2.1 Mastitis

Mastitis is basically an inflammation of the mammary gland i.e. the udder and teats. It is therefore potentially susceptible. The inflammatory response consists of an increase in blood proteins and white blood cells in the mammary tissue and the milk. The purpose of the response is to destroy the irritant, repair the damaged tissue and return the udder to normal function (Miles et al., 2006).

2.1.1 Symptoms

Inflammation is characterized by Gross abnormalities in the udder (swelling, heat, redness, pain). Persisting inflammation leads to tissue damage and replacement of secretory tissues with of the udder with nonproductive connective tissues. Abnormalities in milk may include flakes, clots or a watery appearance. The flakes in the milk are congealed leukocytes, secretory cells and protein (Lampang et al., 2008).

2.1.2 Mastitis - causative factors

Mastitis is one of the most costly diseases of dairy cattle reported by DeGraves and Fetrow (1993) and many others. It has been estimated that mastitis costs about 150 to 300 dollars per cow per year, totally 1.5 to 3.0 billion dollars annually in the USA (Hogan, 1999). Mastitis is an inflammation of the milk secreting tissue of the udder caused by bacterial infections. Whilst over 200 microbial species, sub species and serotypes have been isolated from bovine mammary gland (Mallikarjunaswamy, 1997) and identified as causative agents of mastitis. Apart of different species of bacteria, several other groups of micro-organisms such as virus, fungi, yeast, algae and chlamydia can cause mastitis in cattle and buffaloes.

Mastitis is caused by many bacteria, which include the coliform group (specifically E.coli, Enterobacter, Klebsiella sp., etc.), Streptococci, Staphylococci, Corynebacteria, Pasteurella, Mycoplasma, Leptospira, Yersinia, Mycobacteria, Pseudomonas, Serratia etc. In India, Staphylococcus, Streptococcus and E.coli generally cause 90-95% of all infections of mammary gland (mastitis). Fortunately, the vast
majority of mastitis cases is caused by a relatively small number of microorganisms that can be grouped into two categories: (1) contagious and (2) environmental.

2.1.3 Contagious: The important organisms of this group are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Corynebacterium bovis* and *Mycoplasma* species.

Many workers from India have been reported that *Staphylococcus* spp. is the chief etiological agent of mastitis in cattle (Sharma *et al.*, 2007; Sharma, 2008; Singh *et al.*, 2005). The organism is ubiquitous and can colonize the skin as well as the udder. Antibiotic treatment is not always successful for certain isolates. *Staphylococcus aureus* is capable of causing preacute, acute, subacute, chronic, gangrenous and subclinical types of mastitis.

*Streptococcus agalactiae* was a major cause of chronic mastitis in pre-antibiotic era. It multiplies in the milk and on the mammary epithelial surfaces, generally causing a subacute or chronic inflammatory reaction with periodic acute flare ups. The affected tissue eventually is destroyed resulting in reduced milk production or agalactia.

2.1.4 Environmental: Environmental mastitis is associated with a relatively high incidence of clinical (related to subclinical) mastitis in the herd that may fluctuate according to season (Hogan, 1993). The bacteria isolated from the clinical cases are usually gram negative or *Str. uberis*. Cases of E. coli mastitis seldom become chronic (Hogan *et al.*, 1992). With herds suffering from *Str. uberis* infections that often occur during the dry period the bacteria can be isolated from nearly all infected quarters. The sources of infection are generally the cow and its immediate environment, particularly the bedding. Klebsiella infections are typically very difficult to treat, and the infected animals usually have to be culled. Before the mastitis problem is found to be caused by Klebsiella, a few cows may already have been lost. Humid sawdust and chippings favour the growth of Klebsiella. The problem is often solved when sawdust storage is
improved (dry storage) or preferably sawdust is replaced with some other bedding material. With Pseudomonas infections, contaminated water is the most likely infection source. The detergents used for cleaning the milking equipment are potential sources as Pseudomonas can grow in the detergents.

2.1.5 The process of infection

When bacteria do enter the udder, the number of immune cells increases rapidly, as the immune system attempts to overcome the infection. Once the infection has been cleared, the SCC levels gradually drop to normal. This can sometimes take weeks, however. In cases of chronic infection, where the bacteria persist in the udder, the SCC levels can remain high throughout the lactation. High SCC levels in the milk cause deterioration of the milk quality. It has been shown that levels above 500,000 cells/ml decrease cheese yield and affect yoghurt making. The shelf life of milk is also affected, but at a higher level of SCC. Consistently high SCC levels in a herd are usually a sign of high levels of subclinical mastitis. Most cases of subclinical mastitis are caused by contagious mastitis bacteria (**S. aureus** or **Str. agalactiae**), even though **Str. uberis** is increasingly considered to cause chronic mastitis as well.

Intramammary infections of dairy cows with Gram-positive bacteria such as **Staphylococcus aureus** (major cause of mastitis) have received a lot of attention because of their major economic impact on the dairy farm through production losses induced by an increase in somatic cell count. Management strategies, including greater awareness for efficient milking and hygienic measures, have limited the spread of Gram-positive bacteria and resulted in a significant decrease of proportion of **S. aureus** isolates and subclinical mastitis worldwide. Other organisms such as coliform subspecies and **Streptococcus uberis**, both environmental bacteria that cause clinical mastitis, have received less attention. **Escherichia coli** causes inflammation of the mammary gland in dairy cows around parturition and during early lactation with striking local and sometimes severe
systemic clinical symptoms. This disease affects many high producing cows in dairy herds and may cause several cases of death per year in the most severe cases. Many studies, executed during the last decade, indicate that the severity of *E. coli* mastitis is mainly determined by cow factors rather than by *E. coli* Pathogenicity.

### 2.2 Types of Mastitis

There are several ways of classifying mastitis. A simple classification recognizes mastitis as two major groups:

1. **Contagious Mastitis**: Caused by bacteria live on the skin of the teat and inside the udder. Contagious mastitis can be transmitted from one cow to another during milking.

2. **Environmental mastitis**: Describes mastitis caused by organisms such as *Escherichia coli* which do not normally live on the skin or in the udder but which enter the teat canal when the cow comes in contact with a contaminated environment. The pathogens normally found in feces bedding materials, and feed. Cases of environmental mastitis rarely exceed 10% of the total mastitis cases in the herd. Contagious mastitis can be divided into three groups: Clinical mastitis, Sub-clinical mastitis and Chronic mastitis.

1a) **Clinical mastitis**

It is characterized by the presence of gross inflammation signs (swelling, heat, redness, pains).

1b) **Pre-acute mastitis**

Characterized by gross inflammation, disrupted functions (reduction in milk yield, changes in milk composition) and systemic signs (fever, depression, shivering, loss of appetite and loss of weight).

1c) **Acute mastitis**

Similar to preacute mastitis, but with lesser systemic signs (fever and mild depression).

1d) **Sub-acute mastitis**

In this type of mastitis, the mammary gland inflammation signs are minimal and no visible systemic signs.
1e) **Sub-clinical mastitis**

This form of mastitis is characterized by change in milk composition with no signs of gross inflammation or milk abnormalities. Changes in milk composition can be detected by special diagnostic tests.

