5. Discussion
Mastitis, the intramammary infection of dairy cows with Gram-negative bacteria such as *Escherichia coli* 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. It is one of the most dreaded diseases for dairy farmers because of reduced milk production, increased treatment costs, labor, milk discarding following treatment, death and premature culling (Yang *et al.*, 2011). *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. During *E. coli* mastitis, the host defense status is a cardinal factor determining the outcome of the disease. De and Mukharjee (2009) have been reported the overall prevalence of clinical mastitis and subclinical mastitis were 15.18% and 42.93% respectively during the month of July and August in Uttar Pradesh. Annual losses in the dairy industry due to mastitis have been approximately 2 billion dollars in USA and 526 million dollars in India due to subclinical mastitis where as clinical mastitis is responsible for approximately 70% and 30% respectively of dollar losses (Varshney and Naresh, 2004).

Early detection is essential for successful treatment. Antimicrobial therapy is pivotal for its containment and recovery. Despite the wide spread use of these drugs, antimicrobial treatment of mastitis has been less effective than desirable. However, antibiotic treatment of mastitis is not completely effective against environmental pathogens (Pyorala, 2002). Another concern relates to the overuse of drugs such as antibiotics, which may increase the probability of bacteria developing resistance, and may result in a rise in the number of humans and animals infected by antibiotic-resistant bacteria (Molbak, 2004). Animal welfare under clinical mastitis, as well as the pressure to reduce the use of antibiotics, has prompted a search for alternative treatments (Gabriel Lietner *et al.*, 2013).
Recent research showed that chicken egg yolk antibodies will act as promising alternative for diagnosis and treatment of mastitis causing organisms. Indeed, the immunization of hens is an excellent method to efficiently generate large quantities of antibodies because antibody production is non stressful and non invasive, and the isolation and purification of IgY are relatively simple and high yielding (Zhang, 2003).

The present investigation was to raise specific polyclonal antibodies in chicken against mastitis causing *E.coli* isolated from clinical cases of mastitis in cow.

*E.coli* was isolated from mastitic milk samples collected from clinical cases of cows from veterinary dispensaries in and around Coimbatore. From the isolated organisms *E.coli* and *S.aureus* were predominant. The higher incidence of *E.coli* and *S.aureus* were in accordance with the work done by Nadeem Akram *et al.* (2013) who reported higher proportion of *E.coli* (37.5%) and *S.aureus* (31.94%) in clinical cases of mastitis in and around Lahore. Awandkar *et al.* (2009) reported that the major prevalent pathogens isolated from clinical cases of mastitis in and around Udgir were *E.coli* and *S.aureus*. Sumathi *et al.* (2008) also reported the higher prevalence of *E.coli* and *S.aureus* in clinical cases of mastitis in dairy cattle in and around Bangalore.

To generate anti-*E.coli* antibodies the formalin killed *E.coli* antigen was injected intramuscularly at the multiple sites of the breast muscles of 24 week old white leg horn chickens. Booster injection with increasing concentration of antigen was given to chicken at 7 days interval. The presence of anti-*E.coli* antibodies in serum and egg yolk was observed. The agglutinating antibodies were detected in serum two weeks after the primary immunization (Table 5). Then specific antibodies were detected in egg yolk after a month (Table 6). The concentration of antibodies increased in the egg yolk with subsequent booster doses with an average yield of 80mg per egg yolk at 180th day of immunization period.
The IgY characterization studies like effect of pH, temperature, ionic strength of buffer and various organic solvents on antigen-antibody interactions was studied. IgY was inactivated at below pH 4 and above pH 9. Shimizu et al. (1988, 1992, 1993b) reported that at pH 3.5, IgY activity decreases and is almost completely lost at pH 3 because of rapid conformational changes. When incubated at various temperatures, IgY was inactivated at temperature above 60°C. Hatta et al. (1993a) and Shimizu et al. (1988, 1993b) reported that minimal loss of activity was observed following heating between 60°C and 65°C but was decreased markedly by heating for 15 min at 70°C or higher. The interaction of IgY with antigen was maximum at molarity around 35mM and above 50mM the activity of IgY was minimized. The study with various solvents revealed that 5%-30% of these solvents does not show any adverse effects on antigen antibody interactions.

