I. INTRODUCTION

India is the largest milk producer in the world with an annual production of 104 million tones in 2008 whereas world milk production reached at 684 million tones in 2008. The total milk production in India accounts for approximately 15 per cent of total world milk production (Indian Dairyman, 2008). With its status as the largest milk producer in the world, India has assumed an important position in the global dairy industry. Many international dairy organizations are viewing India with an eye to tap its vast growing market for dairy products as the Indian dairy industry offers opportunities to galore entrepreneurs’ world wide. The 11th Five Year Plan (2007-2012) focuses on faster and more inclusive growth of the economy. The goal for agricultural sector as a whole is a growth rate of about 4 per cent and for the dairy sector, a growth rate of about 5 per cent in milk production. The dairying has been identified as an important component for diversification for the agricultural sector. Furthermore, with the current trends of increasing milk production, India will be producing more than 130 million tones of milk by 2015 (Sadana, 2006). In order to maintain its position as the largest milk producer, India should combat the costliest and complex disease like ‘mastitis’. In terms of economic loss, mastitis is undoubtedly the most important disease with which Indian dairy industry has to contend. The total economic losses due to mastitis alone accounts up to Rs. 6053.21 crores per annum in India (Dua, 2001).

Mastitis results when pathogenic bacteria are able to gain entry in to the udder, overcome the cow’s immune defenses, establish an infection and produce inflammation of udder secretary tissue. The disease is often expressed as an increased somatic cell
counts (SCC) in the milk which leads to poor quality milk in the affected animals. Mastitis is a major cause of economic loss in dairy farming. This loss is primarily due to reduced milk yield, rapid spoilage of milk, discarding of milk with antibiotics, treatment and replacement costs, lower price of poor quality milk, increased culling rate or death from infection and decreased fertility (Bradley, 2002).

The vast majority of etiological agents of mastitis is of bacterial origin and just five species of bacteria viz. *Staphylococcus aureus* (*S.aureus*), *Streptococcus uberis* (*S.uberis*), *Streptococcus dysgalactiae* (*S.dysgalactiae*), *Streptococcus agalactiae* (*S.agalactiae*) and *Esherichia coli* (*E.coli*) account for almost 80 per cent of all mastitis cases (Anon., 2001, Ali *et al*., 2008). Classically, mastitis pathogens have been classified as either contagious or environmental. Contagious pathogens are considered as organisms adapted to survive within the host in particular within the mammary glands. They are capable of establishing sub clinical infection which is typically manifested by elevation in the SCC of milk from the affected quarter. In contrast, the environmental pathogens are best described as opportunistic invaders of mammary gland not adapted to survive within the host and they invade, multiply and cause clinical infections and are rapidly eliminated. They typically spread from cow to cow around or at the time of milking (Bradley, 2002).

*Staphylococcus aureus* is one of the most frequently (45 % - 60 %) isolated (Verma, 1988; Kaya *et al*., 1998; Wani and Bhat, 2003 and Ali *et al*. 2008) and a major contagious mastitis pathogen that cause either clinical or subclinical or chronic bovine mastitis with high economic losses to the farmers. In cows, intramammary infections
(IMI) due to *S. aureus*, which account for 25-30 per cent of total IMI, are generally subclinical. This type of mastitis impairs alveolar functions, reduces milk yield and has deleterious effect on milk composition, one of which is an increase in milk SCC (Leitner *et al.*, 2000; Dego and Tareke, 2003 and Ali *et al.*, 2008). Its treatment necessitates the extensive use of antibiotics in dairy herds in contrast to increasing public concern over food safety expressed as the desire to minimize antibiotic residues in milk. Moreover, the presence of *S. aureus* in raw milk used by dairy industries is a public health problem (Leitner *et al.*, 2008).