1f) **Chronic mastitis**

An inflammatory process that exists for months, and may continue from one lactation to another. Chronic mastitis for the most part exist as sub-clinical but may exhibit periodical flare-ups sub-acute or acute form, which last for a short period of time. The number of somatic cells in the milk, an indicative of the inflammatory response, may be elevated. Subclinical mastitis may progress and develop into clinical cases, or they may persist for a long time at a subclinical level.

2.3 **Etiological Factors**

*Staphylococcus aureus* and *Escherichia coli* were the common cause of bovine intra-mammary infection (IMI) around the world which causes important economic losses for dairy farmers and industry. Antimicrobial therapy is one of the basis of control programs for mastitis caused by this organism. However, several factors at cow, pathogen, and antibiotic treatment levels affect the probability of cure of the disease. On continuous administration of antibiotics, the pathogenic organisms may develop disease resistance against them. Chicken egg yolk antibodies are used as an alternative for antimicrobial in dairy industry.

2.3.1 **Escherichia Coli**

*Escherichia coli* is come under Enterobacteriaceae family. It is a gram negative, straight rod measuring 1-3 x 0.4-0.7 μm arranged singly or in pairs. It is motile by peritrichous flagellum. It is an aerobe and facultative anaerobe. Good growth occurs on ordinary media at a temperature range of 10-40°C (optimum 37°C). Colonies are large, thick, grayish white, moist, smooth, opaque or partially translucent.
On Blood agar they show haemolysis and on MacConkey agar bright pink colour colonies are observed due to lactose fermentation.

2.3.1a Antigenecity of *E. coli*:

Serotyping of *E. coli* is based on three antigens – the somatic antigen O, the capsular antigen K and the flagellar antigen H. So far 170 types of O antigens, 100 K antigens and 75 H antigens have been recognized. The antigenic pattern of a strain is recorded as the number of particular antigen it carries, as for example O111:K58:H2. The K antigen is the acidic polysaccharide antigen located in the envelope or micro capsule (K for Kapsel, germen for capsule). It encloses the O antigen and renders the strain in agglutinable by the O antiserum. It may also contribute to virulence by inhibiting phagocytosis. Formerly K antigens were subdivided into three kinds – a thermo labile L antigen, the thermo stable A antigen and the B antigen found on entero-pathogenic strain associated with infantile diarrhoea. Later it was shown that the B antigen was not a separate entity. K antigens are therefore currently classified into two groups, I and II, generally corresponding to the former A and L antigens.

Several different serotypes of *E. coli* are found in the normal intestine. Most of them do not have K antigen. The normal colon strains belong to the early O groups (1, 2, 3, 4 etc), while the enteropathogenic strains belong to the later O groups (26, 55, 86,111 etc).

2.3.2 Colibacillosis:

*Escherichia coli* infection is responsible for the significant losses in the global poultry industry. Although the organism is a normal inhabitant of the intestinal tract of commercial poultry, toxigenic strains are responsible for both localized and systemic reactions (Barnes *et al.*, 2008). Strains of *E. coli* serotype O78 have been found to cause numerous extra and intra intestinal clinical symptoms in various hosts (Adiri *et al.*, 2003).

This subversive disease is the primary cause of morbidity, mortality and condemnation of carcasses in the poultry industry.
worldwide (Ewers et al., 2004). In the past few years, both the incidence and the intensity of colibacillosis have rapidly increased and may continue to become an even greater problem in the poultry industry (Altekruse et al., 2002).

The infection is usually secondary to viral or mycoplasma infections or environmental stresses and most frequently involves Avian pathogenic *E. coli* of sero groups O1, O2 and O78 (Gomis et al., 2001), with the later two constituting about 80% of the case (Adiri et al., 2003).

(Vandekerchove et al., 2004) reported that *E. coli* strains isolated from the majority of the flaws belonged almost exclusively to the O78 serotype. Approaches to prevent and control APEC infections in the poultry industry include improved hygienic methods, vaccination, and use of competitive exclusion products and finally antimicrobial chemotherapy, with increase of resistance to antimicrobial agent affecting limited success (Kwaga et al., 1994; Gomis et al., 2003; Knezevic et al., 2008).

Mahdavi et al., (2010) reported that the investigation of antibacterial properties of IgY powder against specific dangerous bacterial species such as *E.coli* O78: K80, as an important cause of economically devastating diseases, is necessary. They were demonstrated that IgY powder can be applied as a feed additive to confer novel protection against *E.coli* O78 : K80 in protecting humans, chickens and farm animals from gastro intestinal infections, associated septicemia and bacterial resistance to antibiotics caused by *E.coli* O78:K80.

Yu-Hong Zhen et al., (2007) conducted an experiment to characterize the specific IgY against *E.coli* O111 In particular to evaluate its ability to inhibit mastitis – causing *E.coli* in vitro and to assess the potential of this approach for controlling bovine mastitis. They concluded that specific IgY produced by hens immunized with *E.coli* O111 and five other mastitis causing *E.coli* strains
Respiratory tract infections and associated septicemia due to *E.coli* in broiler chickens cause major economic losses, due to mainly carcass condemnation and to reduce to growth and feed conversion efficiency (Gross, 1994; Barnes, 2008). Disease occurs most commonly as air saculitis but may involve septicemia with pericarditis, perihepatitis, synovitis, osteomyelitis. Mortality is usually about 5% and morbidity around 50% but mortality can reach 20%, especially under poor management conditions and in the presence of concurrent infections (Wray *et al*., 1997). Disease prevention using anti bacterial agent is expensive and always effective due to rapid development of genetically transferable resistance to antibiotics (Heller, 1973; Bongers *et al*., 1995; Blanco *et al*., 1997). Therefore there have been many attempts to develop vaccines to protect chickens from the diseases and infections caused by *E.coli*.

(Kariyavasam *et al*., 2004) demonstrated an experiment where egg yolk antibodies induced by immunizing hens with selected *E.coli* antigens were evaluated their ability to protect the broiler chickens against respiratory/ septicemic disease caused by APEC. They came out with the result of anti-Pap G IgY provided the greatest protection against the three sero groups of *E.coli* used for challenge. Hence vaccination of broiler breeders to induce anti-Pap G and anti-LutA antibodies may provide passive protection of progeny chicks against respiratory / septicemic disease caused by APEC.

*E.coli* is also found to be the primary environmental pathogen causing bovine mastitis, which reduces milk yield leading to considerable economic losses in the dairy industry (Shpige *et al*., 1997). Antibacterial therapy is a dominating strategy for controlling mastitis, even though the milk produced from the dairy cows often needs to be discarded for a period of time after treatment (Nair *et al*., 2005). Furthermore, the increasing prevalence of antibacterial – resistant bacteria has reduced the effectiveness of antibacterial therapy (Diarra *et al*., 2002).
Mammalian immunoglobulins (IgG) were also used to treat many diseases. (Sherman et al., 1983) demonstrated an experiment on protection of calves against fatal enteric colibacillosis by orally administrated E.coli K-99 specific monoclonal antibody. They were able to reduce the economic loss to cattle owners when outbreaks of enteric colibacillosis caused by K-99+ ETEC occur in unvaccinated herds through the oral administration of K-99 specific MCA to calves.