The cytotoxic effect of antibodies on Murine embryonic fibroblasts remained almost same as that of control. This suggested that the chicken egg yolk antibodies had no toxic effect on cells viability. Jiang et al. (2001) tested acute and chronic toxicity of IgY and reported that egg yolk IgY has no toxicity to the body. Luzia et al. (2014) reported that the IgY antibodies, when subjected to in vitro culture medium containing human lymphocytes, did not produce damage in the cellular structures. The percentage of cellular viability did not decrease in any of the tested concentrations.

Based on the in vitro tests IgY sprayer was developed and the preliminary studies showed from the day one the IgY sprayer reduced the total bacterial load up to 20%. This can be improved by developing affinity purified antigen specific antibodies and by intramammary immunization procedures for effective neutralization. Iqbal et al. (2013) compared the effect of intramammary infusions of egg yolk antibodies and antibiotics in 40 S.aureus mastitic buffaloes. The clinical and microbiological cure rates were 50% better in the egg yolk treated buffaloes than antibiotic treated buffalo groups. The milk yield of 90% and 40% buffaloes was found increased in the groups that received egg yolk antibodies and antibiotic, respectively.
The yolks of eggs laid by immunized chicken have been recognized as an excellent source of polyclonal antibodies for over a decade (Polson et al., 1980). Hen therefore produces a more hygienic, cost efficient, convenient and a plentiful source of antibodies as compared to traditional methods in obtaining antibodies from mammalian serum (Gassmann et al., 1990). IgY production is also less invasive, requiring only the daily collection of eggs compared to blood collection in mammals (Karlsson et al., 2004). The antibody productivity of an egg-laying hen is much greater than that of a similar sized mammal (Jann Hau, 2005). The genetic differences between chickens and mammals make it possible to produce antibodies against highly conserved mammalian proteins, which otherwise would not be possible in mammals, and much less antigen is required to produce an efficient immune response (Larsson et al., 1998).

The present experimental results indicated that the chicken egg yolk antibodies (IgY) effectively inhibited the mastitis causing *E.coli* strains. The antibodies generated by an alternative mean were potent enough to inhibit the growth of *E.coli*. Our results clearly showed that highly purified chicken egg yolk antibodies could be used for therapy in bovine mastitis. Chicken egg yolk antibodies will play a crucial role in research, diagnostics and immunotherapy in future.
The eggs were obtained and the antibodies were purified from egg yolk using Polyethylene glycol and Ammonium sulphate precipitation method described by Polson et al. (1980). The egg yolk antibodies were further purified by DEAE Cellulose ion exchange column chromatography and the fractions were concentrated by polyvinyl pyrolidone (PVP) powder. The DEAE cellulose column purified fractions were further analyzed for their purity by SDS PAGE. The high molecular weight band (180KDa) was observed using Coomassie Brilliant Blue stain which confirmed the purity of IgY. The total protein concentration in the column purified IgY fractions varied in the range of 0.5 – 6.45mg/ml of yolk throughout the immunization period. The specific IgY was found to be 24.1% against *E.coli*.

The Microagglutination Test (MAT) and Microscopic Slide Agglutination Test (MSAT) were performed to check the specificity of IgY against *E.coli* antigen. In MAT vigorous clumping was observed and in MSAT the agglutination was observed upto 1:1280 dilution. Both these results suggest that specific and high titre antibodies were generated against *E.coli*. ELISA reports revealed that there was a gradual increase in the antibody titer in egg yolk and reached a plateau and remained stable till 24th week of observation. The booster doses administered at weekly intervals increased and maintained the antibody level in yolk. A high titer was observed at dilutions of more than 1:10000.

The present investigation showed that the chicken egg yolk antibodies (IgY) inhibited the growth of mastitis causing *E.coli*, when the specific egg yolk antibodies were added to the *E.coli* culture. The growth was completely inhibited when 500µl of IgY was added to the culture. Zhen et al. (2008a) reported that the specific IgY against mastitis-causing *Staphylococcus aureus* inhibited the growth of *S. aureus*. The growth of *S. aureus* was inhibited by the specific IgY at concentrations of 1–5 µg/ml (Marco et al., 2009). In inhibition ELISA there was a decrease in absorbance with increasing concentration of IgY. It indicates that chicken egg yolk antibodies (IgY) effectively neutralize the antigens in pre incubation period.