For defense purposes, bacteria have developed an interesting system. After adhering to the epithelial surface, they begin to multiply while emitting chemical signals that "intercommunicate" the bacterial cells. Once the signal intensity exceeds a certain threshold level, the genetic mechanisms underlying exopolysaccharide production are activated (Costerton *et al.*, 1999). In this way, the bacteria multiply, embedded within an exopolysaccharide matrix, thus giving rise to the formation of a Biofilm / microcolony. “Biofilms (BF) are microbially derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a self produced matrix of extracellular polymeric substances / exopolysaccharide matrix and exhibiting an altered phenotype with respect to growth rate and gene transcription” (Costerton *et al.*, 2003). Such BF are adherent to an inert or living surface, which constitutes a protected mode of growth that allows survival in hostile environment. Within a BF, bacteria are able to interact with each other through intercellular communication and thus rapidly adapt to changing environments. The organisms within BF are resistant to the host immune response and antibacterial agents, compared to their
free-living planktonic counterparts. The biofilm matrix plays a key role in the protection of biofilm bacteria from host defenses. However, it is important to point out that these exopolysaccharides are both chemically and physically distinct from those forming the bacterial capsule (McKenney et al., 1998).

Although bacterial infections are widely reported in animals, their association with BFs is rarely discussed. *Staphylococcus aureus* is the common cause of IMI, which frequently become chronic, associated with the ability of this bacteria to produce biofilm (Cucarella et al., 2001). Recently, the ability of *S.aureus* to form biofilm *in vivo* is considered to be a major virulence factor influencing its pathogenesis in mastitis. The implication of biofilm in chronic bacterial infections in many species has triggered an increasing interest in the characterization of genes involved in biofilm formation. The *bap* gene is a newly identified gene that encodes the biofilm-associated protein (BAP). The biofilm associated protein is a novel cell wall associated protein that promotes the primary attachment of bacteria to surfaces and intercellular adhesion forming biofilms which is ultimately involved in pathogenesis of mastitis, causing a persistent intramammary infection (Cucarella et al., 2001; Gotz, 2002; Cucarella et al., 2004 and Vautor et al., 2008).

A multitude of strategies have been applied to compare gene and protein expression patterns in biofilms with those in planktonic cultures. When assessed by DNA microarrays, gene expression in biofilms differed from that of planktonic cultures in *Bacillus subtilis* and *Pseudomonas aeruginosa* (Whiteley, 2001 and Stanley, 2003). Biofilm formation in *S.aureus* is considered to be a two step process in which the bacteria
first adhered to a surface, followed by multiplication and cell to cell adhesion forming multilayered biofilm /microcolonies. This is mediated by polysaccharide intercellular adhesin (PIA) and biofilm associated protein (Bap), a surface associated protein. The intercellular locus consisting of the genes icaA,D,B and C encode the proteins mediating the synthesis of PIA in  *Staphylococcus* species (Cramton *et al*., 1999). Among the ica genes, icaA and icaD have been reported to play an important role in biofilm formation in  *S. aureus* and  *Staphylococcus epidermidis* (S.epidermidis) (Yazdani *et al*., 2006). On the other hand, the biofilm associated protein locus encodes a novel cell wall associated protein that promotes the primary attachment of bacteria to surfaces and intercellular adhesion forming biofilms which ultimately involve in pathogenesis of mastitis, causing a persistent intramammary infection (Cucarella *et al*., 2001 and Cucarella *et al*., 2004).

Biofilms adopt their own strategy of survival by way of altering their cell wall proteins and other components. Microcolony or sessile bacterial cells under BF mode of growth, may release low level of antigen, stimulating immune response and induce antibody production. However, these low levels of antibodies may not be effective in killing bacteria inside the BF. Therefore, by stimulating the immune response effectively by exogenous administration of BF antigens, early humoral responses can be induced against the exopolysaccharides responsible for biofilm formation, thereby avoiding the appearance of these microcolonies and controlling the infections associated with BF or preventing establishment of BF on the mucosal or epithelial surfaces. This could be achieved by *in vitro* growth of the bacteria in BF mode, which simulate natural *in vivo* conditions to express novel immunogenic proteins. Hence, such BF grown bacteria can be exploited as potential vaccine candidate against mastitis causing organisms in bovines.
Experimental and field trials conducted in the Department of Microbiology, Veterinary College, Bangalore using these BF based vaccines against important avian bacterial pathogens have shown promising results (Shivaraj and Krishnappa, 2002; Veeregowda, 2003; Prakash, 2004; Prakash, 2006 and Ramesh, 2006).