2.3.3 Staphylococcus aureus

The genus Staphylococcus (Gr. staphylo, bunch of grapes) consists of gram-positive cocci, 0.5 to 1.5 μm in diameter, usually in irregular clusters, within which pairs and tetrads are commonly seen. They are nonmotile and nonsporing. Members of this genus are facultatively anaerobic. Colonies are round, convex, mucoid, and adherent to the agar. They are chemoorganotrophic, requiring nutritionally rich media. Staphylococci have respiratory and fermentative metabolism, producing acid but no gas from carbohydrates. They are able to grow on nutrient agar with 5% NaCl.

Staphylococci are usually positive for catalase. Oxidase negative members contain cytochromes and are Voges-Proskauer positive. Most species reduce nitrate to nitrite. Optimum growth is at 37°C. Staphylococci are commensals on the skin and in the human mouth and upper respiratory tract. They can be human pathogens. S. aureus (L. aureus, golden) is the most important clinical member of this genus. It may be isolated from the skin or mucous membranes of the body. It can cause various infections (e.g., carbuncles, abscesses, pneumonia, endocarditis, food poisoning, toxic shock syndrome) throughout the body. In addition, some of these staphylococci are resistant to penicillin.

This resistance comes about when the bacteria produce penicillinase (ß-lactamase), which hydrolyzes the ß-lactam ring of penicillin. S. epidermidis (Gr. epidermidis, the outer skin) is a nonpathogenic member of this genus. It is part of the normal microbiota of the skin. Additionally, S. saprophyticus (Gr. sapros,
putrid) can be isolated and may be responsible for urinary tract infections especially in females.

2.3.3a Infections caused by *Staphylococcus aureus*

*Staphylococcus aureus* is a bacterium commonly found on the skin and nose of healthy animals and humans (Jablonski, 2001; Moreillon *et al.*, 2005). The major reservoirs of the organism are infected udders, teat canals, and teat lesions, but these bacteria have also been found on teat skin, muzzles, nostrils, and vagina (RobersNickerson *et al.*, 1995). The bacteria are spread to uninfected quarters by teat cup liners, milker’s hands, washcloths, and flies (Chambers, 2001; Marshall *et al.*, 2000; Roghmann *et al.*, 2005). *S. aureus* produces toxins that destroy cell membranes and can directly damage milk-producing tissue (Balaban, 2000). This infection occasionally causes inflammation in humans and animals (Jablonski *et al.*, 2001). Mastitis is one of the most costly problems in the dairy industry (Crossley *et al.*, 1997; RobersNickerson *et al.*, 1995).

Staphylococci are inherently susceptible to most antibiotics (Lindberg *et al.*, 2004), but antibiotic residues are undesirable in milk products for a number of reasons (McEwen *et al.*, 1992). Such residues may be detrimental to the manufacture of certain cultured milk products, and antibiotic use in animal production contributes to microbe resistance (Belschner *et al.*, 1996; Kirk *et al.*, 2002). A wide range of alternatives to antibiotics have emerged as a result of their ban (organic acids, probiotics, herb extracts, and others), of which passive immunization with antibodies is an approach with great potential due to its high specificity (Sol *et al.*, 1997).

*S. aureus* is a frequent etiological agent of food poisoning (Jablonski, 2001). On the other hand, the *S. aureus* isolates from human sources may be considered the most dangerous strains of public health significance (Isigidi *et al.*, 1992). In fact, poultry meat
has been frequently associated with foodborne illness in which initial contamination is traceable to food handlers (Dohnalek, 1989).

### 2.4 Pathogenesis

Because the teats of the cow are continually exposed to bedding, manure, and stable floors, in which coliform bacteria are found, it is probable that the natural route of infection is through the teat duct. Infection can take place in either the dry or lactating cow. It is possible that an infection could be latent in the dry cow and become active upon parturition, thereby producing the high incidence of the disease immediately after calving. *E. coli*, *Aerobacter aerogenes* and their intermediate types have been found in dry cows without producing clinical mastitis (Balaban et al., 2000). When the mammary gland becomes active at the time of parturition and begins to secrete milk, this provides an excellent medium for the multiplication of resident bacteria (Akita et al., 1993). Invasion of the lactiferous ducts and sinuses follows this rapid multiplication. Of the 29 cases occurring midway in their lactation period, 19 had a history of recent teat injury. Presumably this could predispose to infection, either by direct entrance of the organism through the injury or by contamination of the teat or the wound by the farmer during treatment. Most of the injuries were healed before mastitis developed. Pathogenic strains of *E. coli* are powerful producers of endotoxin. These endotoxins are absorbed into the systemic circulation if sufficient in quantity and cause a severe toxaemia and the clinical syndrome observed in pre-acute cases. Because the affected quarter is very often normal in size, and not grossly swollen as in pre-acute staphylococcal mastitis, it would appear that vascular involvement does not occur unless there is a secondary invasion by other organisms, especially *Clostridium* and *Staphylococcus sp*.

Invasion by these organisms often results in the development of a gangrenous process and a grossly enlarged quarter. This is in keeping with the histopathological findings i.e. that no coliform organisms were found in the interstitial tissues of mammary glands.
affected with a pure culture of coliform bacteria, the organisms remaining in the lactiferous ducts and sinuses (Allison, 1985).

An enteritis was associated with the mastitis in seven of the pre-acute cases. The possibility exists that the mastitis followed the enteritis, due to faecal contamination of the udder. However, serotyping of the *E. coli* isolated from the two sites was not carried out. Haematogenous infection of the udder from the enteritis seems unlikely because of the very few cases (Aaristrup *et al.*, 2000) in which all quarters were affected initially in the disease. Spread from the affected quarter to other quarters can occur presumably by contamination with secretions from the affected quarter. Lethal doses of *E. coli* endotoxins injected into dogs and cats will produce vomiting, retching and a profuse, fluid diarrhoea but comparable experiments in adult cattle have not been recorded. The possibility exists, however, that the endotoxin absorbed into the blood stream from the affected quarter may cause the severe diarrhoea observed (Radostits, 2000).

**2.4.1 Development of the disease**

Mastitis begins after bacteria pass through the teat duct and enter the cisternal area. Invasion of teat usually occurs during milking organisms present in the milk or teat end enter the teat canal and cistern when there is admission of undesired air in the milking unit after milking the teat canal remains dilated for 1-2 hours while the canal of a damaged teat may remain partially open permanently. This makes it easier for organisms from the environment or those found on injured skin to enter the teat canal. Adherence of bacteria to tissues lining cisterns and ducts may prevent flushing out during milking and help establish infections. Bacteria eventually enter the glandular tissues where they affect alveolar cells. Toxins produced by bacteria causes death of or damage to milk-secreting epithelial cells, and these cells produce substances to the blood stream that increase blood vessel permeability. This allows leukocytes to move from the blood into the alveolus where they function by engulfing bacteria (Oliver *et al.*, 1992).
2.4.2 Transmission of pathogens

The major reservoirs of *S. aureus, E.coli* are infected udders, teat canals, and teat lesions, but these bacteria also have been found on teat skin, muzzles, and nostrils. The bacteria are spread to uninfected quarters by teat cup liners, milkers’ hands, washcloths, and flies. Staphylococci do not persist on healthy teat skin but readily colonize damaged skin and teat lesions. The organisms multiply in infected lesions and result in increased chance of teat canal colonization and subsequent udder infection. Heifers infected during gestation that carry infections through calving represent an important reservoir from which *S. aureus, E.coli* spread to uninfected herd mates. There is considerable debate surrounding the route of *S. aureus, E.coli* infection in heifers prior to first calving, but calves fed colostrum from an *S. aureus, E.coli* infected dam is a likely source. Early work suggested *S. aureus*-infected colostrum was not a culprit for first-calf heifers calving with the infection (Barto *et al.*, 1982). However, later work did show a positive correlation between feeding *S. aureus*-infected colostrum to a calf (RobersNickolson *et al.*, 1995). Though the data is limited, if an *S. aureus, E.coli* problem exists on a farm, careful colostrum selection, e.g., pasteurization, is certainly one area to consider. Clearly, good mastitis control programs will address the presence of this disease in heifers (Wolf *et al.*, 2010).

