt; 0.02 ml</td>
</tr>
<tr>
<td>merosal</td>
<td>0.00005 g</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>0.00005 g</td>
</tr>
<tr>
<td>PBS</td>
<td>Q.S.</td>
</tr>
</tbody>
</table>

Appendix-XI: Composition of plain bactrin *S. aureus* vaccine (PSAV)

<table>
<thead>
<tr>
<th>Each 5 ml dose contained</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Phylococcus aureus 6736153</em></td>
<td>$5 \times 10^{10}$ cells</td>
</tr>
<tr>
<td>Crude Toxin Extract</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>formalin</td>
<td>&lt; 0.02 ml</td>
</tr>
<tr>
<td>merosal</td>
<td>0.00005 g</td>
</tr>
<tr>
<td>Sodium Azide</td>
<td>0.00005 g</td>
</tr>
<tr>
<td>PBS</td>
<td>Q.S.</td>
</tr>
</tbody>
</table>
thorough mixing of sensitized sheep RBC suspension the plate was kept at 37 °C for 30 minutes.

RBC control

th test dilution causing 50% agglutination of sheep RBCs was taken as end point.

Serum antibody response to different concentrations of *S. aureus* at different days in rabbits.