Intramammary infections caused by *S. aureus* in bovines are very difficult to cure. In the context of the high prevalence and economic consequences of *S. aureus* IMI and the relative inefficiency of control measures, the development of a vaccine against *S. aureus* IMI is of great interest. Vaccination has been employed as an adjunct to therapy as well as a preventive measure for *S. aureus* mastitis. Several 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 which have been evaluated for protection against *S. aureus* mastitis (Ruegg, 2001). The outcome of these studies has been inconsistent and confusing. Although, *S. aureus* bacterins like Somatostaph® / Lysigin® and ‘Mastivac I’ (Leitner et al., 2008) are commercially available in the United States of America (USA) and Israel respectively, these vaccines have limited ability to prevent new IMI infections. A three-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). Experimental vaccines for *S. aureus* composed of pseudocapsule-enriched bacterins supplemented with α – and/or β - toxoids appear promising, but none of these have been commercialized (Yancey et al., 1999). Many other conventional vaccines are also commercially available against *S. aureus* mastitis. The efficacy of such vaccines in reducing the severity of clinical disease has been demonstrated (Nordhaug et al., 1994a; Giraudo et al., 1997;
Leitner et al., 2003b and Lee et al., 2005) but the vaccines seem unable to prevent new intramammary infections. As yet, no commercial vaccines are currently available in India and other developing countries and it is unlikely that vaccines themselves will give the whole answer to bovine mastitis for sometime to come. Recently, bovine mastitis causing *E.coli* BF and free cell (FC) based vaccines were compared by vaccination trials in pregnant rabbits (Kavitha, 2008 and Jyothi, 2009) and in lactating cows (Chandrashekhara, 2009). These studies have indicated the superiority of biofilm vaccine as serum and milk IgG and IgA levels detected by ELISA were significantly higher in BF vaccinated than FC vaccinated and control animals.

Rabbits have been considered to be good animal models for mastitis studies as the lactating mammary gland of the rabbit is susceptible to natural infection by staphylococci and the disease could be reproduced in the laboratory by injecting low numbers of organisms into the mammary tissue. The type of disease produced varied with the strain used to infect the lactating mammary gland in rabbits (Adlam et al., 1977 and Adlam et al., 1980). Further, they are economical and have more number of teats than ruminants. Hence, they may help to reduce the cost and the number of animals involved. They are also larger than mice and may thus be easier to handle them for intramammary injections or infusions (Amorena et al., 1991 and Reinoso et al., 2002) and collection of milk.

Keeping this information in background, the present study was undertaken to evaluate the bovine mastitis causing *S.aureus* biofilm vaccine in rabbits.
The objectives of the study were:

- Molecular Characterization of *S.aureus* isolates derived from subclinical and clinical bovine mastitis cases with reference to Biofilm associated protein (*Bap*) gene.

- Analysis of Proteins of *S.aureus* grown under biofilm and planktonic mode with reference to their immunogenicity and cross reactivity.

- Production and experimental evaluation of *S.aureus* biofilm based vaccine in rabbits.

The following parameters were used in the present study;

- *Bap* specific Polymerase Chain Reaction of *S.aureus* isolates.

- Sub clinical mastitis test - California Mastitis Test (CMT) and Somatic cell count.

- Western blot analysis of proteins of *S.aureus* grown under biofilm and planktonic mode.

- Immunoglobulin G (IgG) based serum Enzyme Linked Immunosorbent Assay (ELISA).