2.5 Economical loss

Mastitis causes heavy economic losses to the dairy industry worldwide. The first report on mastitis caused losses in India was about Rs.52.9 crore annually (Dhanda and Sethi, 1962). These losses increased to Rs.6053.21 crore annually in the year 2001 (Dua, 2001). Nearly 70% of this loss is a result of reduced milk production caused by sub-clinical. Apart from its economic importance it is also a matter of concern of carries public health significance (Vasavda, 1988). Moreover, presence of antibiotic residues in the milk is undesirable due to its public health concern. Traditionally, the mastitis control programmes are focused at use of chemical disinfectants, antiseptic or...
herbal teat dips (Maiti et al., 2003) and antibiotic therapy. In herds without an effective mastitis control program, about 40% of the cows are infected in an average of two quarters. Reduced milk production accounts for about 70% of the total loss associated with mastitis.

The inflammatory response consists of an increase in blood proteins and white blood cells in the mammary tissue and the milk. All of the cases were in lactating cows and 51 of the 80 (64%) were in cows recently calved, the majority within two or three days. Fifty-eight of the cases were encountered in the stabling periods and the remainder were seen in the summer periods when the cows were at pasture and had access to the stables only twice daily to be fed and milked. Because the monthly calving rate in this area is relatively even, this would suggest that more cases occurred in housed cattle than in those on pasture (Pereira et al., 2002). This could be explained by continuous contact with bedding, manure, stalls and pens during the stable feeding period. It is well known that the coliform group of bacteria are usually found in large numbers in areas where cattle are confined. The natural habitat for *E. coli, Aerobacter aerogenes* and their intermediate types is in the alimentary tract and the bacteria are present in large numbers in the faeces. No cases were encountered in dry cows or heifers. Twenty-nine of the cases occurred in cows about half way through their lactation period. The sporadic occurrence of this disease suggests that these saprophytes occasionally become pathogenic, although they are relatively harmless most of the time. Coliform organisms responsible for acute mastitis differ from ordinary strains by possessing a distinct capsule (Nickolson et al., 1995).

### 2.6 Treatment

#### 2.6.1 Dry cow therapy

Dry cow therapy (DCT) is more effective in eliminating infections than lactating treatment. However, DCT is not effective if the infections have become chronic by the end of lactation. When cows are not given DCT, spontaneous cures have been very low. DCT is cost-effective (Kirk et al., 2002). When a cow is dried off, it is recommended to treat
all quarters with a commercially available DCT. Follow these steps when dry treating. Cows must be milked out completely, teats dipped in postmilking teat dip and blotted dry after 30 seconds contact time. Teat ends should be scrubbed with alcohol pads before partially inserting the antibiotic tube into the teat (1/8 inch). Although internal teat sealants do not prevent the spread of \textit{S.aureus} infections, the commercially available product does help to reduce new environmental infections. Internal teat sealants are especially useful during the first two weeks and the last seven to 10 days of the dry period when cows are most susceptible to mastitis pathogens. This increase in susceptibility is in part due to the stress associated with drying and the precalving period (Brooke B. E \textit{et al.}, 1981).

### 2.6.2 Antibiotic treatment

Antibiotic treatment will not control this disease but it may, in certain cases, shorten the duration of the infection. Treatment effectiveness decreases as the cow becomes older and even as the first lactation progresses. Cure rates were 34 percent when 89 cows in 10 Dutch herds were treated for subclinical \textit{S. aureus} mastitis (Sol \textit{et al.}, 1997). The results showed that the probability of cure was lower in older cows with high SCC and in cows infected in hindquarters during early and midlactation. \textit{S. aureus} infections were found in 36 percent of clinical mastitis cases in Finnish herds (Pyorala, 1997). Of these, 39 percent responded to treatment. Cows with an SCC of less than 1 million were more likely to cure an infection compared with those over the cut-off point. Successful treatment during lactation is greater if detected and treated early, whereas the response is lower when treating chronic infections. Use of a strip cup or similar device is strongly recommended for detecting abnormal milk.

New clinical infections should be treated promptly and appropriately, especially in first-lactation cows. Tissue damage can be minimized if animals are treated during the early stages of infection. As always, consult a veterinarian regarding off-label treatment options. The use of DHI program SCC records in addition to visual
CHAPTER 2

REVIEW OF LITERATURE

observation of fore-stripped milk and milk culture results will indicate effectiveness of treatment. Many researchers have looked at the efficacy of pirlimycin treatment both in heifers prior to calving and in all animals as an extended therapy treatment during lactation. According to the manufacturer, pirlimycin is one of the most effective compounds against *S. aureus* because its chemical nature allows it to penetrate mammary tissues. In heifers, a single tube of pirlimycin treatment in each quarter six to 12 days prior to calving significantly reduced *S. aureus* infections at calving (Roy *et al.*, 2009).

Furthermore, mastitis data presented to the FDA suggests that two tubes, administered 24 hours apart to infected quarters of cows during lactation, resulted in a cure rate of 36.6 percent, whereas only 1.1% of non-treated controls recovered spontaneously. In field cases, the rate of cows cured during lactation increased to 49.4 percent. However, trials using the same treatment scheme at Louisiana State University and Iowa State University found cure rates of only 12 percent or less for chronically infected *S. aureus* cows during lactation. Single-quarter, extended therapy with repeated label doses of pirlimycin has been examined as a means of providing drug levels beyond the expected life of the leukocytes that naturally fight off this infection. This protocol has been widely adopted for new intramammary infections with *S. aureus*, as it increases cure rates. Four-quarter extended treatment with repeated label doses will provide adequate therapeutic concentrations for many *S. aureus* bacteria. A cure rate of 50 percent at four weeks after treatment was found in more than 100 treated cows (Belschner *et al.*, 1996).

Whether these cure rates justify the additional expenses and effort, not to mention the potential risk of extra-label use and antibiotic residue, is unknown (Wolfe *et al.*, 2010).

2.7 Diagnosis

2.7.1 Milk Examination

Over a period of years many tests have been developed for the diagnosis of mastitis. For convenience they may be divided into two
Groups, viz., Direct or cultural tests- to determine the presence and identity of mastitis organisms in the milk and, Indirect Tests- which depend upon the development of palpable lesions in the udder or changes in the composition of milk.

2.7.2 Direct/Cultural test:

The gold standard for determining udder infection status is milk culturing. Finding mastitis pathogens in milk is a clear indication of potential problems especially with certain bacterial species.

a) Direct microscopic somatic cell counting (DMSCC)

The direct microscopic somatic cell counting (DMSCC) is the most accurate of the mastitis screening tests when conducted properly. For this reason, regulatory agencies generally use this test for confirmation of high somatic cell counts based on other tests. This test is also the standard by which all other tests are calibrated. Studies identifying cell types in milk have shown that somatic cells in milk are primarily (75%) leukocytes which include macrophages, lymphocytes and polymorphonuclear neutrophil leukocyte (Sandholm et al., 1995).

Leukocytes increase in milk in response to infection (or injury). They are the body's primary defense against microorganisms and disease. Epithelial cells (25%) that are secretary and lining cells, on the other hand, increase as a result of injury (or infection). They indicate that damage to body tissue, particularly udder tissue, has occurred. They are in fact dead cells that have been sloughed from the alveoli and canals within the udder.

Even though the DMSCC is the most accurate, it is also the most time consuming. Stained milk films are microscopically examined and somatic cells are counted. As one can easily see this is a very tedious procedure and requires extensive training.

b) Electronic somatic cell count (ESCC)

The electronic somatic cell count (ESCC) test fulfills several needs which dairymen desire. The ESCC focuses attention on the individual cow. It does not pinpoint the quarter(s) affected but does
monitor udder health of individuals. The ESCC also allows a herd average SCC to be calculated which serves as a monitor of the udder health of the herd.

2.7.3 Streaking and identification-

Isolation and identification of bacteria is done on the basis of morphological, cultural and biochemical characteristics. Bacterial colonies should be subjected to Gram’s stain for identification of Gram negative and Gram positive micro-organisms. Milk samples are plated on MacConkey agar to detect coliforms and gram-negative bacteria, modified Edward media for streptococci and streptococci-like organisms, Vogel-Johnson agar for staphylococci, and modified Hayflick medium for Mycoplasma organisms. Plates are incubated at 37°C for 48 hours. EMB agar for Metallic sheen of *E. coli* and colonies can be seen on on MacConkey agar.

a) Differential cell stain (DCS)

Differential cell stain (DCS) like panoptic staining with Pappenheim, Giemsa, Wright, May-Grünwald or Leishman stains is a standard technique in haematological diagnostic procedures and, based on these, the direct smear method used for observing somatic cells in milk should be similar to blood smear technique (May and Grünwald 1902).

Application of this simple and cheap procedure provides additional information about cell types for understanding the udder health status; treating mastitic cases and several characteristics of cells in milk can be evaluated. This test is also the standard by which the differential cell count test is calibrated (Ostensson, 1990).

b) Differential cell counting (DCC)

Differential cell counting (DCC) of milk somatic cells can be a useful diagnostic tool in bovine mastitis research because each cell type has its own more or less specific function in the immune response. Cell numbers (and types) in milk can be counted by microscopy or by particle counters, such as the Coulter counter and
Fossomatic (Dohoo et al., 1982). Pulse and flow cytometric techniques have also been used for counting milk cells (Ostensson, 1990). Quantification of the different physiologic and biochemical properties of individual cells or cell compartments in a cell population can be determined rapidly with flow cytometry (FCM). FCM is a multiparametric analysis of each cell as it passes through a light beam. Elimination of microorganisms from the mammary gland depends mainly on the combination of humoral components and phagocytosis. Phagocytosis is the function of the cellular defence mechanism of the udder. The flow cytometric method allows a rapid and accurate simultaneous determination of different parameters of phagocytosis, and so the response of the immune system (resistance) of individuals (Saad, 1987).

2.7.4 Milk Sample Collection

Milk samples must be collected before the cow is treated with antibiotics. Samples for culture should be collected immediately before milking. When individual quarters show clinical signs of mastitis or positive California Mastitis Test results, individual samples should be collected from the affected quarters. If the entire herd is being sampled, composite samples (all four quarters in one collection vial) will provide reasonable results. To minimize contamination and maximize the chances of receiving useful information from the milk culturing process (Barnum, 1961).

2.7.5 Indirect tests

2.7.5a California Mastitis Test

For 50 years the California Mastitis Test (CMT) has been the only reliable cow side screening test for subclinical mastitis. The CMT does not identify the type of bacteria that cause mastitis but is used to identify quarters that have subclinical mastitis. The CMT was developed to test milk from individual quarters but has also been used on composite milk samples and bulk milk samples (Schalm, 1957). Fresh, unrefrigerated milk can be tested using the CMT for up to 12 hours, reliable readings can be obtained from refrigerated milk for up
to 36 hours. If stored milk is used, the milk sample must be thoroughly mixed before testing because somatic cells segregate with the milk fat.

The CMT reaction must be scored within 15 seconds of mixing because weak reactions will disappear after that time. The CMT reagent is simply a detergent plus bromocresol purple (used as an indicator of pH). The degree of reaction between the detergent and the DNA of cell nuclei is a measure of the number of somatic cells in milk. The relationship between SCC values and CMT is not precise because of the high degree of variability in SCC values of each CMT score. The use of the CMT to identify quarters infected with contagious mastitis has been extensively evaluated (Painter, 1965; Wesen et al., 1968). In general, as CMT reactions increase, the likelihood of recovering pathogenic bacteria increases.

The ability of the CMT to detect infected quarters of fresh cows has been recently reported (Sargeant et al., 2000). In that study, CMT was performed on quarter milk samples each day after calving until 10 days postpartum. When a positive CMT was defined as a reaction of >1, about 57% of infected quarters were accurately identified (43% were missed). Another study used the CMT to test 7,431 composite milk samples obtained from herds in which about 35% of the cows were sub-clinically infected with Staphylococcus aureus and Streptococcus agalactiae (Brooke, 1981). When a CMT value of trace or greater was used, 92% of infected cows were correctly identified. To minimize the number of false negative results, the test should be read as positive when at least a trace reaction is apparent.

2.7.5b New Cowside Tests

Many mastitis control programs would be improved with the use of a rapid diagnostic test for subclinical mastitis. The Delaval Direct Cell Counter (DCC) is a new device that is designed to be used on farms for rapid enumeration of somatic cells. Small cassettes designed to be used on farms for rapid enumeration of somatic cells. Small cassettes are filled with approximately 1μl of fresh milk, stained
automatically in the cassette and inserted into a small battery operated optical cell counter. The DCC produces a somatic cell count in less than 1 minute within the range of 10,000 to 4,000,000 cells/ml.

2.7.5c Tests of Electrical Conductivity (EC)

The principle behind differential EC is that sources of variation in EC other than mastitis would be the same for all for quarters, so a comparison of EC values between quarters should reduce variation. The use of differential EC has been shown to improve the ability of EC to detect mastitis. When differential values were used rather than an absolute threshold, the rate of false positives decreased from 43% to 32% and the rate of false positives decreased to 4%. The use of differential quarter sample EC values is probably the best current use of this technology.

2.7.5d Wisconsin Mastitis Test (WMT)

This is a laboratory test that is generally conducted on bulk tank samples. The test uses the same reagent as the CMT. However, the reaction is not estimated but measured and thus the WMT is more precise than the CMT. The WMT results are generally reported in mm. The test is usually used as a screening test on producer’s milk due to its simplicity and objectivity.

2.7.5e Strip cup test

This test is useful in field conditions for physical examination of milk. This test can be carried out by laymen at farm itself. In this test, enamel plate divided in four strip cups is used (Sharma et al., 2009). The bottom of the plate is black colored so that it gives a good contrast to easily observe the milk flakes. The milk flakes can be seen by tilting the cups at an angle. This test is very useful in primary screening of animals for mastitis.

2.7.5f Surf field mastitis test

This test is easy, cheap and enough sensitive to detect all cases of sub clinical mastitis. The advantage of this test is that the readily available house hold surf (detergent) is used as reagent. The principle of
this test is the reaction of somatic cells DNA with detergent (surf) and leads to the formation of gel of varying degree depending upon the number of somatic cells in the milk (Mohammed et al., 1998).

2.7.5g White slide test (WST)

This test is also simple and easy to perform. The principal of this test is also similar to CMT. Take one drop of 4% NaOH to 5 drops of milk to be tested on a clean glass slide and mixed vigorously with a glass rod for 20 minutes. The results are graded on the basis of precipitation of milk.

2.8. Hen egg yolk antibodies

It is a prerequisite for antibodies preparation. The almost extreme properties of antibodies to recognize small specific structures on other molecules have made them an indispensable tool in laboratory in various applications such as research, diagnostic and therapy. Antibodies presently available for these purposes are mostly mammalian monoclonal or polyclonal antibodies. The production of these antibodies requires normally the use of laboratory animals. Nowadays, most classical chosen mammals for polyclonal and monoclonal antibodies are rabbits and mice, respectively.

In 1893, Klemperer first demonstrated that the immunization of a hen resulted in the transfer of specific antibodies from the serum to the egg yolk. For over a hundred years there was no scientific application for this knowledge. But when the animal welfare became a matter of serious ethical concern for the scientific community, the results of Klemperer have attracted a great attention, particularly since the 1980s.

In the sense of animal welfare, the use of laying hens for antibody production represents a refinement and a reduction in animal use. It is a refinement in that the painful and invasive blood sampling or scarifying are replaced by collecting eggs. It entails a reduction in the number of animals used because the antibodies productivity in laying hens is nearly 18 times greater than that in rabbits (Schade et al., 1996). Furthermore, because of the high yolk
antibodies concentration, over 100 mg of antibodies can be obtained from one egg (Akita et al., 1993). Since a laying hen produces approximately 20 eggs per month, 2 g of antibody per month may be obtained from a single laying hen.

2.8.1. Molecular properties of IgY

2.8.1a Structure of IgY

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**Fig:** 1 Structure of IgG and IgY (Kovacs-Nolan 2004)

IgY has a molecular mass of ~180 kDa which is heavier than that of mammalian IgG (~150 kDa). The general structure of the IgY molecule consists of two identical heavy (H) chains and two identical light (L) chains, which are linked by Disulfide Bridge. The light chain of IgY consists of one variable (V$_L$), and one constant domain (C$_L$), like mammalian IgG. But, intra-chain disulfide linkage between the V$_L$ region and C$_L$ region of L-chain, which stabilizes the structure of the mammalian IgG L-chain is absent in the IgY L-chain and thus intramolecular forces of IgY are weaker than those of mammalian IgG (Shimizu et al., 1988). The heavy chain of IgY contains one variable domain (V$_H$), four constant domains (C$_{H1}$; C$_{H2}$; C$_{H3}$ and C$_{H4}$), unlike mammalian IgG which has three constant domains (C$_{H1}$; C$_{H2}$ and C$_{H3}$). In the heavy chain of IgG, the C$_{H1}$ and the C$_{H2}$ domains are
separated by a hinge region, which gives considerable flexibility to the Fab fragment (the portion which contains the antigen-binding activity). In contrast, the heavy chain of IgY does not have a hinge region, but there are regions near the boundaries of the $C_{H1}$-$C_{H2}$ and $C_{H2}$-$C_{H3}$ domains that contain proline and glycine residues. These regions have the potential to confer limited flexibility on the molecule.

Comparisons of C-domain sequences in IgG and IgY have shown that the $C_{H2}$ and $C_{H3}$ domains of IgG are the equivalents of the $C_{H3}$ and $C_{H4}$ domains of IgY, respectively. The equivalent of $C_{H2}$ domain of IgY is absent in the heavy chain of IgG. The content of $\beta$-sheet structure in C domains of IgY is lower than that of mammalian IgG; therefore, the conformation of IgY domains is more disordered in comparison to mammalian IgG. As for IgG, the Fc part of IgY is the site of most biological effector functions. It contains two carbohydrate side-chains, in contrast to only one in IgG.

**Table 1 - Comparison of the characteristics of mammalian IgG and avian IgY (Schade et al., 1996).**

<table>
<thead>
<tr>
<th></th>
<th>Mammalian IgG</th>
<th>Avian IgY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody sampling</td>
<td>Invasive</td>
<td>Non-invasive</td>
</tr>
<tr>
<td>Antibody amount</td>
<td>200mg IgG per bleed (40 ml blood)</td>
<td>50-100mg IgY per egg (5-7 eggs per week)</td>
</tr>
<tr>
<td>Amount of antibody per month</td>
<td>200 mg</td>
<td>~ 1500 mg</td>
</tr>
<tr>
<td>Amount of specific antibody</td>
<td>~ 5%</td>
<td>2-10%</td>
</tr>
<tr>
<td>Protein-A/G binding</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Interference with mammalian IgG</td>
<td>yes</td>
<td>No</td>
</tr>
<tr>
<td>Interference with rheumatoid factor</td>
<td>yes</td>
<td>No</td>
</tr>
<tr>
<td>Activation of mammalian complement</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>
2.8.1b Biochemical properties of IgY

The valency of IgY is two, same as for mammalian antibodies. In place of the hinge region of mammalian IgG, IgY has a sequence that is more rigid, giving IgY limited flexibility. This probably is the reason for many of the different properties of chicken IgY in comparison to mammalian IgG. The restricted mobility of the hinge region (Cu2) in IgY heavy chain makes the antibody more rigid. This affects the capability of the antibody to precipitate or agglutinate antigens. Only part of chicken antibodies is precipitated at physiological salt concentrations and approximately 25% of the antibodies remain in the supernatant at maximum precipitation. The precipitation curve resembles the curve obtained with horse antibodies with a rapid decline with antigen excess. The precipitation improves at 1.5 M NaCl. The poor precipitation properties might be due to steric hindrance of the Fab arms to crosslink epitopes of two large antigens.

The conditions permitting precipitation might loosen the restricted movement of the Fab arms and give functional independence to the binding sites. Orally given IgY is generally not immunogenic but IgY injected intravenously is an immunogen and elicits a typical anti-IgY, IgM and IgG response in mice (Walsh WE, et al., 2000). IgY applied to other endothelial surfaces then the gastrointestinal tract is probably immunogenic but not yet sufficiently tested. The stability of IgY under acidic conditions and toward pepsin digestion is slightly lower than that of bovine IgG (Shimizu et al., 1988). However, IgY is fairly stable against digestion by internal proteases such as trypsin and chymotrypsin. There appears to be one subpopulation of IgY resistant to papain digestion (Lopez et al., 1993). Immune complexes formed with chicken antibodies are slightly different to those formed with rabbit antibodies. The precipitation curve is steeper and the antigen excess effect on immune complex formation is more pronounced.
2.8.1c Physico-chemical properties of IgY

IgY and IgG differ not only in structure, but also in their stability to pH, heat, and proteolytic enzymes. The isoelectric point of IgY is lower than that of IgG (Polson et al., 1980). It is in the range of 5.7 to 7.6, whereas that of IgG lies between 6.1 and 8.5. Although the stability of both immunoglobulins was similar when subjected to alkaline conditions, IgY showed much less stability than that of rabbit IgG to acid denaturation. Shimizu et al. (1992) found that the activity of IgY was decreased by incubating at pH 3.5 or lower, and completely lost at pH 3. The rabbit IgG antibodies, on the other hand, did not demonstrate a loss of activity until the pH was decreased to 2, and even then some activity still remained. Similar results were also observed by Hatta (1993), using IgY produced against human rotavirus. Similarly, the IgY was significantly more sensitive to heating than the rabbit IgG. Shimizu et al. (1992) found that the activity of IgY was decreased by heating for 15 minutes at 70°C or higher, whereas that of the IgG did not decrease until 75±80°C or higher. Shimizu et al. (1988), however, described the addition of sugar to an IgY solution, and found high concentrations of sugar allowed the IgY to maintain activity when subjected to high heat (75±80°C), low pH (3), or high pressure (5000 kg/cm²). IgY, like IgG, has been found to be relatively resistant to trypsin and chymotrypsin digestion, but sensitive to pepsin digestion (Shimizu et al., 1988). Hatta (1993) found that almost all of the IgY activity was lost following digestion with pepsin, however activity remained even after 8 hours incubation with trypsin or chymotrypsin. The proteolytic digestion of antibodies is a common technique, used to remove the cross-reacting Fc portion of the antibody molecule. Since the Fc fragment (the most hydrophobic moiety of the antibody molecule) of IgY is bigger than that of IgG, the IgY molecule is more hydrophobic than the IgG molecule (Davalos et al., 2000).
2.8.1d Stability of IgY

i) pH stability

The stability of IgY to acid and alkali has been studied under various conditions. It was found that the activity of IgY was decreased at pH 3.5 or lower and almost completely lost with irreversible change at pH 3 (Shimizu et al., 1992). Rapid decrease of the IgY activity at low pH(s) indicated conformational changes and damage in the Fab portion including the antigen-binding site.

Under alkaline conditions, the activity of IgY did not change until the pH increased to 11. However, it was markedly diminished at pH 12 or higher (Shimizu et al., 1988).

ii) Proteolysis stability

IgY is relatively resistant to trypsin or chymotrypsin digestion, but is fairly sensitive to pepsin digestion. Hatta (1993) demonstrated that almost all of the IgY activity was lost following digestion with pepsin, but 39% and 41% of the activity remained even after 8 h of incubation with trypsin or chymotrypsin, respectively. The stability of IgY against pepsin appears to be highly dependent on pH and the enzyme/substrate ratio. At pH 5 or higher, IgY was fairly resistant to pepsin and retained its antigen-binding and cell-agglutinating activities. However, at pH 4.5 or below, both activities were lost (Shimizu et al., 1988). The results of Hatta 1993 who also observed the IgY behavior with pepsin under different incubation times and pH confirmed the susceptibility of IgY to pepsin at low pH. Digestion of IgY with pepsin at pH 2 resulted in complete hydrolysis of the antibody molecule, leaving only small peptides. However, IgY digested with pepsin at pH 4 retained 91% and 63% of its activity after 1 h and 4 h incubation time, respectively.

After tryptic digestion, IgY retained its antigen-binding and cell-agglutinating activities in spite of a definite breakdown of the polypeptides. Unlike the trypsin digestion, no definite cleavage of the IgY chains was observed for chymotryptic digestion and the activities of IgY remained high for these digestes (Shimizu et al., 1988).
iii) Temperature and pressure stability

IgY has been thermally treated at various temperatures for different periods of time. The binding activity of IgY with antigen decreased with increasing temperature and heating time. According to Hatta (1993) IgY is stable at temperature ranging between 60°C and 70°C. The activity of IgY decreased by heating for 15 min at 70°C or higher (Shimizu et al., 1992) and IgY denatured seriously when thermally treated at temperatures higher than 75°C (Chang et al., 1999). IgY is relatively stable to pressure as reported with no detectable inactivation of IgY by pressure up to 4,000 kg per cm² (Shimizu et al., 1988).

iv) Freeze and spray-drying stability

Freezing and freeze-drying are low temperature processes that are usually considered to be less destructive. However, proteins may suffer from a loss of activity as a result of conformational changes, aggregation or adsorption (Skrabanja et al., 1994). There have been some reports on the stability of IgY in regard to these methods. Freezing and freeze-drying did not affect the activity of IgY unless repeated several times (Shimizu et al., 1988). However, Chansarkar (1998) showed that frozen or freeze-dried IgY resulted in some loss of antigen-binding activity and a significant drop in the solubility under the conditions of high salt and protein concentrations. The same findings were observed by Sunwoo (2002).

Yokoyama et al., (1992) analyzed some properties of IgY powders obtained by spray-drying or freeze-drying the water-soluble fraction of egg yolks from Escherichia coli immunized hens. As compared to the freeze-dried powder, the spray-dried powder did not show a significant alteration in antibody titers and yields, even when several spray-drying temperatures were tried (140 to 170°C). However, a higher moisture content of powder was observed in the powder prepared by spray-drying than by freeze-drying.
2.8.2 Immunization of hens

Specific IgY development and production can be achieved by immunizing laying-hens with the target antigen. However, the resulting immune response of the immunized hens cannot be very predictable. Mainly five factors influence this response: the antigen (dose and molecular weight), the type of adjuvant used, the route of application, the immunization frequency, and the interval between immunizations (Schade et al., 1996).

2.8.3 Production and Purification of IgY

Chicken IgY is usually produced against a greater number of antigenic epitopes on a mammalian antigen thus giving an amplified signal and greater test sensitivity. Another advantage lies in the possibility of developing high titer chicken antibodies even though low doses of mammalian antigen (0.001 - 0.01 mg per dose) are applied (Gassmann et al., 1990).

Vaccination frequency and interval depends on the immunogenic potential of antigen and adjuvant used. Often, reported interval is two to four weeks. The presence of yolk antibodies should be checked two weeks after the second immunization. When the antibody titer decreases booster immunization can be given during the whole laying period. A laying hen produces 5 to 6 eggs per week. Average volume of egg yolk (15 ml) contains 50 to 100 mg IgY per egg (Bollen et al., 1996). Of which 2 to 10 percent are specific antibodies (Schade, 1996). Comparing to mammals chickens significantly yield greater amount of antibodies (Hau et al., 2005). The egg yolk contain larger amount of lipids. To avoid some problem in immunoassay popular methods are carried out which enable a successful purification. Akita and Nakai (1993) compared four methods of purifying IgY, they include Water dilution method, Polyethylene glycol method, Dextransulphate method, Xanthan gum method based on purity, ease of use, potential scaling up and immuno activity. Among the four methods chloroform - polyethylene glycol method yielded 2 to
5 times more IgY than PEG method, but PEG method (Polson et al., 1980) was more convenient and efficient as well.

2.9 Application of IgY

As described above, chicken eggs are a rich source (25 g IgY/year/chicken) of excellent antibodies, which in several regards surpass mammalian ones. The relatively inexpensive production of large quantities suggests that IgYs could be used for purposes where a high amount of immunoglobulins is necessary to reach the intended therapeutic effect. Prophylaxis and/or acute passive immunization are relevant fields. Common mammalian antibodies were applied for these purposes only exceptionally to treat cases of emergency, because of their cost. That is why, there is a broad area for application of chicken antibodies. IgY are extensively tested to be used for prophylaxis purposes such as against bovine rotavirus causing death of newborn calves (Kuroki 1994), Salmonella enteritidis or S. typhimurium infections (Lee et al., 2002), pathogenic strains of E. coli of piglets (Jin et al., 1998), for deactivation of urease of Helicobacter pylori (Chang et al., 1999) or Streptococcus mutans that is considered to be the main oral microorganism responsible for tooth cavity formation (Hamada et al., 1991). In most cases, there is no need for purification of the IgY fraction - it is possible to use dried egg yolks of immunized chicken. On the other hand, for the acute treatment of intoxication caused by microbial or snake toxins, antibodies must be purified as much as possible to reduce side effects (Devi et al., 2002).

Antibodies raised against staphylococcal enter toxin B were tested in the respect of passive vaccination of nonhuman primates. All rhesus monkeys treated with the IgY specific for enter toxin B up to 4hr after challenge survived lethal toxin aerosol exposure, suggesting potential therapeutic value of specific IgY (LeClaire et al., 2002). Using of chicken antibodies is absolutely necessary in cases when application of mammalian antisera might result in anaphylactic shock. Rather new way of application of IgY technology is a medical area of xeno-transplantation (Fryer et al., 1999). Antiporcine
endothelial cell antibodies effectively block human antiporcine xeno antibody binding that is expected to inhibit xenograft rejection by endogenous antibodies. The purpose of this approach is based on the failure of IgY to activate mammalian complement system (see below) (Tini et al., 2002).

Immunodiagnostics in clinical chemistry is another widespread area of chicken antibody application. In this respect, all the advantages of IgYs, e.g. high titers against conserved mammalian antigens and no reactivity with rheumatoid factor, complement system, Fc receptors, can be fully utilized. Mammalian antibodies used for ELISA frequently give rise to false positive response in tests with mammalian sera because of the interference caused by the aforementioned protein systems (Carlander et al., 2002). Since IgYs, in contrary to IgGs, do not bind protein A (produced by Staphylococcus aureus) chicken antibodies are well suited also for detection of antigens (pathogens) in stool samples. Another promising application of IgY consists in human haemo classification (Calzado et al., 2001).

Recently, chicken antibodies have been widely used as primary antibodies for ELISA, Western blotting and immunohistochemistry techniques (Hatta 1997). For example, immunodetection of Botritis-specific invertase present in infected grapes is carried out with specific IgYs (Rutz, 2002). Since it is possible to conjugate IgY with horseradish peroxidase, FITC or biotin, the resulting conjugates can be used for common immunochemical procedures (Larsson et al., 1998). IgY possessing a lower pI value than mammalian IgG, is applicable for rocket electrophoresis to quantify immunoglobulins of mammalian sera (Altschuh et al., 1984). Thus, there is no need for IgY carbamylation to differentiate values of isoelectric point in analyzing and developing antibodies as is common when using rabbit IgG. The only limitation of chicken antibody application consists in the lower ability of IgY to precipitate antigens. However, using optimized reaction conditions, formation of precipitate can be
facilitated (e.g. by using a higher ionic strength).

Using of immobilized IgY (bound to gel resin) for immunoaffinity purification of low and high molecular weight compounds has been described in several publications (Hatta 1997). Bound antigen is usually eluted with high yields (97%) under milder conditions (pH 4) than from columns based on IgG, hence this process is suitable for purification of acid-labile antigens.

2.10 Advantages of Chicken Antibodies

From the point of view of animal welfare and bio-ethics, the production of antibodies using a chicken followed by their purification from eggs is more acceptable than preparation of mammalian antisera from blood. Moreover, immunization of the chicken is usually well-tolerated without the formation of abscesses and/or serious health problems, as is common for rabbits. The amount of injected mammalian antigen is often much lower than it is necessary for immunization of rabbits to assure an adequate immune response. Since the antibody is purified from egg yolks, not blood, the stress of the experimental animal is reduced to only the injection of the immunization doses (Thalley, 1990). Animals do not need to be sacrificed to obtain blood for antisera production. The enormous biosynthesis of immunoglobulins, stored in yolks, makes the chicken a progressive experimental animal for antibody manufacture.

Another advantage of using the chicken results from the evolutionary distance between birds and mammals. Hens are able to produce antibodies with high titers against conserved mammalian proteins. Rabbits used for the same purpose are seldom successful in the formation of antisera with acceptable titers. Moreover, IgY against mammalian antigens usually recognizes several antigen surface regions resulting in a stronger final signal in immunological assays (Tini 2002). As mentioned above, because of lack of reactivity with rheumatoid factor, Fc receptors and complement system, IgYs overcome interference problems known with the use of IgG for immunodetection (e.g. ELISA) in human sera. Chicken antibodies
have been successfully used in our laboratory for more than 10 years for various tasks during the study of cytochromes P450, enzymes playing a major role in the metabolism of drugs and activation of carcinogens. Since studied cytochroms P450 are of mammalian origin, the chicken was used to produce antibodies against these antigens.

Prepared IgYs are used as primary antibodies in ELISA and Western blotting and also as secondary antibodies when conjugated with peroxidase. Antibodies raised against rat recombinant CYP1A1 were able to cross-react with human CYP1A1 showing detection limit as low as 0.005 pmol of the enzyme (Stiborova et al., 2002). At present our research is focused on immobilization of IgY in order to prepare an immunosorbent for affinity purification of cytochromes P450.

2.11 IgY use for passive immunization

Passive immunity is the transfer of active humoral immunity in the form of ready-made antibodies, from one individual to another. Passive immunity can occur naturally, when maternal antibodies are transferred to the offspring. It can also be induced artificially, when high levels of antibodies specific for a pathogen or a toxin are recovered from immunized individual or from patient recovering from the infection and administered to non-immune individual. The antibodies transfer may be carried out via systemic, intravenous or oral route. This latter, is the route of choice for localized treatment of the digestive tract infections. Immunity derived from passive immunization lasts for only a short period of time as long as the antibodies remain in the organism, but it provides immediate protection.

In both humans and animals, the administration of preformed specific antibodies is an attractive approach to establish protective immunity against viral and bacterial pathogens (Kim et al., 1999). It is becoming a more and more interesting alternative to control the increasing number of antibiotic-resistant organisms. Passive
immunization can also be used against organisms that are non-responsive to antibiotic therapy.

Antigen-specific IgY can be produced on a large-scale from eggs laid by chickens immunized with selected antigens (Hatta 1997). Therefore, the use of IgY for passive immunization has been studied extensively, demonstrating its effectiveness in preventing or treating infectious diseases caused by various pathogens in animal models.

2.12 Present investigation

The present study deals with generation of egg yolk antibodies against two bacterial pathogens namely *Escherichia coli* and *Staphylococcus aureus* causing poultry infections. Because of the evolution of antibiotic resistant bacteria (Ragunath, 2008), IgY antibodies are used as viable alternative to make healthy chickens and clean eggs without even a reminiscent of antibiotics in the eggs. This procedure is not involving immunization of chickens or administration of any vaccines.